From the
DEPARTMENT OF CLINICAL SCIENCE, INTERVENTION
AND TECHNOLOGY
DIVISION OF TRANSPLANTATION SURGERY
Karolinska Institutet, Stockholm, Sweden

STRATEGIES TO IMPROVE MACROENCAPSULATED ISLET GRAFT SURVIVAL

Anne Sörenby

Karolinska Institutet

Stockholm 2007

Published by Karolinska Institutet.

ISBN 978-91-7357-304-7

Printed by
REPROPRINT AB
Stockholm 2007
www.reproprint.se
Gårdsvägen 4, 169 70 Solna

To My Family

Yes.....!!

ABSTRACT

Chronic immunosuppressive therapy may have severe side-effects. In cell transplantation, the graft can be encapsulated within a membrane chamber, providing a physical barrier against the immune system. The cell graft then becomes dependent on the diffusion of nutrients and oxygen from the surrounding microcirculation. A major drawback has been the formation of avascular fibrotic tissue around the chamber. The immunoprotective device studied (TheraCyte™) has an outer membrane inducing neovascularization. However, major parts of the encapsulated graft are still lost soon after transplantation, probably because of relative hypoxia and malnutrition. The overall aim of this thesis was to assess various strategies to improve islet graft survival in the device, using rodent models.

The purpose of the first paper was to improve the method for histological evaluation of the vascularization around the device. Vascular profiles within various distances from the membrane surface were counted at different times and then correlated with glucose kinetics. We found that the vascular profiles within 100 µm had the highest correlations with glucose kinetics and concluded that vessels within this distance are important for the exchange of small molecules between the circulation and the device's lumen. Therefore, we recommend that 100 µm should be used in histological evaluations of the membrane vascularization.

In the second paper we hypothesized that preimplantation of the device should improve encapsulated islet graft survival. Previous studies have indicated that it takes up to 3 months for recovery of the microcirculation after membrane implantation. Therefore, we implanted empty devices and transplanted islets 3 months later in these chambers. This approach significantly improved the cure rates of diabetic animals, and the islet dose required for cure was reduced by about 10 times. Morphometry evaluations confirmed increased graft survival in preimplanted devices.

The third paper aimed at evaluating the effects of exendin-4 treatment on the metabolic outcome after islet transplantation. Exendin-4 inhibits islet apoptosis, stimulates islet differentiation and regeneration and has beneficial effects on peripheral tissues. We found that exendin-4 treatment significantly improved the metabolic outcome after free islet transplantation to the renal subcapsular site. The benefit lasted longer than the treatment, suggesting that exendin-4 had long-standing effects on the islet graft. This substance seems to be an interesting new approach to improve the survival also of encapsulated islet grafts.

In the last paper we evaluated the risk of recipient sensitization using macroencapsulated islets. A heterotopic heart graft was transplanted one month after free or encapsulated islet transplantation. The time-to-rejection was significantly shorter in the free islet group, while it did not differ between encapsulated islet graft recipients and naive animals. We therefore conclude that the device protects against sensitization, at least during the first month after transplantation.

Today, side-effects of the immunosuppressive therapy are one of the main limiting factors for the use of islet transplantation. If immunoprotection could be achieved by encapsulation of the islet graft, it should be possible to widen the indications. This thesis describes promising strategies to improve the survival of macroencapsulated islet grafts, which might contribute to make macroencapsulation a clinical reality.

LIST OF PUBLICATIONS

This thesis is based on the following papers which will be referred to by their Roman numerals:

I. Improved histological evaluation of vascularity around an immunoisolation device by correlating number of vascular profiles to glucose exchange. Sörenby A, Rafael E, Tibell A, Wernerson A, Cell Transplantation 2004, Vol 13, 713-19.

II. Preimplantation of an immunoprotective device can lower the curative dose of islets to that of free islet transplantation – studies in a rodent model. Sörenby A, Kumagai-Braesch M, Sharma A, Hultenby K, Wernerson A, Tibell A. Submitted.

III. Exendin-4 treatment improves metabolic control after rat islet transplantation to athymic mice with streptozotocin-induced diabetes. Sharma A, Sörenby A, Wernerson A, Efendic S, Kumagai-Braesch M, Tibell A, Diabetologia, 2006, 49(6), 1247-53.

IV. Macroencapsulation protects against sensitization after allogeneic islet transplantation in rats. Sörenby A, Wu G, Zhu S, Wernerson A, Sumitran-Holgersson S, Tibell A. Transplantation 2006, Vol 82(3), 393-7.

CONTENTS

LIST OF ABBREVIATIONS

APC	Antigen-presenting cells
AUC_{0-100}	Area-under-the-curve, 0-100 minutes
AUC_{0-40}	Area-under-the-curve, 0-40 minutes
AUC_{40-100}	Area-under-the-curve, 40-100 minutes
Bw	Body weight
CAT	Catalase
C_{max}	Maximum concentration
CyA	Cyclosporine
DA	Dark Agouti rats
DSG	15-deoxyspergualine
Encap.	Encapsulated
Ex-4	Exendin-4
GLP-1	Glucagon-like peptide-1
GPX	Glutathione peroxidase
H&E	Hematoxylin and eosin
HbA1c	Glycosulated hemoglobin
IBMIR	Instant blood-mediated inflammatory reaction
IEQ	Islet equivalents
IPGTT	Intraperitoneal glucose tolerance test
IPTR	International Pancreas Transplant Registry
IVGTT	Intravenous glucose tolerance test
MMF	Mycophenolate mofetil
NA	Nicotinamide
NO	Nitric oxide
ns	Not significant
PBS	Phosphate-buffered saline
PET	Positron-emission tomography
PI	Preimplanted
SD	Sprague Dawley
SOD	Superoxide dismutase
T1DM	Type 1 Diabetes mellitus
T2DM	Type 2 Diabetes mellitus
TTP	Time-to-peak
VEGF	Vascular endothelial growth factor
Vv's	Volume densities

1 BACKGROUND

Throughout history people have tried to replace diseased or damaged organs and tissues. During the early 20th century, various surgical methods were developed to transplant different organs. However, the immune reactions against the grafts were not taken into account and the results were poor. At the same time, the field of immunology was explored and during the 1950s the first HLA-molecules were found. In 1954, Joseph Murray did the first successful kidney transplantation between genetically identical twins, for which he received the Nobel Prize in 1990. After improvements in immunosuppressive drug therapy, long-term survival of renal allografts was achieved with kidneys from close relatives and diseased donors in the mid-1960s. Nowadays, the transplantation of kidneys, livers, pancreases, lungs and hearts is the treatment of choice for a variety of conditions with end-stage organ failure. (*1*)

Autotransplantation of various tissues, such as skin and vascular grafts, have been successfully used in clinical medicine for many years. Autologous parathyroid cells have frequently been reimplanted in the arm after parathyroidectomy. The first human allogeneic bone marrow transplantation was performed in 1957 by Thomas, who shared the Noble Prize with Murray in 1990. However, the results were poor until HLA-matching was introduced in the early 1970s. (*1*) Now, hematopoietic stem cell transplantation is a standard treatment for malignant hematologic diseases. At present, the transplantation of neurological cells to cure diseases such as Parkinson's and Alzheimer's remain in an experimental stage. The transplantation of insulin-producing cells to cure diabetes has been studied since the early 1970s. Such treatment is now

being introduced clinically, but the procedure must be developed further because of problems with inefficiency and long-term graft failure.

1.1 β-CELL TRANSPLANTATION

The pancreas gland consists of an exocrine part producing digestive enzymes, and an endocrine part, the islets of Langerhans, containing the insulin-producing β-cells. The islets are scattered throughout the exocrine tissue and constitute only about 1-2% of the total volume. In addition to the β-cells, islets also contain α-cells secreting glucagon, δ-cells secreting somatostatin and pancreatic polypeptide-producing cells. Type 1 diabetes mellitus (T1DM) is caused by autoimmune destruction of the β-cells, usually before adulthood, triggered by genetic and environmental factors (*2, 3*). The total number of cases with diabetes worldwide in the year 2000 was estimated to be 171 million, corresponding to a prevalence of 2.8% (*4*). About 10% of these patients have T1DM. The disease forces the patients to follow a diet and take exogenous insulin throughout their lives. Despite this treatment, the disease can cause long-term secondary complications, such as neuropathy, retinopathy, nephropathy and vascular disease. A strict metabolic control reduces the risk of developing such secondary complications (*5*) Complete normalization of the metabolic control can be obtained only by transplantation of new functioning β-cells.

1.1.1 Pancreas transplantation

Experiences from transplantation of a whole vascularized pancreas graft show that the metabolic control is usually normalized (*6*). The International Pancreas Transplantation Registry (IPTR) had more than 23 000 reported pancreas transplantations worldwide at the end of 2004. In the US, the overall 3-year graft survival was at least 62% and the patient survival rate exceeded 88% between 2000 and 2004. The effects on long-term

complications are beneficial with an increase in life-expectancy (*7, 8*) and a reduction in neuropathy (*9, 10*). Transplantation of the pancreas is usually performed simultaenously with a renal transplantation. The normalized metabolic control protects the transplanted kidney from developing diabetic nephropathy (*11-13*). Some studies also suggest stabilization or even improvement in retinopathy (*14-16*). However, pancreas transplantation involves the risks connected with major surgery and lifelong immunosuppressive therapy. Patients undergoing kidney transplantation are in any case exposed to the risk of immunosuppressive therapy. For them the increase in surgical risk of pancreas transplantation is frequently outweighed by the benefits. However, the major cause of pancreas graft loss is early posttransplant complications, especially thrombosis, pancreatitis and infection (*17*). As these complications are related to the vascular supply or the exocrine tissue, they would be avoided by transplantation of only the islets of Langerhans.

1.1.2 Islet transplantation

The islets of Langerhans can be separated from the rest of the pancreatic tissue by collagenase digestion and density gradient centrifugation (*18*). The isolated islets can then be transplanted to the liver by a percutaneous intraportal infusion. The major surgery of pancreas transplantation is thereby avoided, which is the main advantage of the procedure.

1.1.2.1 Experimental islet transplantation

In 1972, Lacy's group was the first to reverse diabetes by islet transplantation in a rodent model (*19, 20*). However, the findings proved difficult to reproduce. Considerable effort was made to improve the isolation procedure in rodents and establish adequate animal study models (*21, 22*). In larger animals, of which pigs have

been the most extensively studied, the isolation procedure proved more difficult. Wennberg et al. did more than 450 isolations from adult pigs and were able to isolate large numbers of well-functioning islets, but concluded that further standardization would be required to reduce variations in the procedure (*23*).

Various transplant sites apart from the liver have also been evaluated, such as the spleen and kidney capsule, and they were comparable as regards glucose control and vascularization in rats (*24, 25*). Lau et al. recently suggested that the pancreas is a better transplant site than the liver (*26*). In a mouse model, intraportally transplanted islets showed defects in glucose oxidation, insulin biosynthesis and reduced insulin content, while islets that had been directly injected into the pancreatic tissue were comparable to nontransplanted control islets as regards these parameters. Moreover, the glucose-stimulated insulin release was higher in islets from the pancreas than the liver, although less than in control islets.

Early islet graft loss was found to be a considerable problem of the intraportal islet transplantation procedure, making the method inefficient as compared to whole organ pancreas transplantation. Immediately after the injection of islets into the portal vein, a large part of the graft is destroyed by the so-called instant blood-mediated inflammatory reaction, IBMIR (*27*). In a recently published case-report of intraportal transplantation, islets were labeled to be detectable with positron-emission tomography (PET) (*28*). Only 53% of the radioactivity was detected in the liver, indicating that half of the islet graft was damaged during the first minutes after transplantation. Similar results were found by the same group in an experimental study on pigs (*29*). Treatment with low-molecular weight dextran sulfate may be able to block the IBMIR-reaction, as suggested by findings *in vitro* and in a non-human primate model (*30*). Local treatment

by direct coating of the islet surface with heparin seems to have the same effect, but with less risk of bleeding (*31*).

Hypoxia of the islets during pancreas harvesting, isolation and transplantation induces islet apoptosis, which is another cause of early islet loss (*32*). During the engraftment period, exposure to hypoxia continues. Revascularization of islets takes at least 1 week, but the impaired vascular density does not seem to recover fully (*33, 34*). The oxygen tension in rat islets transplanted under the kidney capsule has been shown to be less than that of native islets, 1 month posttransplant (*35*). Culture of islets with angiogenetic factors may counteract this phenomenon (*36*).

In addition to hypoxia, inflammatory cytokines and auto- and alloimmune reactions can induce apoptosis. The inflammatory cytokines also inhibit insulin secretion, presumably via formation of nitric oxide (NO) (*37-39*). Various compounds have been suggested for protection against inflammatory cytokines, such as nicotinamide (NA) and 15-deoxyspergualin (DSG) (*40, 41*). Hyperglycemia may also cause graft failure, at least partly via induction of apoptosis (*42-44*). This may be counteracted by intensive insulin therapy in the early posttransplant period.

1.1.2.2 Clinical islet transplantation

We now know that the islet isolation procedure is more complex for human islets than in rodents (*18*). It was not until an automated method was introduced by Ricordi in 1986 that sufficient islet yield could be obtained for clinical use (*45*). Thereafter, several centers started islet transplantation programs using this method. Today, the isolation technique has been only slightly modified to optimize and further standardize the procedure, and to meet current GMP standards (*46, 47*).

In 1990, Sharp et al. reported a case of temporary insulin independence after intraportal islet transplantation (*48*). This was followed by several other cases, but success rates continued to be low (International Islet Transplant Registry). In 1999, the Giessen Group reported a markedly improved 3-month islet graft function rate of at least 75% in 24 consecutive patients (*49*). In the 1-year follow-up of 37 patients, 24% had achieved insulin independence (*50*).

However, the major breakthrough was reported by Shapiro in the Edmonton Group in 2000, who described successful intraportal alloislet transplantation, defined as insulin independence, in 7 consecutive patients with hyperlabile diabetes and frequent episodes of hypoglycemia (*51*). Six recipients received islets from two donors, and one required a third transplant. Blood glucose levels and glycosylated hemoglobin (HbA1c) values were normalized. The success was partly ascribed to the usage of a new combination of immunosuppressive drugs, consisting of sirolimus, tacrolimus and daclizumab, excluding the diabetogenic glucocorticoids. Now, a slightly modified Edmonton protocol is used worldwide, with reproducible results (*52*). Between 1999 and 2005 about 650 patients were treated worldwide (*53*). Unfortunately, long-term results do not seem that promising. In the 5-year follow-up from the Edmonton group, only about 10% of the 44 patients who had completed the islet transplant were insulin-independent (*54*). However, about 80% were still C-peptide positive, indicating functioning grafts. Although insulin independence remains the ultimate goal, today, stabilization of glucose levels and avoidance of hypoglycemia are considered to be the main indications for islet transplantation.

The causes of the deterioration in glycemic control after islet transplantation are not known. A study by Rickels et al. indicated a reduction in β-cell mass, although

functional defects could not be ruled out (*55*). Allo- and autoimmune destruction may play a role, as well as exposure to toxic immunosuppressive drugs. This is indicated by the better long-term function of islets autografts, which are exposed neither to immunosuppressive drugs nor alloimmune reactions (*56, 57*).

Another serious drawback has recently been reported by the Edmonton group. In a study on the risk of sensitization after islet transplantation, they found that almost one third of the patients who discontinued immunosuppressive therapy after graft failure developed broad panel-reactive HLA-antibodies (*58*).

1.1.2.3 New perspectives

Despite the advances in the field of islet transplantation, the treatment can hardly become a standard procedure until the donor / recipient ratio is brought down to 1, because of the shortage of human donors. In clinical islet transplantation, the liver was chosen as the transplantation site, partly because it seemed to be physiologically superior. However, various factors speak against the intrahepatic site, such as the IBMIR-reaction, defects in metabolic function and the long-term graft dysfunction (*26, 54, 59*). Therefore other sites may be preferred in the future.

The problem of a limited donor pool may be solved by xenogeneic islets, stem cell-derived grafts or *in vitro* expanded allografts (*53, 60, 61*). In the early 1990s, Groth et al. transplanted 10 patients with fetal porcine islet-like cell clusters (*62*). Four patients excreted small amounts of porcine C-peptide in the urine for more than 200 days, and 1 showed biopsy-proven survival of porcine endocrine tissue. However, xenotransplantation was questioned for ethical reasons and potential risks of infections. Clinical studies were therefore stopped in many countries. So far, none of

the patients from Groths's study or any other centers have shown any evidence of infection with porcine endogenous retrovirus (*63*). The breeding of transgenic animals is now an option, which may favor the use of xenotransplantation (*64*). In allotransplantation, islet graft survival may be increased by co-stimulation blockade or mixed chimerism to induce graft tolerance (*53*).

1.2 SIDE-EFFECTS OF IMMUNOSUPPRESSIVE DRUG THERAPY

Although successful transplantations can be performed, potential risks with chronic immunosuppressive therapy remain. Increases in susceptibility to infections and incidence of malignancies are directly related to this therapy (*65-68*).

The various agents also have more specific side-effects. It is well-known that glucocorticoids can cause diabetes, osteoporosis and weight gain and, in children, impaired growth. The anti-proliferative drugs, azathioprine and mycophenolate mofetil (MMF), may induce bone marrow depletion and gastrointestinal problems (*69*). The calcineurin inhibitors, cyclosporine A (CyA) and tacrolimus, cause nephrotoxicity (*70, 71*). Other common side-effects of CyA are hypertension, hyperlipidemia, neurological disorders, gingival hyperplasia and hirsutism (*72, 73*). Tacrolimus has diabetogenic and neurotoxic effects (*72-74*). Sirolimus (rapamycin) causes hyperlipidemia, thromboleukopenia and arthralgia (*75, 76*) as well as adverse renal effects (*77, 78*).

The immunosuppressive drugs also have specific deleterious effects on islet grafts. The success of the Edmonton Group can be partly ascribed to the avoidance of diabetogenic glucocorticoids in the immunosuppressive protocol. However, experimental studies indicate that tacrolimus inhibits insulin gene transcription and is

associated with an increase in morphologic changes of the β-cells (*79, 80*). In a recent randomized multi-center study comparing tacrolimus with CyA, there was a significantly higher incidence of new-onset diabetes or impaired glucose tolerance in the tacrolimus group (*74*). Sirolimus reduces rat and human islet cell viability and induces β-cell apoptosis in rat islets (*81*). It also reduced islet engraftment and impaired β-cell function in a study on syngeneic mice (*82*). The concentrations of tacrolimus and sirolimus are also significantly higher in the portal system than systemically (*57, 83*). However, it is not clear whether a local increased immunosuppressive treatment is beneficial or harmful to islets transplanted to the liver.

1.2.1 Indications for islet transplantation

In the selection of recipients for islet transplantation, the benefits of the transplantation must outweigh the risks of the procedure and the immunosuppressive therapy. Islet transplantations are therefore commonly performed in conjunction with, or after previous kidney transplantation, when immunosuppressive drugs would be given in any case (*84*). Selected patients with metabolic instability or severe problems with hypoglycemia may also be considered (*51*). However, for most patients with T1DM, islet transplantation cannot be recommended at present.

If islet transplantations could be performed without the requirement of chronic immunosuppressive therapy, the indications would of course increase. The treatment could then be offered to otherwise healthy diabetic recipients, before the development of secondary diabetes complications.

1.3 IMMUNOPROTECTION

In cell transplantation, immunosuppressive drugs could be avoided by encapsulation of the graft in a semipermeable membrane (*85-88*). Such membranes protect the graft from the cells of the immune system, but are permeable to small molecules. Encapsulated cells are dependent on the exchange of oxygen and nutrients between the lumen of the device and the surrounding microcirculation. Moreover, the therapeutic product, such as insulin, produced by the cell graft must be able to pass the membrane. Two main immunoprotective strategies are used: macro- and microencapsulation. Using the macrocapsules the whole islet volume is loaded into one or a few devices, as illustrated in Figure 1.3, while each single microcapsule only contains one or a few islets.

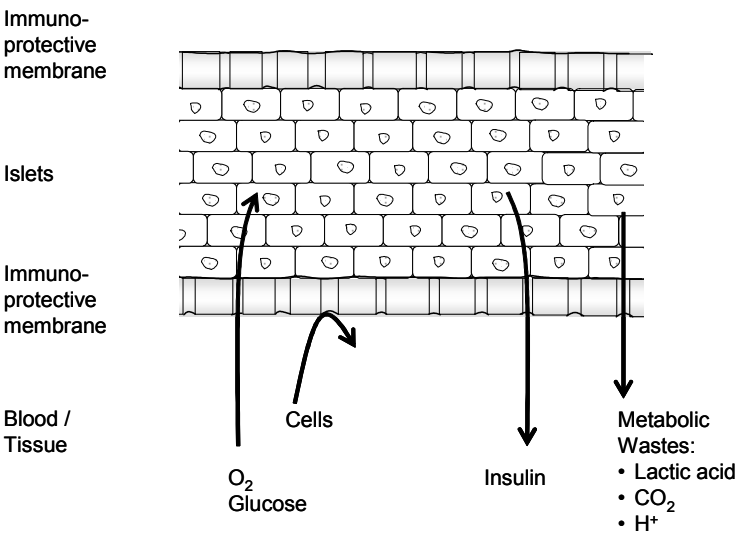
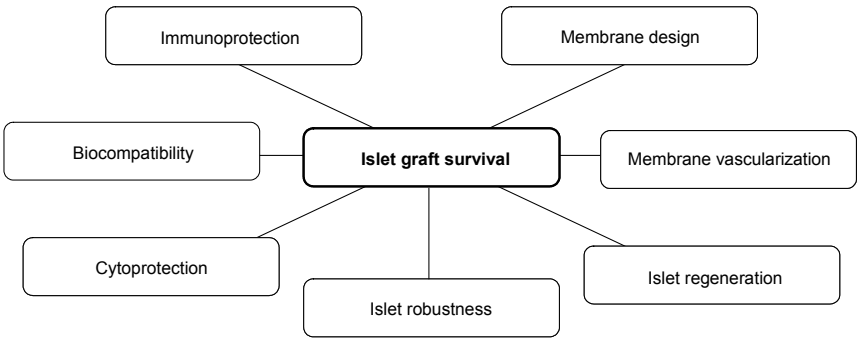


Figure 1.3. Schematic illustration of macroencapsulated islets.

Encapsulated cells have been used experimentally for therapeutic treatments of various diseases such as diabetes, neurological disorders, hemophilia, renal failure and erythropoietin deficiency (*89-95*). Several methods have been described for immunoprotection of islets, which is the main subject in the following sections.

1.3.1 Common problems and improvement strategies

The function and survival of encapsulated islets depend on a few basic principles, as shown below:



To achieve and improve function and survival of encapsulated islets, each of these factors should be considered. *Immunoprotection* - i.e., the ability of the membrane to protect against rejections - is by definition a requirement for the method. However, the physical barrier cannot be entirely impermeable, since it must permit diffusion of nutrients. An adequate pore size is therefore of crucial importance (*94*). Ultrafiltration membranes can protect even xenografts, but at the cost of reduced graft survival because of nutritional limitations (*85, 96*).

The *design* of the immunoprotective device takes into consideration the size and shape of the membrane, which determine the diffusion distance for oxygen and nutrients (*85*). The shape can also affect the *biocompatibility* together with the configuration of the surface, because irregular surfaces tend to increase foreign-body reaction with the formation of avascular fibrotic tissue, which thereby influences the *vascularization* of the membrane (*97-99*). It should be noted that neovascularization after tissue damage takes time. Immunoprotection, design, biocompatibility and vascularity are all factors related to the method of encapsulation, and are therefore discussed below together with the various encapsulation methods.

Cytoprotection includes treatment with various drugs to enhance islet survival during the initial suboptimal physiological conditions caused by hypoxia and inflammation. Scavenging of reactive oxygen species and nitric oxide (NO) can be one approach. When rat islets were compared with various other rat tissues, as regards expression of the scavengers superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX), islets were shown to have the weakest antioxidant enzyme defense system (*100*). Other studies have shown that treatment with SOD or CAT may reduce the β-cell toxicity of alloxan and streptozotocin (*101, 102*). Overexpression of SOD, CAT or glutathione peroxidase (GPX) protects bioengineered insulin-producing cells from the toxic effects of various NO-donors (*103*). A slow-release preparation of SOD and CAT has been described by Giovagnoli et al., who entrapped the scavengers in a biogradable polymeric matrix (*104*). When neonatal pancreatic porcine cell clusters were co-cultured with entrapped SOD and CAT, improvements in viability, function and morphology occurred.

There are also a number of other interesting drugs for islet protection. Nicotinamide preserves β-cell function at the onset of diabetes, reduces lipid peroxidation and promotes β-cell differentiation (*105-107*). 15-deoxyspergualin (DSG) shortens the duration of posttransplant hyperglycemia and improves cure rates and long-term graft function (*40, 108*). Nicotinamide and DSG treatment in combination may be even more effective (*40*). Lazaroids have a potent cytoprotective effect by inhibition of lipid peroxidation (*109, 110*). They reduce ischemia-reperfusion injury (*111, 112*). A lazaroid with the generic name tirilazad mesylate was registered for the treatment of stroke in Sweden (*Freedox™, Pharmacia & Upjohn, Sweden*). Our group performed promising pilot studies indicating that tirilazad mesylate could lower the curative dose of encapsulated islets. Unfortunately, the production of the drug was discontinued before these studies were finished. In addition to the various drugs described above, numerous other cytoprotective drugs have been suggested as potential treatments in islet transplantation.

Islet regeneration may be important for healing of early graft damage and long-term graft survival (*53, 60, 61*). The possible mechanisms for islet regeneration include proliferation, hypertrophy and differentiation of immature cells, which may be induced by various growth factors.

Cytoprotection and islet regeneration may be obtained by treatment with glucagon-like peptide-1 (GLP-1) or its long-lasting analogue exendin-4 (*114-116*). The substances have been described to inhibit cell apoptosis (*117*) and stimulate islet replication and neogenesis (*118, 119*). Moreover, they have acute effects on insulin secretion, gastric emptying and glucose trafficking in the peripheral tissues (*120*). Exendin-4 has been shown to improve the glycemic control in patients with Type 2 diabetes mellitus

(T2DM) (*121*). The pharmacodynamics and safety of exendin-4 have already been tested in phase 2 and 3 clinical trials of T2DM and the drug has now been introduced on the market in the USA (*122, 123*). Studies have also indicated that exendin-4 have positive effects on postprandial blood glucose levels in T1DM (*113*). On the basis of these findings, we hypothesized that exendin-4 would also have beneficial effects on transplanted islets, which at that time, to our knowledge, had not been evaluated. To reduce confounding factors in the first explorative studies, we used a free islet transplant model and planned to continue with encapsulated islets, if positive effects were found.

With the term *robustness* we refer to the ability of islets to survive in a suboptimal milieu. A critical factor deciding islet robustness is of course the quality of the islet graft. As described above, a number of drugs have been shown to increase graft resistance to damage. Genetic modification of islets, to decrease the vulnerability to oxidative stress and inflammatory cytokines may become a clinical alternative in the future.

1.3.2 Microencapsulation

In microencapsulation, one or a couple of islets are enclosed in a spherical capsule (*85, 124-126*). The capsules are usually prepared by suspending the islets in a polyanionic aqueous solution dropped into calcium ions. The gel drops are then mixed with a cationic macromolecule to form a capsule (*126*). The most commonly used ions are alginate and poly L-lycine, although a large number of other combinations have been tested. An automated method has been developed to form uniform capsules with an equal sized capsule wall (*127*).

In 1980, allogeneic rat islets in alginate microcapsules were first reported to reverse diabetes (*128*). A couple of year's later, long-term insulin independence was described in monkeys after transplantation of microencapsulated porcine islets (*129*). However, the results of that study have been difficult to reproduce by others.

One major problem with the microencapsulation method has been the overgrowth of fibroblasts, limiting the diffusion across the capsule wall and thereby impairing islet survival (*130, 131*). Several factors, such as the fraction of individual polysaccharides, the concentration of ion solutions and the purity of the alginate affect the biocompatibility (*126, 132*). The surface to volume ratio in these spherical capsules gives a favorable mass transfer situation, which enhances the survival and function of the cell graft (*125*). Because of the large total volume of the microencapsulated islet graft, implantation is usually done in the peritoneal cavity. However, a recent study by Dufrane et al. on microencapsulated pig islets transplanted to rats showed a higher degree of broken capsules and capsules with severe cellular overgrowth after intraperitoneal transplantation than after renal subcapsular and subcutaneous transplantation (*133*). To shift the transplant site, the total graft volume must be reduced, for example by reducing the size of the individual microcapsules. However, reduction of the capsule size may cause a decrease in islet survival (*124, 134*). When de Vos et al. reduced the capsule size from 800 μm to 500 μm, inadequately encapsulated islets increased from about 6% to 24%. However, Calafiore's group developed 2 capsules of smaller sizes by refinements in the preparative process and composition (*135*). Their findings indicated that the medium-sized capsules with diameters of 300-400 μm were advantageous to the smaller ones, as regards the balance between immunoprotection and volume (*124*). They suggested that islets in smaller capsules, although intact, may be more easily reached by inflammatory cytokines, and therefore

more vulnerable. A recent study described a novel encapsulation technique based on direct binding of polyions to the islets in many layers (*136*). According to the authors, the capsule has the advantage of being smaller than alginate-based capsules and the technique enhance total trapping of the islets.

A phase 1 clinical study is presently being conducted by Calafiore's group (*137, 138*). Ten patients will be included in the study, using human islets in a microcapsule of Na alginate and poly-L-ornithine. The first preliminary results of 2 patients indicate stabilization of glucose levels, decline in exogenous insulin consumption and decrease in HbA1c. The method also seems safe and without side effects. According to a recent study by the same group, the capsule used in the clinical protocol has been further improved as regards physical strength and longevity in a rat model (*139*).

In recent years, microencapsulation has frequently been described as the best method for immunoprotection of islets (*86, 140, 141*). Advantages using the microencapsulation method include the minor implantation surgery, the surface-to-volume-ratio and the possibility to use various transplant sites. Some potential disadvantages of this method include incomplete encapsulation and insufficient long-term material stability, both leading to rejection and the risk for sensitization.

1.3.3 Macroencapsulation

Using macroencapsulation, the whole graft is placed in one or a few chambers made of semipermeable membranes (*85, 86*). Devices for macroencapsulation may be grouped into intravascular and extravascular chambers. The extravascular chambers can be further grouped into cylindrically-shaped tubular chambers and planar devices with a flat configuration.

1.3.3.1 Intravascular encapsulation devices

An intravascular chamber consists of a semipermeable membrane, directly connected to the circulation as an intravascular shunt. The advantages of this method are the large oxygen supply, which enhances islet survival, and the rapid glucose-stimulated insulin response (*86, 87*).

However, the introduction of an intravascular shunt is a major surgical procedure, with the risk of bleeding after implantation (*142*) and a high risk of thrombosis in the device or in the anastomosis, which in most cases would require life-long anticoagulation therapy (*85-87*). Therefore, the risks of the treatment are seldom outweighed by the benefits.

1.3.3.2 Extravascular diffusion devices

In diffusion chambers, oxygen and nutrients are provided by diffusion from the surrounding microcirculation. Sites of implantation may include the peritoneal cavity, the omentum and subcutaneous fat. The main advantages of these chambers are that they only require minor surgery to implant and are easy to retrieve.

Tubular diffusion chambers are usually made of acrylic-copolymer fibers (*85, 143, 144*). The cylindrical shape and the acrylic-copolymer material provide a smooth surface which induces hardly any fibrotic reaction (*99, 144*). This type of device was successfully used in a study by Scharp et al. to encapsulate allogeneic islets for transplantation to diabetic patients (*144*). After 2 weeks, the islet viability was more than 90%. However, the disadvantage of using tubular chambers is the suboptimal mass-transfer situation, because widening the tube to reduce the length leads to necrosis of the central graft (*85*). In Scharp's study, the islets were suspended in alginate to

avoid islet aggregation and central graft necrosis, which resulted in a low loading density. The loading dose was only 50 islet equivalents (IEQ) in a 1.5 cm long fiber, which would correspond to enormous lengths for curative doses. However, tubular chambers may be advantageous in the transplantation of other cell types, when less volume is required.

The flat configuration of planar diffusion chambers can provide a more efficient configuration for islet encapsulation. However, the majority of early studies using planar devices reported islet graft failure and fibroblastic overgrowth of the chamber (*87*). According to a review by Colton, a theoretical model of flat devices shows that oxygen supportive vessels must be in close contact with the membrane surface for oxygen to reach the entire graft (*85*). Brauker et al. screened various membranes for foreign body reaction and vascularization (*145*). They found that membranes with larger pores, that permitted cells to penetrate, could induce vascularization at the membrane-tissue interface. The induced vessels were referred to as close vascular structures, defined as vessels within 15 μm from the membrane surface. To obtain immunoisolation, the vascular-inducing membrane was laminated to another membrane with smaller pores. This bilayer membrane was later used for construction of the Boggs™ chamber and the TheraCyte™ device (*TheraCyte Inc., Irvine, CA, USA*) which were used for the studies of this thesis and are described in more detail below (*94*).

1.3.4 Previous studies of the bilayer membrane

1.3.4.1 Immunoprotection

The bilayer membrane prevents host cell entry, but allows the passage of larger molecules, such as IgG. In studies by Brauker et al., allogeneic embryonic lung tissue survived for 1 year in rats, when implanted in intact devices (*146*). The tissue was

destroyed within 3 weeks if holes were made in the device. Xenografts survived no more than 3 weeks in intact devices, and the surrounding tissue showed severe inflammation and reduced vascularization. When allo- and xenogeneic tissues were mixed in the chamber, all tissue was destroyed. Again, the inflammatory reaction was pronounced, and was thought to be the cause of the tissue death. The study also suggested a mechanism underlying the xenograft rejection. In a following study, Loudovaris et al. showed that xenografts from monkeys could survive in $CD4^+$ T-cell-depleted mice (*147*). Since the membrane is impermeable to cells, they concluded that $CD4^+$ T-cells probably indirectly recognize antigens from the xenograft, and then stimulates a local inflammatory response. It should be noted that a recent, small, xenograft study on neonatal pig islets transplanted to nonobese diabetic mice and nondiabetic cynomolgus monkeys showed contradictory findings, since the TheraCyte™ device protected the graft (*148*). The authors suggested that the islets express only a few surface antigens with alpha-1 galactose, which is the main inducer of acute rejection of porcine xenografts in primates.

In humans, the alloprotective properties of the bilayer membrane were confirmed by Tibell at al. (*149*). Patients with hypoparathyroidism were transplanted with encapsulated allogeneic parathyroid tissue in subtherapeutic doses using the Boggs™ Chamber. The devices were implanted s.c. in the forearm. At explantation after 9-13 months, histological intact endocrine tissue was found in all devices. However, fibroblast overgrowth inside the devices was marked.

1.3.4.2 Biocompatibility

The number of close vascular structures - i.e., within 15 μm from the membrane surface - has commonly been used as one evaluation criterion of the biocompatibility of

membranes (*145, 150, 151*). In a study by Rafael et al., the vascularization in the s.c. tissue within 15 µm from the surface of the bilayer membrane was evaluated histologically (*152*). No correlation was found between the number of vascular profiles within 15 µm and the measured glucose recoveries inside the device during IVGTT. However, when a longer distance from the membrane was arbitrarily chosen (250 µm) the number of vascular profiles was significantly correlated to the glucose exchange. We then decided to search for a more precise distance, within which vessels can support the transport of glucose to the lumen of the device. This should improve histological evaluations of the membrane, since the supportive vessels should reasonably be the ones to consider in such evaluations.

1.3.4.3 Vascularization

The physiological conditions inside the device are dependent on the exchange of nutrients and oxygen from the surrounding microcirculation, which, in turn, depends on the vascularity and maturity (function) of new vessels. To study this relationship, the *in vivo* exchange of insulin and glucose across the membrane was previously evaluated by members of our group, using the microdialysis technique (*152, 153*). Insulin was injected into implanted devices in rats at various times after implantation. The injection of insulin directly into the s.c. tissue was used as control. At 1, 2 and 4 weeks, insulin recovery after injection in the devices was lower than that after s.c. injection. At 3 months, the insulin permeability had increased, and recovery after injection in the device was similar to that in the s.c. controls (*153*). The *in vivo* glucose recoveries inside the devices were then studied during an intravenous glucose tolerance test (IVGTT) in rats (*152*). Up to 4 weeks after implantation, glucose recoveries were significantly lower in the devices than the s.c. controls. After 3 months, the difference had disappeared.

The microcirculation around empty devices implanted in rats was then studied using the laser Doppler technique. Compared to day 1, the microcirculatory flow was lower at 4 weeks, but had recovered at 2 and 3 months. The level was maintained for at least 12 months after implantation (*154*). These studies all indicated that the impaired physiological conditions after implantation were nearly normalized after 3 months. Therefore, a study was done to evaluate the effects of preimplantation of devices. Empty devices were implanted s.c. on one side of the back of non-diabetic rats. Three month later, these devices were filled with islets. At the same time, freshly prepared devices filled with islets were implanted on the other side of the back. All devices were then left for 2 weeks before explantation. The study was evaluated by morphometry and showed a higher survival of endocrine tissue in the preimplanted than in the freshly implanted devices (*155, 156*). However, a diabetic model is needed to show the effects on metabolic outcome after transplantation. Therefore, we wished to analyze the concept of preimplantation using a diabetic model, which would permit dose finding studies.

Another approach for improvement of the vascularization may be treatment with angiogenetic factors, such as the vascular endothelial growth factor (VEGF). Trivedi et al. implanted the bilayer device in rats and continuously infused VEGF for 10 days (*151*). High doses of VEGF resulted in a two- to threefold increase in blood vessel formation within 200 μm from the membrane surface, as assessed by morphometry, and a modest increase in insulin diffusion. Using a tubular encapsulation system, Sigrist et al. evaluated a clinically more relevant approach, by transplanting islets immobilized in a collagen gel supplemented with VEGF (*157*). They found an increase in the

number of vessels and a decrease in the distance between the device and the closest vessels, as well as improvement in islet survival and insulin secretion.

1.3.4.4 Other applications of the bilayer membrane

Encapsulated genetically-engineered cell lines that express e.g., human growth factor or the coagulation factor IX, have been shown to secrete their products for more than 3 months, as compared to only a few days if freely implanted (*158-160*). A recent study on the treatment of osteoporosis by encapsulated human parathyroid cells, showed an increase in bone mineral density in ovariectomized rats (*161*).

1.3.4.5 Other planar devices

As mentioned above, early experiences of planar devices included the development of avascular fibrotic tissue around the device. The bilayer membrane was therefore developed. Only a few studies of other types of planar devices have been done recently. Qi at al. described a flat-shaped device based on biocompatible polyvinyl alcohol (*162*). This material has a weak mechanical strength, but was combined with a polyethylene terephthalate mesh for stabilization. Rat islets were embedded in the gel and the macrocapsules prepared by a freeze-thaw process. Devices were implanted in the peritoneal cavity of diabetic mice, which significantly reduced the blood glucose levels.

1.3.5 Protection against sensitization of the recipient

Before clinical trials of macroencapsulated islet transplantation can be considered, it is important to evaluate the potential risks for the recipients. One factor is the risk of sensitization - i.e., the development of anti-donor antibodies - which may cause hyperacute rejection of a subsequent vascularized graft (*163*). In allotransplantation, the direct antigen presentation pathway is thought to be the main inducer of the immune

response (*88, 164-166*). The immunoprotective membrane physically blocks this pathway, because it requires migration of antigen-presenting cells (APC) from the donor to the lymph nodes of the recipient. However, passage of small peptides across the membrane is likely, as the immunoprotective membrane is permeable to small molecules. This could instead activate the indirect pathway by antigen-presentation on APCs of the host (*147*). Sensitization by encapsulated islets therefore cannot be ruled out. Patients with diabetes should preferably be given islets early in their disease. However, as these patients are at risk of developing diabetic nephropathy, they may become candidates for kidney transplantation later on. Sensitization would then reduce graft availability and jeopardize graft survival. Against this background, we wished to evaluate whether the bilayer membrane influence the risk for sensitization after islet transplantation.

2 SPECIFIC AIMS

The specific aims of these studies were:

- To improve the histological evaluation of vascularity around the bilayer immunoprotective chamber, by correlating vascularization within various distances from the device surface with glucose exchange.

- To evaluate whether preimplantation of the immunoprotective chamber lowers the dose of islets needed to cure diabetes.

- To determine whether the effects of exendin-4 improve the metabolic control after islet transplantation.

- To assess the risk of sensitization after allogeneic islet transplantation in the bilayer immunoprotective chamber.

3 MATERIALS AND METHODS

The local Animal Ethics Committee approved all studies. The animals were maintained in accord with the requirements of the Animal Welfare Act and the National Institutes of Health guidelines for the care and use of laboratory animals.

3.1 MATERIALS, TECHNIQUES AND MODELS

3.1.1 The immunoprotective membrane

The immunoprotective chamber is made of a two-layered polytetrafluoroethylene membrane. The inner immunoprotective membrane (*Millipore, Bedford, MA, USA*) has a pore size of 0.45 μm. It is permeable to small molecules, but not to cells. The outer membrane with a pore size of 5 μm has the ability to induce neovascularization (*Gore, Flagstaff, AZ, USA*). To ensure stability, a non-woven polyester mesh is attached to the outer membrane. Before implantation, the membranes were hydrophilized by wetting in 95 % ethanol for 1-2 minutes and washed 3 times in sterile saline.

3.1.2 The Boggs™ chamber

The Boggs™ chamber (*TheraCyte Inc., Irvine, CA, USA*) was the first type of device developed from the two-layered membrane described above (Figure 3.1.2). It consists of 2 round pieces of the membrane separated by a silicon ring, creating a lumen of 5 μl where the islets are loaded, before sealing of the sheets with a titanium ring. The various parts of the device are put together at the time of islet loading.

The Boggs™ chamber was used in the last study of this thesis for transplantation of allogeneic rat islets. The chambers were implanted s.c. on the back of the rats.

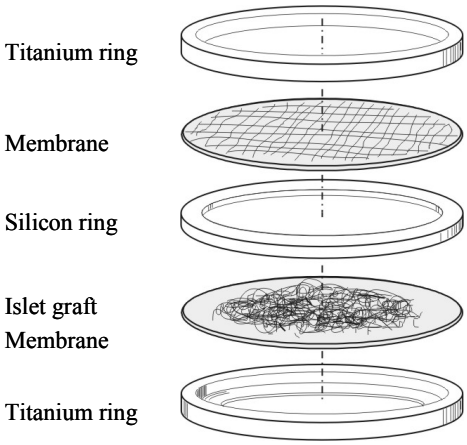


Figure 3.1.2. The Boggs™ chamber.

3.1.3 The TheraCyte™ device

The TheraCyte™ device is a planar diffusion chamber prefabricated in the shape of a tea-bag (*TheraCyte Inc., Irvine, CA, USA*) (Figure 3.1.3-1 page 64). It consists of the inner immunoprotective membrane laminated with the vascularization membrane, the non-woven polyester mesh and an outer woven polyester mesh (Figure 3.1.3-2). It is a further development of the Boggs™ chamber, to simplify the loading procedure and minimize the risk of contamination. For loading of the device, there is a port in one end, which is sealed after the cell injection. The device is available in 3 sizes with volumes of 4.5, 20 and 40 µl.

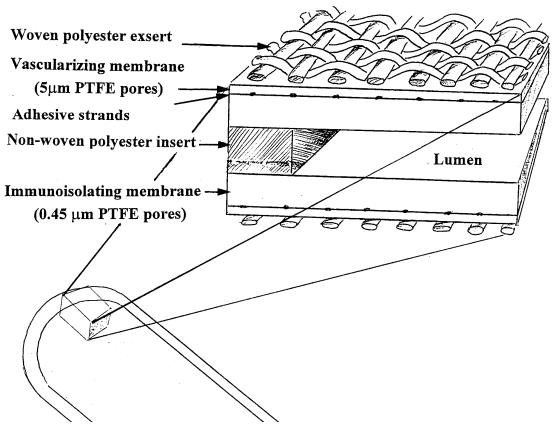


Figure 3.1.3-2. Structure of the TheraCyte device.

3.1.4 Rat islet isolation

The same standard procedure was used for rat islet isolation in Papers II-IV. Islets were obtained from Male Sprague Dawley rats (SD) in study II and III, and Dark Agouti (DA) rats in Paper IV (*all provided by Scanbur BK, Sollentuna, Sweden*), weighing about 300 g. The pancreases were harvested after anesthetizing the animals with Enflurane in Paper II and III, and Hypnorm™ (1 ml/kg of fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml s.c., *Janssen Pharmaceutica, Beerse, Belgium*) in Paper IV. Animals were euthanized by heart exsanguinations. Islets were prepared by the intraductal collagenase digestion technique using 2 mg/ml collagenase V (*Sigma-Aldrich Co, St Louis MO, USA*) or 0.7 mg/ml collagenase P (*Roche Diagnostics GmBh, Mannheim, Germany*). The islets were then purified by discontinuous density gradient centrifugation (*Histopaque-1119, Sigma-Aldrich Co and Lymphoprep* ^{TM}*, Axis-Shield PoC AS, Oslo, Norway*) and hand picked. Isolated islets were cultured overnight in RPMI 1640 medium with pH 7.4 (*Gibco, BRL, Life Technology Ltd.,*

Paisley, Scotland), supplemented with 10% fetal calf serum, 2 mmol/l L-glutamine, 50 U/ml penicillin and 50 mg/ml streptomycin (all provided by *Gibco*), in a humidified atmosphere of 5% CO_2 in air at 37°C. The following day, the islets were washed with Hanks Balanced Salt Solution (*Karolinska University Hospital, Stockholm, Sweden*), hand-picked and transplanted (Figure 3.1.4-1 page 64). Islet purity was about 90%, as estimated semiquantitatively under microscope.

3.1.5 Streptozotocin induction of diabetes

Athymic mice (*nu/nu Black 6, Taconic M&B, Ry, Denmark*) weighing about 25 g were used as islet recipients. These immuno-incompetent nude mice cannot reject cellular grafts, thus the survival of the encapsulated islets is not influenced by any rejection reaction. Diabetes was induced by the injection of streptozotocin (*Sigma-Aldrich Co*) in a dose of 250 mg/kg body weight. Male mice were injected into the penile vein under inhalation anesthesia using Enflurane, while female mice underwent tail vein injection under Hypnorm™ anesthesia (*Janssen Pharmaceutica*). An animal was considered diabetic if its blood glucose level exceeded 20 mmol/l (>360 mg/dl) for 2 or more consecutive days.

3.1.6 Transplantation models

3.1.6.1 Islet transplantation

Islet transplantations were done 3 days after the streptozotocin injection under anesthesia using Enflurane in Paper II and III, and Hypnorm™ (*Janssen Pharmaceutica*) in Paper IV. The islets were packed in a 24 G venflon (*Becton, Dickinson and Company, NJ, USA*) on a Hamilton syringe. Free islets were transplanted under the kidney capsule. In Paper IV, evaluating the risk for sensitization, free islets were also transplanted subcutaneously into the back of the animals. For

encapsulation of islets, immunoprotective chambers were implanted in a subcutaneous pocket on the back of the animals. For fresh implantation, islets were inserted *in vitro* into the device's lumen just before implantation. In animals with preimplanted chambers, an incision was made distal to the port of the chamber, and the cap was removed for insertion of islets. The port was then sealed again with glue and the wound sutured.

3.1.6.2 Heterotopic heart transplantation

To assess the time-to-rejection on a subsequent vascular graft in Paper IV, abdominal heterotopic rat heart allotransplantations were performed using the microsurgical technique described by Ono and Lindsey (*167*). The hearts were obtained from DA rats *(Scanbur BK)* weighing 250-300 g. The animals were anesthetized with Hypnorm™ (*Janssen Pharmaceutica*) and the thoracic cavity of the donor exposed. The abdomen of the recipient was then opened and the infrarenal aorta and vena cava were dissected. The donor hearts were flushed with cold saline solution and the caval and pulmonary veins ligated before removal. The donor aorta was sutured end-to-side to the recipient's infrarenal aorta, and the donor pulmonary artery was sutured end-to-side to the recipient's infrarenal vena cava using 8-0 running sutures. The grafts were observed during the first 30 minutes after reperfusion. The same surgeon performed all operations, and the success rate was 95 %. Evaluation by heart palpation was performed every hour during the first 12 hours, and then twice daily.

3.1.7 Islet recipient management

Nonfasting blood glucose levels and weights of the animals were determined daily during the first week. Thereafter, cured animals were assessed twice a week, while

those with hyperglycemia were checked daily. Animals with a high blood glucose level were given a mixture of short- and long-acting human recombinant insulin (*Actrapid and Ultratard, Novo Nordisk AS, Bagsvaerd, Denmark*) in equal proportions s.c. after the blood glucose measurement. A total of 2 U was given for blood glucose levels of 11-20 mmol/l and of 4 U for levels >20 mmol/l. If the blood glucose level normalized, insulin was discontinued.

An intraperitoneal glucose tolerance test (IPGTT) was done on all cured animals. Before the IPGTT, mice were fasted for at least 6 hours. Glucose (20%) was injected i.p. (10 µl/g) and the blood glucose level determined before injection and then at 5, 10, 15, 20, 30, 45, 60, 90 and 120 minutes after the injection. Healthy mice were used as normal controls and tested simultaneously. After the IPGTT, graftectomy was done to rule out a recurrence of native pancreatic function. The mice were then sacrificed by cervical dislocation. Animals that could not maintain their general condition on insulin treatment due to severe diabetes were sacrificed earlier, in accord with regulations pertaining to animal welfare.

3.1.8 Determination of anti-donor antibodies

In Paper IV, evaluating the risk of sensitization after encapsulated islet transplantation using the TheraCyte™ device, anti-donor antibodies were determined by flow cytometry analysis. For the flow cytometric assay, DA spleen lymphocytes were isolated and used as target cells. The staining procedure was described before (*168*). Briefly, $5x10^5$ DA spleen lymphocytes were incubated with 50 µl of Lewis rat serum for 1 hr at 22 ºC, and then washed 3 times with phosphate-buffered saline (PBS). Ten µl of 1:4 diluted fluoresceinated goat anti-rat IgG (H+L) (*Southern Biotechnology*

Associates Inc., Birmingham, AL, USA) antibodies were added and incubated at 4 ºC on ice in darkness for 25 min. The cells were then washed and analyzed with a Becton Dickinson flow cytometer (*FACSorter, Becton Dickinson, San José, CA, USA)*. A shift in the mean fluorescence of 20 channels in the test sample as compared to the negative control was considered positive (*168*). Serum from a normal nontransplanted Lewis rat served as the negative control.

3.1.9 Histological studies

3.1.9.1 Fixation and preparation

All explanted grafts and native pancreases were immediately fixed in Histofix™ (*Histolab Products AB, Göteborg, Sweden*) or 4% phoshate-buffered formalin and dehydrated. After embedding in paraffin the blocks were cut on a microtome (*Leica Microsystems AB, Sollentuna, Sweden or Rotary Microtome, Microm International, Walldorf, Germany*). The sections of membranes were about 5 µm thick and the ones of graft-bearing kidneys, hearts and pancreases about 3 µm. Routine staining was done with hematoxylin and eosin (H&E). The heart grafts were also stained with Ladewig for detection of fibrin. In Paper III, immunohistochemical staining was performed for the detection of insulin-containing cells (*guinea pig anti-insulin antibody, DAKO, Carpinteria, CA, USA*). All histological evaluations were performed with a light microscope by the same investigator.

3.1.9.2 Counting of vascular profiles

For the counting of vascular structures in Paper I, the membranes that had been implanted for various times in rats were divided longitudinally before embedding. The semiquantitative evaluation of vascular profiles was performed on a central section. Micrographs were taken from a 1 cm long part in the middle of the membranes, on both

membrane surfaces at a final magnification of 325 (i.e., an average of 24 micrographs per membrane). Distances of 15, 25, 50, 75, 100, 200, 300 and 400 µm from the surface of the membrane were marked on the micrographs. Vascular profiles were identified by their shape, the presence of an endothelial cell nucleus at the inner surface of the profile, or by the presence of erythrocytes inside the profile, and were counted within the various distances from the membrane surface. The intraobserver variability was less than 5%, as estimated by repeated evaluations. For studies of vascularization asymmetries between the two sides of the chamber - ie, the side facing the skin and the side facing the muscle - additional membranes were implanted and stained with ink at the skin side at explantation. The amount of vascular profiles along the two sides was then determined semiquantitatively (0, + and ++). Male Sprague Dawley rats (*Scanbur BK*) weighing 200-250 g were used for all membrane implantations in this study. The animals were anesthetized using pentobarbital (*Pentobarbitalnatrium, 60 mg/ml, Apoteket Produktion & Laboratorier, Umeå, Sweden*) in a dose of 0.1 ml/100 g b.w. After explanting the device, animals were sacrificed under anesthesia with CO_2 inhalation.

3.1.9.3 Morphometry of encapsulated islet grafts

Morphometry is a systematic method for determination of tissue volumes from 2-dimensional sections. This method was used in Paper III, to compare graft volumes in preimplanted and fresh implanted chambers. The chambers including grafts were serially sectioned, and sections were taken at a distance of 25 µm for staining with hematoxylin and eosin. Fractional volumes - i.e., volume densities (Vv's) - and absolute volumes were evaluated by morphometry. To calculate the total volume of tissue inside the chamber, digital photos were taken of all tissue-containing sections at 2 times magnification using a light microscope. Thus, about 30-80 printed pictures

from 30-40 sections were evaluated for each membrane. The final magnification of these pictures was 44. The total tissue areas were then calculated by using a semiautomatic interactive image analyzer (*Videoplan, Zeiss, Oberkochen, Germany*) and the total volumes calculated according to Cavalieri´s principle (*169*). To estimate the Vv's of the various tissue components, at least 10 tissue-containing sections were also photographed at 10 times magnification. These sections were taken randomly - i.e., every third section if enough tissue, otherwise every other or all sections - to obtain at least 10 sections with tissue. The photos were printed on paper copies at a final magnification of 220. Point-counting was performed using a square lattice (1 cm) randomly placed over the print-outs. Conventional morphometric principles were then used to estimate Vv's of viable endocrine cells, necrosis and fibrosis. The absolute volumes of each tissue type were then calculated by multiplying the Vv's with the total tissue volumes (*170*). All point-counting was done by one investigator and the intra-observer variation was less than 5%.

3.2 DESIGNS OF THE INDIVIDUAL STUDIES

3.2.1 Paper I

In this study our aim was to determine the minimal relevant distance from the membrane surface within which vessels should be examined histologically. We thought that the vessels of interest in such evaluations should be the ones that can support the encapsulated graft with oxygen and nutrients. Therefore, correlation analyses were performed between various variables describing glucose kinetics inside the device at 1, 2 and 4 weeks and 3 months after implantation, and the number of vascular profiles within 15, 25, 50, 75, 100, 200, 300 and 400 μm from the surface of the membrane.

The glucose recoveries of each membrane device used for the correlation analyses had previously been determined during an IVGTT, using the microdialysis method (Table 3.2.1) (*152*). The 100 minutes of dialysis and the corresponding area-under-the-curve ($AUC_{0\text{-}100}$) were divided into two phases, as the blood glucose reduction showed a biphasic pattern. The first 40 minutes of the IVGTT was defined as the early phase ($AUC_{0\text{-}40}$), and the remaining 60 minutes as the late phase ($AUC_{40\text{-}100}$). The maximum concentration of the dialysate was called C_{max} (mmol/L) and the time-to-peak (TTP, min) was defined as the time taken to reach C_{max}.

Table 3.2.1. Glucose kinetic data inside the TheraCyte™ device during an IVGTT, mean values ± standard deviations (n=7 in each group).* Data from Rafael et al. (*152*). Reproduced, with permission from Cognizant Comm Corp.

Time after implantation	$AUC_{0\text{-}40}$	$AUC_{40\text{-}100}$	$AUC_{0\text{-}100}$	C_{max}	TTP
1 week	140 ± 35	100 ± 34	240 ± 65	4.3 ± 0.9	19 ± 5.6
2 weeks	140 ± 41	120 ± 44	260 ± 76	4.6 ± 1.2	25 ± 6.5
4 weeks	150 ± 13	170 ± 38	320 ± 44	4.7 ± 0.4	23 ± 9.5
3 months	170 ± 21	170 ± 43	340 ± 59	5.8 ± 0.8	18 ± 5.7

* $AUC_{0\text{-}40}$ – area-under-the-curve in the early phase - i.e., the first 40 minutes (mmol / l x min)

$AUC_{40\text{-}100}$ – area-under-the-curve in the late phase - i.e., the remaining 60 minutes (mmol / l x min)

$AUC_{0\text{-}100}$ – area-under-the-curve during 100 minutes of measurements (mmol / l x min)

C_{max} – peak glucose concentration (mmol / l)

TTP – time-to-peak concentration (min)

The longitudinal neovascularization process was also studied, including potential differences in vascularization between the side of the membrane facing the skin and that facing the muscle when implanted subcutaneously. For this purpose, fresh devices were implanted for 1 and 2 weeks and 3 months and marked with ink at the side facing the skin at explantation.

The relations between the number of vascular profiles and glucose kinetics were evaluated by regression analyses. The vascularization process was evaluated by comparing the number of vascular profiles at 1 week with that at 3 months, using the unpaired Student's t-test. Differences were considered significant if $p<0.05$.

3.2.2 Paper II

In this study our hypothesis was that preimplantation of the immunoprotective chamber could lower the curative dose of encapsulated islets. In the first part of the study, we transplanted a suboptimal number of islets - i.e., 375 or 125 islets - in fresh or preimplanted membrane chambers. By doing this, our hypothesis that preimplantation would lower the islet dose required for cure was confirmed. The findings were further strengthened by morphometric evaluations of the islet mass. In the second part of the study we therefore continued with preimplanted devices and reduced the doses to 100, 75 and 50 islets, to determine the lowest curative dose. On follow-up, sustained nonfasting blood glucose levels of ≤10 mmol/l were defined as cure and >10 mmol/l as no cure. At 4 weeks, cured animals underwent an IPGTT followed by graftectomy.

The Chi-Square 2-sided exact test was used to compare the frequencies of cured animals in each group and those of animals requiring insulin during the last 5 days of

observation. Since some animals were sacrificed before the end of the study, blood glucose levels were compared by fitting blood glucose change from baseline for each animal to linear regression models. The mean slopes were then calculated for each group and compared using the Mann-Whitney U-test. The same test was used to compare body weight gains, while data concerning morphometry were compared with the unpaired 2-sided Student's t-test. Differences were considered to be statistically significant if $p<0.05$.

3.2.3 Paper III

The aim of this study was to determine whether exendin-4 treatment improves the metabolic outcome after transplantation of a suboptimal mass of islets in diabetic recipients. Since the effects of exendin-4 on transplanted islets had not been evaluated at that time, we performed this study on free islets, with the intention of doing another study on encapsulated islets if the findings were positive. The study was divided into 3 different parts. First, the islet numbers were reduced from 75 to 50 and then 30, to determine the suboptimal dose - i.e., the dose just between cure and failure (Table 3.2.3). Fifty islets cured diabetic mice in most cases, but 30 islets did not. Partial metabolic control was achieved by transplantation of 35-45 islets (data not shown). On the basis of these findings, 30 islets were used for the two following studies of exendin-4; one evaluating the short-term effects, and the other one long-term effects.

In the short-term study, animals were followed for 8 days. Five groups were studied: 1) 30 islets with exendin-4 treatment (*AnaSpec Inc., San José, CA, USA*); 2) 30 islets without treatment; 3) 75 islets without treatment (quality controls); 4) non-transplanted

diabetic mice treated with exendin-4; and 5) non-transplanted diabetic mice without treatment. Exendin-4 was given as a single i.p. injection of 100 ng (about 24 pmol) per day from day 0 to 7.

Table 3.2.3. Determination of the suboptimal islet number. Reproduced with permission from Springer-Verlag.

	75 islets	50 islets	30 islets
n	4	5	4
Cure	4	4	0
Partial function	0	1	0
No cure	0	0	4

In the long-term study, animals were observed for 4 weeks. Exendin-4 was given for 8 days, as in the short-term study. Three groups were studied: 1) 30 islets with temporary exendin-4 treatment; 2) 30 islets without exendin-4 treatment; and 3) 75 islets without treatment (quality controls). As usual, islets were cultured for 20 hours before transplantation and in group 1, exendin-4 (0.1 nmol/l) was added to the culture medium. At 4 weeks, the cured mice underwent an IPGTT. Thereafter, grafts and native pancreases were removed for histological examinations. One section from each graft and pancreas was evaluated semiquantitatively for comparison of insulin-positive islets.

On follow-up, sustained nonfasting blood glucose levels of 10 mmol/l or below were defined as a cure, 11-20 mmol/l as partial function of transplanted islets and levels above 20 mmol/l were considered as a graft failure. The two-tailed Fisher's exact test

was used for comparisons of the cure rates in each group. Insulin need and body weight gains were compared using the unpaired two-sided Student's t-test. Differences were considered to be statistically significant if $p<0.05$.

3.2.4 Paper IV

In this study, our hypothesis was that the bilayered membrane would prevent sensitization, and that transplantation of encapsulated alloislets should therefore not accelerate the rejection of a subsequent vascularized graft. To test this hypothesis we transplanted a heterotopic heart after the implantation of encapsulated or free islets and determined the time-to-rejection and the development of anti-donor antibodies.

Islets and hearts were obtained from Dark Agouti (DA) rats *(Scanbur BK)* and Lewis rats (200-250 g, *Charles River Laboratories, Sulzfeld, Germany*) were used as the recipients of the grafts. The recipients were divided into 5 groups according to the islet dose and type of transplantation: *1)* no islets, receiving a saline sham injection; *2)* 200 free islets; *3)* 200 encapsulated islets; *4)* 1000 free islets; and *5)* 1000 encapsulated islets. Encapsulated islets 4 weeks after the transplantation are shown in Figure 3.2.4, page 64. The abdominal heterotopic heart transplantations were performed 4 weeks after the transplantation of islets. Time-to-rejection was defined as the cessation of heart contractions, as assessed by heart graft palpation. The various study groups were compared as regards the time-to-rejection using the unpaired Student's t-test.

4 RESULTS AND DISCUSSION

4.1 PAPER I

To improve histological evaluations, we counted vascular profiles at various distances from the membrane surface and correlated these with blood glucose kinetics. Seven devices were evaluated for each time-point. All devices were well vascularized at 3 months (Figure 4.1-1 page 64). Significant increases in the number of vascular profiles from 1 week to 3 months were noted at 50, 75, 100 and 200 µm distances ($p<0.05$) (Table 4.1-1). Within each distance, the number of vascular profiles showed the greatest variations at 2 weeks. At all examinations, the largest variations in vascularization were noted at 15 µm.

The correlations between the number of vascular profiles at various distances and glucose kinetics are shown in Figure 4.1-2. The highest correlations for AUC_{0-40}, AUC_{40-100}, AUC_{0-100} and C_{max} were found at 75 and 100 µm ($p<0.05$). They were significant for AUC_{0-40} from 15 µm to 300 µm, for AUC_{40-100} from 25 µm to 200 µm, for AUC_{0-100} from 25 µm to 300 µm, and for C_{max} from 50 µm to 300 µm. In contrast, the correlations with TTP were significant only at 300 and 400 µm.

Table 4.1-1. Number of vascular profiles within various distances from the surface of the membrane, mean (number of vascular profiles / cm of membrane length) ± standard deviation and coefficient of variation (%). Seven devices were evaluated for each time-point. Reproduced with permission from Cognizant Comm Corp.

Distances	1 week	2 weeks	4 weeks	3 months
15 µm	6.1 ± 4.8 79%	22 ± 32 150 %	35 ± 17 50 %	21 ± 10 48 %
25 µm	18 ± 12 67 %	34 ± 46 140 %	66 ± 21 32 %	44 ± 20 45 %
50 µm	35 ± 20 57 %	56 ± 63 110 %	110 ± 28 26 %	96 ± 28 29 %
75 µm	47 ± 25 53 %	68 ± 74 110 %	130 ± 33 25 %	130 ± 24 19 %
100 µm	64 ± 31 48 %	79 ± 84 110 %	150 ± 33 23 %	150 ± 22 14 %
200 µm	150 ± 62 42 %	150±110 74 %	240 ± 45 19 %	260 ± 43 16 %
300 µm	210 ± 91 44 %	190±120 65 %	280 ± 57 20 %	340 ± 81 24 %
400 µm	360±130 34 %	230±150 68 %	370 ± 83 23 %	410±140 34 %

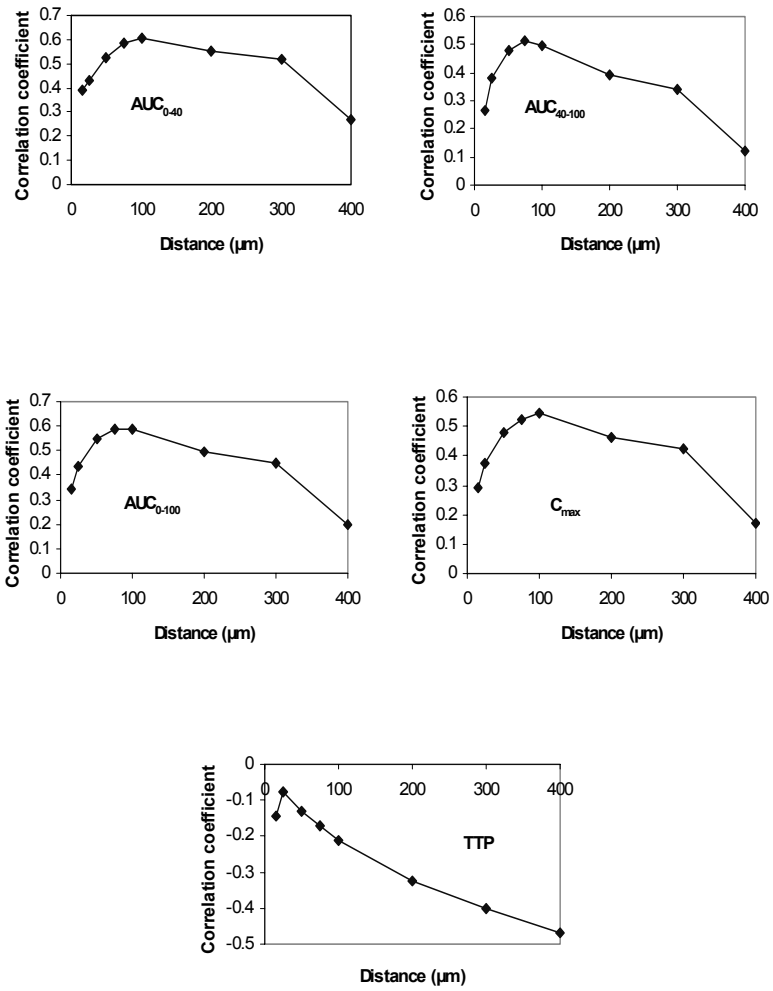
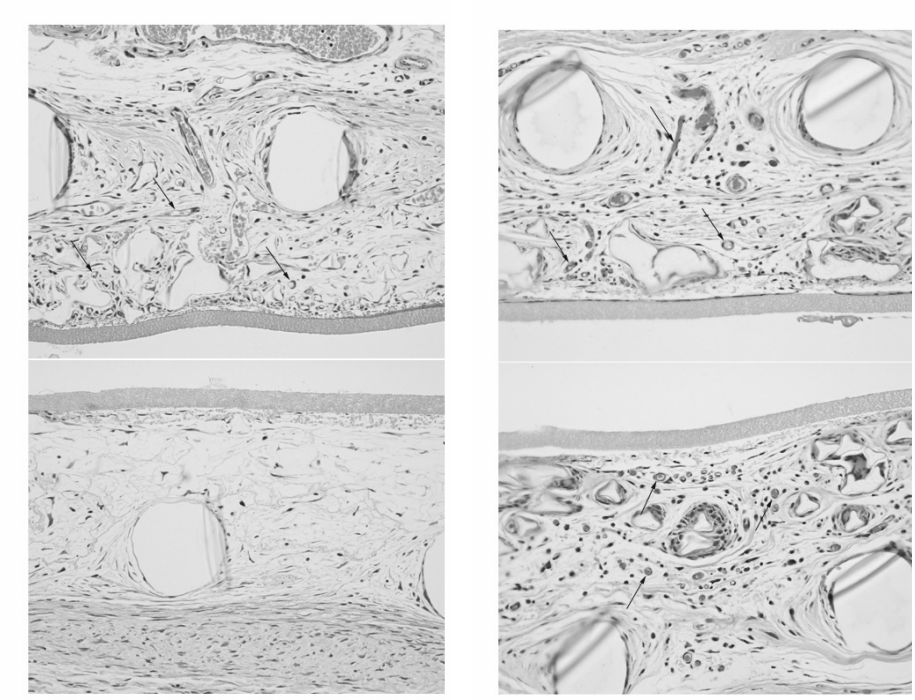


Figure 4.1-2. Correlations between the number of vascular profiles within various distances from the immunoisolation membrane and glucose kinetics, shown by the correlation coefficients at various distances. Reproduced with permission from Cognizant Comm Corp.

When we compared the two sides of the membrane, differences in the degree of membrane vascularization were noted at 1 and 2 weeks. At 1 week, there was a striking difference in the number of vascular profiles between the side facing the skin and that facing the muscle in 4/8 devices, whereas 1 showed a moderate difference. The 3 remaining devices, for which no differences were noted, had few vascular profiles on both sides, unlike those with marked differences, which had more vascular profiles on the side facing the skin (Figure 4.1-3a). The findings were similar at 2 weeks (n=9), but at 3 months (n=10) all devices were well vascularized on both sides (Figure 4.1-3b).



a) **b)**

Figure 4.1-3. a) Device explanted at 1 week. More vascular profiles are seen on the side facing the skin (upper micrograph). **b)** Device explanted at 3 months. The 2 sides of the membrane are more equally vascularized than at 1 week. Stained with H&E, original magnification x20. Reproduced with permission from Cognizant Comm Corp.

4.1.1 Discussion Paper I

The aim of this study was to improve the method for histological evaluations of the biocompatibility and neovascularization of the bilayered membrane device. In summary, the highest correlations between glucose kinetics and the number of vascular profiles were noted at 75 and 100 μm. We believe that the vessels within these distances are important for the diffusion of small molecules, such as glucose, into the device. It seems likely that the vessels capable of supplying nutrition to the device's lumen should be the ones of interest in histological evaluations. Therefore, we suggest that vascular profiles within 100 μm should be the ones examined in evaluations of the vascularity around the bilayered membrane device.

In several previous studies, the number of vascular profiles within 15 μm from the surface of the vascularizing membrane was determined on histological examinations (*145, 150, 151*). However, at times the vascular profiles were more than 100 μm apart from one another. If vessels lying that far apart could sustain the encapsulated cells between them, those further from the membrane surface should also be able do that, which is shown by the findings of this study. We therefore suggest that close vascular structures may improve the transfer of nutrients and oxygen across the membrane, but they are not the only ones to supply the encapsulated graft.

As the goal of islet transplantation is to improve the metabolic control, it is important that the device allows near normal physiological glucose kinetics. The total amount of insulin released corresponds to the AUC, and the peak of the insulin response to C_{max}. TTP is important for the insulin response over time. In contrast to other glucose kinetics, significant correlations between vascular profiles and TTP were noted at 300-

400 µm. We cannot explain this difference, but one reason may be inexact measurements of TTP. Due to the small sample volumes, the dialysate was collected in 5-minute aliquots. Actual values of TTP can be obtained only by making continuous measurements.

In the present study, we also found that the vascularization process seems to begin at the side facing the skin. Differences in the degree of vascularization between the 2 membrane sides were noted up to 4 weeks after implantation, but had largely disappeared at 3 months. It is noteworthy that the time of vascularization varied between different membranes. Particularly at 2 weeks, striking differences were seen in the extent of vascularization between various devices, which is indicated by the high coefficients of variation. These findings together with the ones described above are in accordance with other studies by members of our group, all indicating impaired vascularization during the first month after transplantation and recovery at 3 months (*152-154, 156*).

4.2 PAPER II

The purpose of this study was to evaluate the effects of preimplantation on cure rates, graft survival and islet doses. We began by comparing groups with freshly and preimplanted chambers transplanted with 375 and 125 islets (Table 4.2-1). All animals in the group with 375 islets in preimplanted chambers were cured (n=8), as compared to 1/6 animals in the group with 375 islets in freshly implanted chambers (p=0.003). The corresponding findings in animals transplanted with 125 islets were similar, with 6/6 cured animals in the preimplanted group and no cured animals in the group with freshly implanted chambers (n=7) (p=0.001). The body weight gains of the

preimplanted group with 125 islets significantly exceeded those in the group with freshly implanted devices with 125 islets (p=0.003).

Table 4.2-1. Cure rates in animals with freshly or preimplanted devices.

Group	375 fresh	375 PI	p-value	125 fresh	125 PI	p-value
N	6	8		7	6	
Cure	1	8	0.003	0	6	0.001
No cure	5	0		7	0	
Bw (g)	0.17 ± 3.5	3.1 ± 1.1	ns	-2.9 ± 2.6	3.8 ± 1.6	0.003

PI – preimplanted Bw – body weight gain ns – not significant

Mean blood glucose levels of these 4 groups are shown in Figure 4.2-1. The groups with preimplanted chambers with 375 and 125 islets had significantly greater capacities to reduce the blood glucose levels than the corresponding groups with freshly implanted chambers (p=0.010 and p=0.032, respectively). The 2 preimplanted groups had similar mean blood glucose levels with cure within a week after transplantation, and when compared statistically, no significant difference was noted. In the groups with freshly implanted devices, some animals were euthanized before the end of the study, due to deterioration in their general condition caused by severe diabetes. This partly explains the decrease in mean blood glucose levels over time in the freshly implanted group with 375 islets, as 1 of the 3 remaining animals was cured.

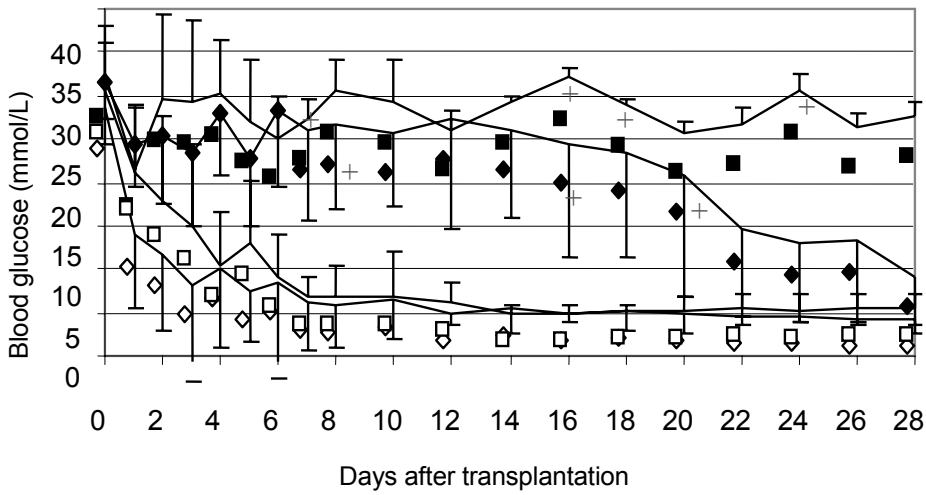


Figure 4.2-1. Mean blood glucose levels with SD. Freshly implanted membrane with 375 islets is indicated by closed diamonds, freshly implanted membrane with 125 islets by closed squares, preimplanted membrane with 375 islets by open diamonds and preimplanted membrane with 125 islets by open squares. Some mice were sacrificed during the observation period because of poor general condition due to severe diabetes. Red + = sacrificed recipients.

The graft survivals of the 125 islet groups were also evaluated by morphometry. The endocrine tissue had significantly higher Vv's in the preimplanted group than in the fresh group ($p<0.001$) (Table 4.2-2). On the other hand, the necrotic tissue Vv's were lower in the preimplanted group ($p=0.026$). When fibrotic tissue Vv's were evaluated no differences could be detected between the two groups.

Table 4.2-2. Volume densities (%), mean ± standard deviation, of the 125 islet groups.

	Freshly implanted (n=5)	Preimplanted (n=5)	p-value
Endocrine tissue	0.117 ± 0.073	0.735 ± 0.144	< 0.001
Necrotic tissue	0.470 ± 0.370	0.016 ± 0.032	0.026
Fibrotic tissue	0.411 ± 0.435	0.202 ± 0.151	ns
Other	0.001 ± 0.003	0.047 ± 0.105	ns

ns – not significant

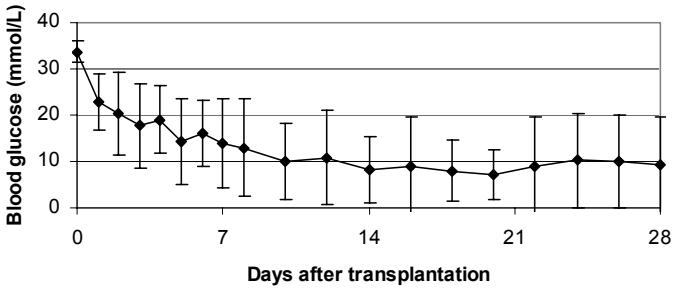
Table 4.2-3 shows the absolute volumes of the 125 islet groups. The endocrine tissue volumes (mm^3) were higher in the preimplanted group than in the fresh group (p=0.035), although the total tissue volumes were similar. On comparison of necrotic and fibrotic tissues in the two groups no statistically significant differences were noted. However, the volumes tended to be lower in the preimplanted devices.

Table 4.2-3. Absolute volumes (mm^3), mean ± standard deviation, of the 125 islet groups.

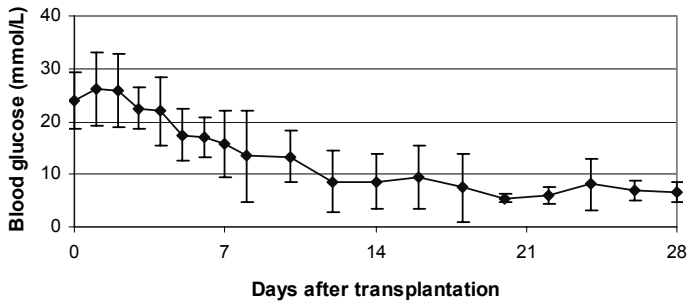
	Fresh (n=5)	Preimplanted (n=5)	p-value
Total tissue	0.151 ± 0.067	0.146 ± 0.114	ns
Endocrine tissue	0.018 ± 0.014	0.095 ± 0.066	0.035
Necrotic tissue	0.066 ± 0.065	0.001 ± 0.001	ns
Fibrotic tissue	0.067 ± 0.085	0.040 ± 0.046	ns
Other	0.000 ± 0.001	0.011 ± 0.023	ns

ns – not significant

(a)



(b)



(c)

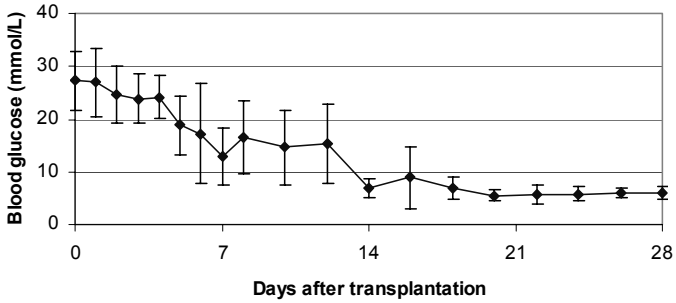


Figure 4.2-2. Mean blood glucose levels with SD. Various numbers of islet cells transplanted into preimplanted devices: (a) 100 islets, (b) 75 islets and (c) 50 islets.

As the cure rates improved after preimplantation in the first part of the study, we continued to reduce the islet doses in preimplanted chambers. The aim was to determine the lowest islet number required for cure. The cure rates of the 100 and 75 islet groups were similar with 7/8 and 6/7 animals cured, respectively. All animals were cured in the 50 islet group (n=6). Figure 4.2-2 shows the blood glucose values of the various groups. No statistically significant difference was noted when the capacity to lower blood glucose in the 100 islet group was compared to that of the preimplanted group with 375 islets from the first part of the study.

The cured animals showed a normal response in the IPGTT, except for the only cured animal in the fresh group transplanted with 375 islets and one animal in the preimplanted group with 125 islets with slightly delayed responses.

4.2.1 Discussion Paper II

The findings of this study indicate that the dose needed for cure using macroencapsulated islets can be reduced by preimplantation of the immunoprotective chamber. The experimental findings are supported by morphometry evaluations which show significantly more endocrine tissue in preimplanted than in freshly implanted membrane chambers.

In our previous studies using freshly implanted devices, 750-1000 islets were needed for a cure. Similar findings were reported by others using the same model (*171*). However, when free islets were transplanted to the renal subcapsular site, 75 islets repeatedly resulted in a cure, and 50 islets cured in most cases (*172*). We thought that the great difference between free and encapsulated islets could be at least partly

ascribed to sub-optimal physiological conditions inside the chamber in the early post-transplant period. Previous studies by members of our group using the TheraCyte™ device supported this hypothesis. When the diffusion of insulin and glucose across the membrane was studied, significantly impairments were noted at 4 weeks after subcutaneous membrane implantation (*152, 153*). However, at 3 months the diffusion had improved and was the same as that on day 1. The microcirculation flow showed the same pattern, with reduced perfusion at 4 weeks and recovery at 2 months, when evaluated by laser Doppler technique (*154*). Moreover, Paper I of this thesis showed that the number of vascular profiles increased between 1 week and 3 months (*173*). The general conclusion of these studies was that the early physiological impairments seem to be largely normalized at three months after implantation. Thereby, it was reasonable to believe that preimplantation of the membranes a few months before transplantation would benefit graft survival. An initial explorative study on preimplanted devices was performed using rats without diabetes. It was evaluated by morphometry and showed significantly higher volumes of viable endocrine cells in preimplanted than in fresh chambers (*155, 156*). However, because of the study design using non-diabetic animals, the effects on the curative dose could not be evaluated. In the present study, we therefore assessed the value of preimplantation, using a model with diabetes, and showed dramatic reduction of the curative islet dose.

Other studies have also suggested preimplantation as a method to improve vascularization before islet transplantation (*171, 174*). One of these studies aimed to induce neovascularization in the peritoneal transplantation site by implantation of a polytetrafluoroethylene solid support (*174*). Four weeks after implantation, non-encapsulated islets were transplanted into the solid support. This approach significantly

improved cure rates in comparison to islets transplanted into the unmodified peritoneal cavity. In another study, using an animal model similar to ours, TheraCyte™ devices (20 µl) were implanted two weeks before loading of 1200 rat islets, and compared with 1200 islets in fresh implanted devices (*171*). The study was designed to cure all animals, therefore the effect of preimplantation was evaluated by the time-to-cure, but no differences were detected. One reason could be that the transplantations were performed too early, before complete development and maturation of new vessels, as indicated by our previous studies (*152-156, 173*).

There are several reasons for reducing the islet dose required for a cure. Considering the limited donor pool it is important to keep the donor-recipient ratio as low as possible. According to the five-year follow-up of the Edmonton group, 44 patients achieved temporal insulin independence with a mean islet dose of 11 900 IEQ/kg, usually from two donors (*54*). In our study, 50 rat islets repeatedly resulted in a cure using preimplanted chambers, while we later have noted that 30 islets do not. We estimate that 50 islets approximately correspond to 100 IEQ in our model. Thus, the islet dose required for a cure using preimplanted devices was about 4000 IEQ/kg, which is less than that in clinical islet transplantation. However, freshly implanted devices required about ten times higher doses, which would not be acceptable in a clinical setting. Another reason for limiting the number of donors is to decrease the risk for sensitization, although Paper IV of this thesis indicate that this risk is low using the TheraCyte™ device (*175*). Moreover, important from a practical point of view is that reduction of the islet dose should also result in reduction of the device size, facilitating the selection of a suitable implantation site.

In summary, we have shown that the curative doses of macroencapsulated and free islet transplantation may be similar, using the preimplantation method. The experimental findings are supported by morphometric evaluations showing significantly more endocrine tissue in preimplanted than in fresh implanted membrane chambers. On the basis of these findings, we believe that preimplantation may be one approach to facilitate the clinical use of macroencapsulation devices.

4.3 PAPER III

In this study we wished to determine whether exendin-4 treatment could improve the metabolic control after islet transplantation. Results of the short-term study, evaluated on day 8, are shown in Table 4.3-1. In the exendin-4 treated group, 63% of the animals showed some function of transplanted islets as compared to 21% in the control group ($p= 0.033$). Five of 16 animals were completely cured in the treated group while 5/16 showed partial graft function. The mean insulin requirement was significantly lower in the treated than in the untreated mice (1.9 ± 1.1 versus 3.2 ± 1.0 U/day, $p=0.001$). The body weight gains of the treated group were significantly higher than in the control group (2.0 ± 1.3 versus 1.1 ± 0.9 g, $p=0.031$). Animals treated with exendin-4 and insulin, but not transplanted, remained diabetic. The histological examinations of pancreases from these exendin-4 treated mice (n=5) were similar to those of diabetic untreated mice (n=4) with no insulin positive cells, except for 1 stained islet in 1 section in the treated group. In pancreases from non-diabetic mice (n=3), more than 15 stained islets were noted per section.

Table 4.3-1. Short-term effects of exendin-4 on transplanted islets. The mean ± standard deviation are given for insulin and weight gain. Reproduced with permission from Springer-Verlag.

	30 islets + exendin-4	30 islets	p
n	16	14	
Cure	5	0	
Partial function	5	3	
Graft function[a]	63%	21%	0.033
Insulin/day (U)	1.9 ± 1.1	3.2 ± 1.0	0.001
Weight gain (g)	2.0 ± 1.3	1.1 ± 0.9	0.031

[a]Graft function= cure + partial function

The findings of the long-term study are shown in Table 4.3-2. At 4 weeks, 7/8 exendin-4 treated animals had graft function as compared to 2/9 in the control group (p=0.015). In the treated groups, 6 animals were cured and 1 showed partial graft function, while 1 animal was cured and 1 had partial function in the control group. All cured animals showed a normal response in the IPGTT, similar to that of normal healthy mice (Figure 4.3-1). The body weight gains in the exendin-4 treated group were significantly greater than in the control group (4.1 ± 1.0 versus 1.6 ± 1.7 g, p =0.001).

Table 4.3-2. Long-term effects of exendin-4 on transplanted islets. The mean ± standard deviation are given for weight gain. Reproduced with permission from Springer-Verlag.

	30 islets + exendin-4	30 islets	p
n	8	9	
Cure	6	1	
Partial function	1	1	
Graft function[a]	87%	22%	0.015
Normal IPGTT	6	1	0.015
Weight gain (g)	4.1 ± 1.0	1.6 ± 1.7	0.001

[a]Graft function = cure + partial function

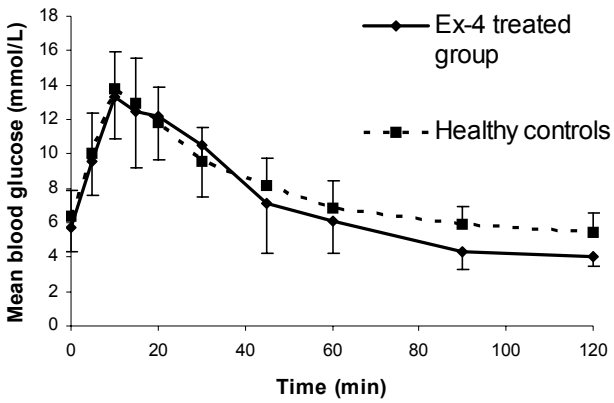


Figure 4.3-1. IPGTT in exendin-4 (Ex-4) treated cured animals and in healthy controls. Reproduced with permission from Springer-Verlag.

Histological examinations of the grafts from cured animals showed prominent, viable, insulin-stained cells under the kidney capsule. Representative insulin-stained grafts from cured (n=3) and non-cured animals (n=3) are shown in Figure 4.3-2, page 64. The pancreas sections from all recipient mice showed few, if any, weakly insulin-stained islets (data not shown).

4.3.1 Discussion Paper III

In the short-term study, when exendin-4 treatment was given throughout the observation period, the treated group obtained significantly better metabolic control than the controls. The findings accord with those of previous studies showing that exendin-4 can cause glucose-dependent stimulation of insulin secretion, suppress glucagon secretion, slow down gastric emptying, inhibit food intake and modulate glucose trafficking in the peripheral tissues (*120*). However, studies have also suggested that exendin-4 can affect the β-cell mass by inhibition of apoptosis, differentiation of islet-like cell clusters and proliferation of existing β-cells (*117-119*). Since the animals were continuously treated with exendin-4, we were unable to distinguish positive effects on β-cell survival from peripheral actions and stimulation of insulin secretion. Therefore, we proceeded with the long-term study, in which animals were again treated for 8 days, but the evaluation was done at 4 weeks. This study showed that the improvements in metabolic control lasted longer than the exendin-4 treatment. We therefore believe that the effects of exendin-4, at least to some extent, are due to improved functional β-cell mass. These experimental findings were further supported by histological evaluations showing more prominent staining of insulin-positive cells in the exendin-4 treated group than in the controls. Likewise, a recent

study also suggested direct effects on the islet graft, because preculture treatment of the islets with exendin-4 significantly shortened the time-to-cure in a syngeneic mouse model (*176*). However, in contrast to our short-term study, no effects of recipient treatment were noted in that study.

Exendin-4 treatment of non-transplanted mice with established streptozotocin-induced diabetes, did not improve the metabolic control. Thus, in this setting, the acute effects of exendin-4 in combinations with insulin were insufficient for control of blood glucose. The findings also indicated that there was no significant regeneration of the native pancreases, which was confirmed by histological evaluations.

The fact that all animals cured by islet transplantation became diabetic after graftectomy, also indicated efficient destruction of the native endocrine pancreases. Moreover, histological examination of the native pancreases from the long-term study showed only a few weakly stained islets as compared to the large numbers of well stained islets in the normal pancreas.

During islet isolation and the peritransplant period, hypoxia, hyperglycemia and inflammatory cytokines may induce islet apoptosis. A study on cultured human islets found that GLP-1 inhibited apoptosis during culture for 5 days (*177*). Another *in vitro* study showed that pretreatment of mouse insulinoma cells with GLP-1 prevented apoptosis, but the drug was unable to rescue already apoptotic cells (*178*). These findings suggest that early graft treatment with GLP-1 or its analogue Exendin-4 is required to prevent apoptosis during and after islet transplantation, which indicates the

importance of *culture treatment*. As previously mentioned, GLP-1 and exendin-4 can increase the β-cell mass through enhanced proliferation and neogenesis (*119*). Thus, it seems likely that *recipient treatment* with exendin-4 after the islet transplantation should increase / preserve the graft mass still more. These two treatment strategies were indeed combined in the long-term study. Although we cannot distinguish their effects from one another, they both probably contributed to the improved metabolic control that lasted after the exendin-4 treatment.

This study indicates that exendin-4 has beneficial effects on the metabolic control after transplantation of a suboptimal number of islets. To evaluate various exendin-4 treatment protocols further, and the relative importance of culture and recipient treatment, we performed another study using the same treatment length and follow-up time as in the long-term study (*179*). The combination of islet preculture and recipient treatment with exendin-4 was better than no treatment in the controls, while preculture alone or recipient treatment alone did not significantly differ from the untreated controls. The combined protocol was then evaluated in animals transplanted with 500 islets in freshly implanted devices. Graft outcomes were again significantly better in the animals treated with exendin-4.

4.4 PAPER IV

The aim of Paper IV was to evaluate the risk of sensitization by islets encapsulated in the TheraCyte™ device. For this purpose, free or encapsulated islets were transplanted in various doses and the time-to-rejection of a subsequent transplanted heterotopic heart was determined. The heart graft survival data are shown in Table 4.4.1. The control animals, with no islets had a mean heart graft survival of 6.4 days (n=10), while the heart graft survivals were significantly shorter in the free islet groups ($p < 0.001$) - i.e.,

4.8 days for the 200 free islets (n=10), and 1.0 day for the 1000 free islets (n=8). In contrast, the encapsulated islet groups had mean heart graft survivals similar to those of the group which had not been transplanted with islets - i.e., 6.4 and 6.0 days for the 200 (n=10) and 1000 (n=8) encapsulated ones, respectively.

Table 4.4-1. Heart graft survival times. Reproduced with permission from Lippincott Williams & Wilkins.

Islet dose	Days of survival	Mean ± SD	p-value vs control
No islets (n=10)	6, 6, 6, 6, 7, 7, 7, 7, 6, 6	6.4 ± 0.5	
200 free (n=10)	3, 5, 5, 4.5, 5.5, 5, 4.5, 5, 5, 5	4.8 ± 0.7	< 0.001
200 encap. (n=10)	6, 6, 7, 6, 6.5, 7.5, 7, 6, 6.5, 5.5	6.4 ± 0.6	1
1000 free (n=8)	0.2, 0.2, 0.3, 0.4, 1, 1, 2, 3	1.0 ± 1.0	< 0.001
1000 encap. (n=8)	5, 6, 6, 6, 6, 6, 6, 7	6.0 ± 0.5	0.1

Encap. - encapsulated

Data from the flow cytometry analyses are given in Table 4.4.2. In samples taken before the heart transplantations, none of the animals in the control group, which had not been previously transplanted with islets (n=8), had anti-DA antibodies. On the contrary, 7/10 animals that had been transplanted with 200 free islets, and all of the 8 animals that had been transplanted with 1000 free islets were positive for anti-DA antibodies before the subsequent transplantations of hearts. In the encapsulated groups, 1/10 which had received 200 islets and 3/8 which had received 1000 islets had

developed anti-DA antibodies. At the time of rejection of the heart graft, all animals had anti-DA antibodies. Two animals had detectable alloreactive antibodies before islet transplantation, of which 1 belonged to the 1000 free and 1 to the 1000 encapsulated islet group. The latter had no detectable antibodies when the analysis was performed after the islet transplantation.

Table 4.4-2. Number of animals with anti-DA antibodies determined by flow cytometry analysis. Reproduced with permission from Lippincott Williams & Wilkins.

Islet dose	Before islet transplantation	Before heart transplantation	At heart rejection
Control, no islets (n=8)	-	-	8
200 free (n=10)	-	7	10
200 encapsulated (n=10)	-	1	10
1000 free (n=8)	1	8	8
1000 encapsulated (n=8)	1	3	8

Histological evaluations of the heart grafts on H&E stained sections showed various degrees of nuclear splitting, edema and interstitial bleeding in all samples. Thrombi were found in a few grafts in each group, confirmed by Ladewig staining. Most grafts had been slightly infiltrated by lymphocytes and neutrophils. A few grafts, especially in the 1000 free islet group, were mainly infiltrated by neutrophils.

4.4.1 Discussion Paper IV

The findings of this study indicate that the bilaminar device can protect against sensitization by alloislets, at least during the first month after transplantation. Heart graft rejections were not accelerated by primary transplantation of 200 or 1000 encapsulated islets, as compared to non-transplanted controls. On the contrary, all heart grafts of animals given free islets were rejected earlier than in the controls ($p < 0.05$). In addition, all animals transplanted with free islets had antibodies before the heart transplantation, whereas that was found in only a few of the animals in the encapsulated groups. It is noteworthy that the graft survivals in the encapsulated groups were not shortened in the animals with antibodies, which will be discussed below.

The immunoprotective membrane used in our studies protects allografts, but usually not xenografts from rejection (*146-148*). Severe local inflammation around the device has been suggested as the mechanism for xenograft rejection (*146*). In allotransplantation, the immune response is mainly induced by the direct antigen presentation pathway, which requires cell-to-cell contact between donor APC (antigen presenting cells) and host T-cells. The pathway can therefore be inhibited by a cell-impermeable barrier. In contrast, the indirect route plays a major role in the response to xenografts, where small donor peptides are presented to the T-cells by the APC of the host (*88, 146, 164-166*). However, the indirect route is also thought to play a role in allograft recognition. The peptides are then primarily derived from donor-MHC, as these molecules differ most between individuals of the same species. Since the immunoprotective membrane permits diffusion of nutrients and insulin, small peptides can probably be shed across the membrane to be presented on host-APC. In that sense, it seems more appropriate to use the term “immunoprotection” instead of the previously often used “immunoisolation”. As an indirect evidence of peptide shedding, it has been

shown that CD4+ T-cells that recognize foreign peptides on MHC class II are required for the rejection of xenografts in the bilaminar membrane, but CD8+ T-cells, NK-cells and B-cells are not (*147*).

Theoretically, sensitization by encapsulated alloislets should also be possible. However, no antibodies were detected in most of the animals given encapsulated islets in this study. Since only the allo-peptides of MHC-origin can induce a T-cell response via host-APC, the response was probably too weak to cause antibody production of detectable levels. This accords with the findings of Shiroki et al. who transplanted encapsulated human alloislets in hollow fibers to patients with diabetes (*180*). They found higher levels of alloreactive antibodies only in patients who had been presensitized to a specific HLA and then given a transplantation of islets sharing the same HLA. Thus, the preformed antibodies probably contributed to induce a stronger immune response. However, it cannot be ruled out that a larger islet dose could have triggered a primary response. In our study, the shorter survivals of the heart grafts after primary transplantation of 1000 than of 200 free islets indicate that there may be a correlation between the strength of the immune response and the islet dose. It is also important to consider the time of antibody detection and subsequent graft transplantation. In Shiroki's study, the grafts were explanted at 2 weeks, which may have been long enough to induce a secondary, but not a primary immune response (*180*). In our study, we transplanted the hearts 4 weeks after the islets, and cannot rule out that sensitization may occur later. However, in Tibell's 1-year study of encapsulated parathyroid tissue, no antibodies developed (*149*). Therefore, it seems possible that the bilaminar membrane can provide long-term protection against a humoral response.

Several studies on the humoral response to encapsulated implants have analyzed the effects of antibodies on the encapsulated graft (*147, 181*). However, it is also clinically relevant to switch the perspective and evaluate the effects of antibodies on a subsequent non-encapsulated graft. This has been done in a different setting using microencapsulated xenoislets, and the survival time of a subsequent skin graft as evaluation criteria (*182*). In that setting, the subsequent skin graft was rejected faster than a primary skin graft, indicating that the microcapsules could not prevent sensitization by xenoislets. For our sensitization studies on the bilayered membrane, we used a subsequent vascularized graft, to resemble the clinical situation of a diabetic patient undergoing islet transplantation and later needing a renal graft. Transplantation of a heterotopic heart is also a feasible method for evaluation of the time-to-rejection, as the cessation of heart contractions constitutes a definite end-point. Histological examinations in our study confirmed that all the heart grafts were rejected by acute cellular or hyperacute rejection (*183-185*). Most of the grafts in the 1000 free islet group showed more typical features of hyperacute rejection, but the hearts of both the encapsulated and control groups had mainly features of acute cellular rejection.

A few animals with encapsulated islets developed alloreactive antibodies but still did not show an accelerated heart graft rejection. It may be due to differences between the various immunoglobulin isotypes in activating complement followed by rejection. Other reasons could be differences in titers and affinities of the antibodies, as well as the antibody specificity, since the FACS analyses were done on spleen cells. If the antigens recognized on the spleen cells are not expressed on the endothelial cells in heart tissue the antibodies will not be able to cause rejection of the heart graft. Two animals showed alloreactive antibodies before the islet transplantation, possibly due to a previous infection causing a cross reaction.

In this study, we conclude that the bilaminar membrane protects against sensitization and prevents accelerated rejection of a subsequently transplanted vascularized graft. However, heart transplantations were performed only 4 weeks after the encapsulated islet transplantation. Before studies are extended to humans, long-term studies on antibody development and antibody specificity may be valuable for further risk analyses.

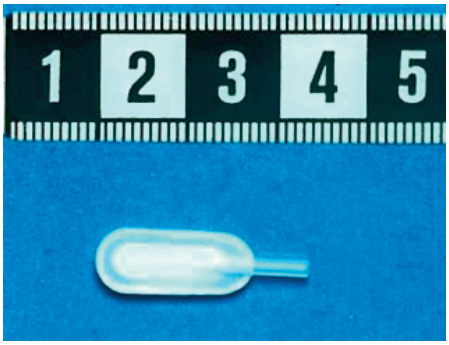


Figure 3.1.3-1. The TheraCyte™ device with a lumen volume of 4.5 µm.

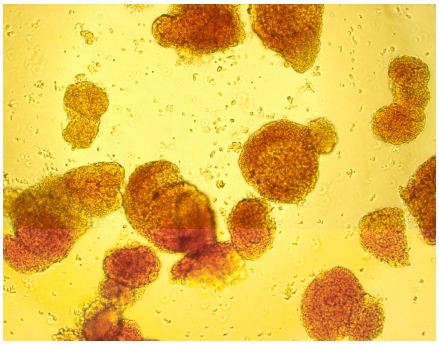


Figure 3.1.4-1. Islet preparation from DA rats, original magnification x40.

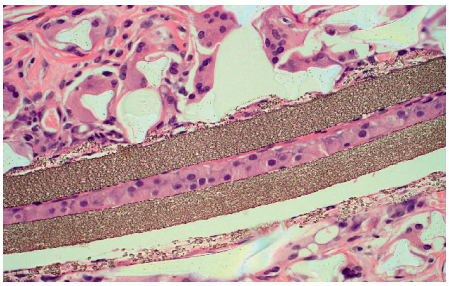


Figure 3.2.4. Encapsulated rat islets 4 weeks after transplantation, original magnification x40.

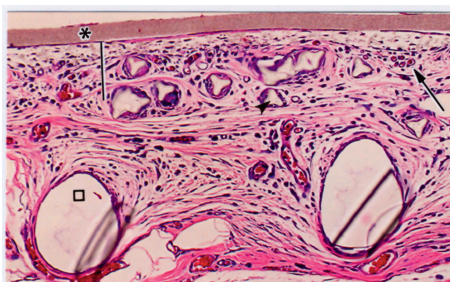
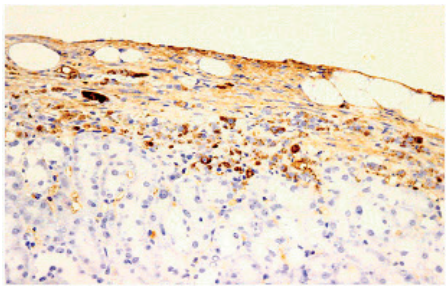
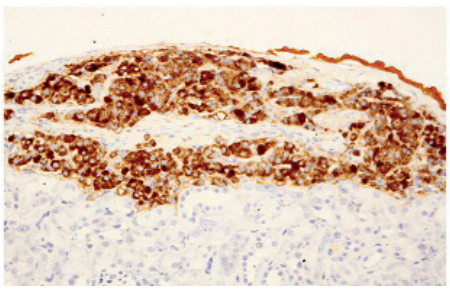


Figure 4.1-1. Device explanted at 3 months. Arrow: cluster of vascular profiles close to the vascularizing membrane; bar: distance of 100 µm from the immunoprotective membrane (asterisk); arrowhead: inner mesh; square: outer mesh. Original magnification x20.



(a)



(b)

Figure 4.3-2. Immunohistochemical staining for insulin from a non-treated animal **(a)** and an exendin-4 treated animal **(b)** showing positive cells (brown colour) under the kidney capsule (original magnification x20).

5 CONCLUSIONS

- The number of vascular profiles within 100 µm of the immunoprotective membrane showed the highest correlations with glucose kinetics. We therefore suggest that these vessels should be considered in histological evaluations of vascularization.

- Preimplantation of the device resulted in higher cure rates when compared to similar islet numbers placed in freshly implanted devices.

- Preimplantation of the device reduced the number of islets needed for cure to that required when transplanting non-encapsulated islets under the kidney capsule.

- Treatment with exendin-4 significantly improved the metabolic control after non-encapsulated islet transplantation. The benefit lasted longer than the treatment period which suggests that exendin-4 had positive effects on the functional graft mass.

- Encapsulation of the islet graft protected the recipients against sensitization, at least during the early posttransplant period.

6 FUTURE PROSPECTS

The overall aim of this thesis was to assess various strategies to improve the survival of macroencapsulated islet grafts. We evaluated 2 main approaches: *i)* optimization of the handling of the membrane device and *ii)* improvement of islet robustness.

Paper I and II, concerned the handling of the device. Preimplantation of the device proved to be an efficient method for increasing the survival of the encapsulated islets and reducing the dose needed for cure. This effect was probably related to the neovascularization of the device surface. Studies that aim at hastening the vascularization process - e.g. by local VEGF-treatment - would be of interest. Histological evaluations in such studies should include the examination of vessels within 100 μm of the membrane surface.

The findings in Paper III and in later studies (*179*) indicate that exendin-4 treatment improves the metabolic control after islet transplantation. More studies of exendin-4 are needed to determine whether longer periods of treatment of the recipient or pretreatment of the donor can further improve the results. We also plan to assess the combination of preimplantation and exendin-4 treatment.

It was recently shown that the risk for sensitization after islet transplantation is greater than previously anticipated (*58*). This is of significant importance since a number of the potential islet graft recipients later will require renal transplantation. Our findings in Paper IV indicate that macroencapsulation may help to reduce the risk of host sensitization. However, our observation period was limited to 4 weeks. It is therefore

important to determine whether the membrane can protect against recipient sensitization also for longer periods.

Another aspect that requires further evaluation is whether the metabolic control will be fully normalized by transplantation of islets in the device. The diffusion barrier created by the membrane as well as the ectopic placement in the s.c. tissue may affect the efficacy of the graft. Theoretically, delayed insulin responses, insufficient blood glucose control but also post-prandial hypoglycemia may occur.

So far, our studies have mainly been performed in rodent models. It is now important to verify that our strategies to improve outcomes after encapsulated islet transplantation are efficient also when human islets are placed in the device. Hopefully, we can then proceed to limited clinical trials to explore the efficacy and safety of macroencapsulated human islet grafts in diabetic patients.

7 ACKNOWLEDGMENTS

Financial support for this thesis was received from Karolinska Institutet, the Swedish Research Council (project number 52120016460), Swedish Medical Association, Stockholm County Council (project numbers 20050448 and 20060560710) and the Foundation for International Surgical Cooperation.

I would like to express my sincere gratitude to all people that gave me support during the work with thesis, especially to:

Annika Tibell, my supervisor, for teaching me a scientific approach, for bright ideas and for endless enthusiasm over my work! I admire you as both a researcher and private person!

Annika Wernerson, my co-supervisor, for guiding me through the world of pathology, and making me realize that it is never simple! I really value our friendship!

Annika & Annika, for being such a perfect combination of supervisors!

Professor Bo-Göran Ericzon and Professor Emeritus Carl-Gustav Groth, for accepting me as a PhD student in your department!

Makiko Kumagai-Braesch, for everything! Without you these studies would never have been finished!

Ehab Rafael, for co-authorship and enthusiastic sharing of your work!

Guosheng Wu, for co-authorship and teaching me the islet isolation method!

Amit Sharma, for co-authorship, fruitful discussions and enormous help with animal care!

Kjell Hultenby, for co-authorship, and showing me the "real" membrane with SEM!

Suchitra Sumitran-Holgersson, for co-authorship and enlightening immunology discussions!

Suad Efendic, for co-authorship and the introduction to exendin-4!

Shunsheng Zhu, for co-authorship and work with heart transplantations!

Anneli Hansson, for skilful sectioning of all the hard membranes at any time!

Katarina Fernström, for well organized administrative work on many unorganized occasions!

Bo Nilsson, for statistical advice and analyses, always within tight deadlines!

Zoe Walsh for excellent English corrections!

TheraCyte™ Inc. and especially Tom Loudovaris, for kindly providing the immunoprotective devices used in this thesis!

Silvia Nava, for helping me during a tough period when studies did not proceed as wished!

Xiaohui Jia for islet isolations and animal care!

Marie Larsson, for improving the layouts and illustrations!

Henryk Wilczek, for transferring the data from a worn-out Mac to my PC!

Aina Östman, Berit Sundberg, Ingrid Lindell and Yodit Kassaye, for help with small, but usually urgent things!

The staff in the Animal Department, for good advice about handling of animals!

All the other people in the Department of Transplantation, Huddinge, for helping me in some way!

My new colleagues in Malmö in the Department of Urology and the Department of Surgery, for understanding my somewhat reduced focus this year!

All friends and family, You are a treasure!

My AT-friends, Anna and Peter, I really enjoyed your company and our discussions on what to do "afterwards".

My Med School friends, Anna, Anna, Britta, Frida and Karin, I miss you. Remember mid November, although I'll spoil it this year! Sahar, you are a fantastic person, who gives me insight into other options in life.

My friends from LTH, Andrea, Anna, Malin, Catarina, Jenny and Anna, the side-track I took via a degree in Chemical Engineering was definitively worth it. Otherwise I wouldn't have known you!

My high school friends, Lotta, Åsa, Jessica, Helena and Emma, thanks for all the great fun times, and continuing friendship with you and your families. An extra thanks to Bjarne, for urgent photo processing when I submitted Paper IV.

My friends from Bandhagen, Camilla, Robin, Eva and Tobias with children, you were like family when we lived in Stockholm! Together with Helena and Peter, you contributed to this work, by taking care of our children and letting me stay with you during my Stockholm visits.

My oldest friends, Malin, whom I've been in touch with for almost 30 years. I can contact you whenever I wish and always feel comfortable with you. Madde, my travel and party mate for many years. I still enjoy your company, although the focus of our lives has changed!

My father- and mother-in-law, Lars and Birgitta, thanks for Andreas and for the care of our family!

My grandmother Stina, the pride you express is mutual!

My brothers, Ola and Max with family, your friendship is invaluable!

My parents, Eva and Nisse, mamma och pappa, you've made my life so easy!

My husband, Andreas, you are unique. Jag älskar dig!

My children, Saga och Lova, ingenting går upp mot er!

My baby, your kicking will comfort me during the dissertation. Längtar efter Dig!

8 REFERENCES

1. Johnsson C TG. Transplantation. Lund: Studentlitteratur, 2002.
2. Hermann R, Knip M, Veijola R, et al. Temporal changes in the frequencies of HLA genotypes in patients with Type 1 diabetes--indication of an increased environmental pressure? Diabetologia 2003; 46 (3): 420.
3. Gillespie KM, Bain SC, Barnett AH, et al. The rising incidence of childhood type 1 diabetes and reduced contribution of high-risk HLA haplotypes. Lancet 2004; 364 (9446): 1699.
4. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care 2004; 27 (5): 1047.
5. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. N Engl J Med 1993; 329 (14): 977.
6. Tajra LC, Martin X, Benchaid M, Dawhara M, Lefrancois N, Dubernard JM. Long-term metabolic control in pancreas transplant patients according to three techniques. Transplant Proc 1998; 30 (2): 268.
7. Tyden G, Tollemar J, Bolinder J. Combined pancreas and kidney transplantation improves survival in patients with end-stage diabetic nephropathy. Clin Transplant 2000; 14 (5): 505.
8. Knoll GA, Nichol G. Dialysis, kidney transplantation, or pancreas transplantation for patients with diabetes mellitus and renal failure: a decision analysis of treatment options. J Am Soc Nephrol 2003; 14 (2): 500.
9. Navarro X, Sutherland DE, Kennedy WR. Long-term effects of pancreatic transplantation on diabetic neuropathy. Ann Neurol 1997; 42 (5): 727.
10. Solders G, Tyden G, Tibell A, Persson A, Groth CG. Improvement in nerve conduction 8 years after combined pancreatic and renal transplantation. Transplant Proc 1995; 27 (6): 3091.
11. Wilczek HE, Jaremko G, Tyden G, Groth CG. Pancreatic graft protects a simultaneously transplanted kidney from developing diabetic nephropathy: a 1- to 6-year follow-up study. Transplant Proc 1993; 25 (1 Pt 2): 1314.
12. Fioretto P, Sutherland DE, Najafian B, Mauer M. Remodeling of renal interstitial and tubular lesions in pancreas transplant recipients. Kidney Int 2006; 69 (5): 907.
13. Fioretto P, Steffes MW, Sutherland DE, Goetz FC, Mauer M. Reversal of lesions of diabetic nephropathy after pancreas transplantation. N Engl J Med 1998; 339 (2): 69.
14. Giannarelli R, Coppelli A, Sartini MS, et al. Pancreas transplant alone has beneficial effects on retinopathy in type 1 diabetic patients. Diabetologia 2006; 49 (12): 2977.
15. Koznarova R, Saudek F, Sosna T, et al. Beneficial effect of pancreas and kidney transplantation on advanced diabetic retinopathy. Cell Transplant 2000; 9 (6): 903.
16. Pearce IA, Ilango B, Sells RA, Wong D. Stabilisation of diabetic retinopathy following simultaneous pancreas and kidney transplant. Br J Ophthalmol 2000; 84 (7): 736.
17. Humar A, Ramcharan T, Kandaswamy R, Gruessner RW, Gruessner AC, Sutherland DE. Technical failures after pancreas transplants: why grafts fail and the risk factors--a multivariate analysis. Transplantation 2004; 78 (8): 1188.
18. Gray DW, McShane P, Grant A, Morris PJ. A method for isolation of islets of Langerhans from the human pancreas. Diabetes 1984; 33 (11): 1055.
19. Ballinger WF, Lacy PE. Transplantation of intact pancreatic islets in rats. Surgery 1972; 72 (2): 175.

20. Ricordi C, Strom TB. Clinical islet transplantation: advances and immunological challenges. Nat Rev Immunol 2004; 4 (4): 259.
21. Gotoh M, Maki T, Kiyoizumi T, Satomi S, Monaco AP. An improved method for isolation of mouse pancreatic islets. Transplantation 1985; 40 (4): 437.
22. Ohzato H, Porter J, Monaco AP, Montana E, Maki T. Minimum number of islets required to maintain euglycemia and their reduced immunogenicity after transplantation into diabetic mice. Transplantation 1993; 56 (2): 270.
23. Wennberg L, Song Z, Rydgard KJ, et al. Experience with 450 adult porcine islet isolation procedures. Transplant Proc 2001; 33 (4): 2537.
24. van Suylichem PT, Strubbe JH, Houwing H, Wolters GH, van Schilfgaarde R. Insulin secretion by rat islet isografts of a defined endocrine volume after transplantation to three different sites. Diabetologia 1992; 35 (10): 917.
25. Carlsson PO, Palm F, Andersson A, Liss P. Markedly decreased oxygen tension in transplanted rat pancreatic islets irrespective of the implantation site. Diabetes 2001; 50 (3): 489.
26. Lau J, Mattsson G, Carlsson C, et al. Implantation site-dependent dysfunction of transplanted pancreatic islets. Diabetes 2007; 56 (6): 1544.
27. Bennet W, Groth CG, Larsson R, Nilsson B, Korsgren O. Isolated human islets trigger an instant blood mediated inflammatory reaction: implications for intraportal islet transplantation as a treatment for patients with type 1 diabetes. Ups J Med Sci 2000; 105 (2): 125.
28. Eich T, Eriksson O, Lundgren T. Visualization of early engraftment in clinical islet transplantation by positron-emission tomography. N Engl J Med 2007; 356 (26): 2754.
29. Eich T, Eriksson O, Sundin A, et al. Positron Emission Tomography: A real-time tool to quantify early islet engraftment in a pre-clinical large animal model. Transplantation in press.
30. Johansson H, Goto M, Dufrane D, et al. Low molecular weight dextran sulfate: a strong candidate drug to block IBMIR in clinical islet transplantation. Am J Transplant 2006; 6 (2): 305.
31. Cabric S, Sanchez J, Lundgren T, et al. Islet surface heparinization prevents the instant blood-mediated inflammatory reaction in islet transplantation. Diabetes 2007; 56 (8): 2008.
32. Paraskevas S, Duguid WP, Maysinger D, Feldman L, Agapitos D, Rosenberg L. Apoptosis occurs in freshly isolated human islets under standard culture conditions. Transplant Proc 1997; 29 (1-2): 750.
33. Vajkoczy P, Menger MD, Simpson E, Messmer K. Angiogenesis and vascularization of murine pancreatic islet isografts. Transplantation 1995; 60 (2): 123.
34. Mattsson G, Jansson L, Carlsson PO. Decreased vascular density in mouse pancreatic islets after transplantation. Diabetes 2002; 51 (5): 1362.
35. Carlsson PO, Liss P, Andersson A, Jansson L. Measurements of oxygen tension in native and transplanted rat pancreatic islets. Diabetes 1998; 47 (7): 1027.
36. Olsson R, Maxhuni A, Carlsson PO. Revascularization of transplanted pancreatic islets following culture with stimulators of angiogenesis. Transplantation 2006; 82 (3): 340.
37. Corbett JA, Sweetland MA, Wang JL, Lancaster JR, Jr., McDaniel ML. Nitric oxide mediates cytokine-induced inhibition of insulin secretion by human islets of Langerhans. Proc Natl Acad Sci U S A 1993; 90 (5): 1731.
38. Kaufman DB, Naidu Y, Norman JG, et al. Functional significance of donor islet interleukin-1 receptor type 1 (IL-1Rt1) expression in islet transplantation. Transplant Proc 1997; 29 (1-2): 772.
39. Langrehr JM, White DA, Hoffman RA, Simmons RL. Macrophages produce nitric oxide at allograft sites. Ann Surg 1993; 218 (2): 159.
40. Kenmochi T, Miyamoto M, Mullen Y. Protection of mouse islet isografts from nonspecific inflammatory damage by recipient treatment with nicotinamide and 15-deoxyspergualin. Cell Transplant 1996; 5 (1): 41.
41. Sandberg JO, Eizirik DL, Sandler S, Tracey DE, Andersson A. Treatment with an interleukin-1 receptor antagonist protein prolongs mouse islet allograft survival. Diabetes 1993; 42 (12): 1845.

42. Biarnes M, Montolio M, Nacher V, Raurell M, Soler J, Montanya E. Beta-cell death and mass in syngeneically transplanted islets exposed to short- and long-term hyperglycemia. Diabetes 2002; 51 (1): 66.
43. Juang JH, Bonner-Weir S, Wu YJ, Weir GC. Beneficial influence of glycemic control upon the growth and function of transplanted islets. Diabetes 1994; 43 (11): 1334.
44. Federici M, Hribal M, Perego L, et al. High glucose causes apoptosis in cultured human pancreatic islets of Langerhans: a potential role for regulation of specific Bcl family genes toward an apoptotic cell death program. Diabetes 2001; 50 (6): 1290.
45. Ricordi C, Lacy PE, Finke EH, Olack BJ, Scharp DW. Automated method for isolation of human pancreatic islets. Diabetes 1988; 37 (4): 413.
46. Goto M, Eich TM, Felldin M, et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation 2004; 78 (9): 1367.
47. Nano R, Clissi B, Melzi R, et al. Islet isolation for allotransplantation: variables associated with successful islet yield and graft function. Diabetologia 2005; 48 (5): 906.
48. Scharp DW, Lacy PE, Santiago JV, et al. Insulin independence after islet transplantation into type I diabetic patient. Diabetes 1990; 39 (4): 515.
49. Bretzel RG, Brandhorst D, Brandhorst H, et al. Improved survival of intraportal pancreatic islet cell allografts in patients with type-1 diabetes mellitus by refined peritransplant management. J Mol Med 1999; 77 (1): 140.
50. Bretzel RG. Current status and perspectives in clinical islet transplantation. J Hepatobiliary Pancreat Surg 2000; 7 (4): 370.
51. Shapiro AM, Lakey JR, Ryan EA, et al. Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. N Engl J Med 2000; 343 (4): 230.
52. Shapiro AM, Ricordi C, Hering BJ, et al. International trial of the Edmonton protocol for islet transplantation. N Engl J Med 2006; 355 (13): 1318.
53. Bretzel RG, Jahr H, Eckhard M, Martin I, Winter D, Brendel MD. Islet cell transplantation today. Langenbecks Arch Surg 2007; 392 (3): 239.
54. Ryan EA, Paty BW, Senior PA, et al. Five-year follow-up after clinical islet transplantation. Diabetes 2005; 54 (7): 2060.
55. Rickels MR, Schutta MH, Markmann JF, Barker CF, Naji A, Teff KL. {beta}-Cell function following human islet transplantation for type 1 diabetes. Diabetes 2005; 54 (1): 100.
56. Robertson RP, Lanz KJ, Sutherland DE, Kendall DM. Prevention of diabetes for up to 13 years by autoislet transplantation after pancreatectomy for chronic pancreatitis. Diabetes 2001; 50 (1): 47.
57. Shapiro AM, Gallant HL, Hao EG, et al. The portal immunosuppressive storm: relevance to islet transplantation? Ther Drug Monit 2005; 27 (1): 35.
58. Campbell PM, Senior PA, Salam A, et al. High risk of sensitization after failed islet transplantation. Am J Transplant 2007; 7 (10): 2311.
59. Jindal RM, Sidner RA, McDaniel HB, Johnson MS, Fineberg SE. Intraportal vs kidney subcapsular site for human pancreatic islet transplantation. Transplant Proc 1998; 30 (2): 398.
60. Anneren C. [Growth of insulin-producing beta cells. Perhaps a new way to treat type 1 diabetes]. Lakartidningen 2002; 99 (21): 2394.
61. Bonner-Weir S, Sharma A. Pancreatic stem cells. J Pathol 2002; 197 (4): 519.
62. Groth CG, Korsgren O, Tibell A, et al. Transplantation of porcine fetal pancreas to diabetic patients. Lancet 1994; 344 (8934): 1402.
63. Heneine W, Tibell A, Switzer WM, et al. No evidence of infection with porcine endogenous retrovirus in recipients of porcine islet-cell xenografts. Lancet 1998; 352 (9129): 695.
64. Groth CG. Prospects in xenotransplantation: a personal view. Transplant Proc 2007; 39 (3): 685.
65. Gupta RK. Opportunistic infections in renal allograft recipients. Transplant Proc 2007; 39 (3): 731.

66. Fishman JA, Rubin RH. Infection in organ-transplant recipients. N Engl J Med 1998; 338 (24): 1741.
67. Villeneuve PJ, Schaubel DE, Fenton SS, Shepherd FA, Jiang Y, Mao Y. Cancer incidence among Canadian kidney transplant recipients. Am J Transplant 2007; 7 (4): 941.
68. Vajdic CM, McDonald SP, McCredie MR, et al. Cancer incidence before and after kidney transplantation. Jama 2006; 296 (23): 2823.
69. Placebo-controlled study of mycophenolate mofetil combined with cyclosporin and corticosteroids for prevention of acute rejection. European Mycophenolate Mofetil Cooperative Study Group. Lancet 1995; 345 (8961): 1321.
70. Bumbea V, Kamar N, Ribes D, et al. Long-term results in renal transplant patients with allograft dysfunction after switching from calcineurin inhibitors to sirolimus. Nephrol Dial Transplant 2005; 20 (11): 2517.
71. Tonshoff B, Hocker B. Treatment strategies in pediatric solid organ transplant recipients with calcineurin inhibitor-induced nephrotoxicity. Pediatr Transplant 2006; 10 (6): 721.
72. Pratschke J, Neuhaus R, Tullius SG, et al. Treatment of cyclosporine-related adverse effects by conversion to tacrolimus after liver transplantation. Transplantation 1997; 64 (6): 938.
73. Singh N, Bonham A, Fukui M. Immunosuppressive-associated leukoencephalopathy in organ transplant recipients. Transplantation 2000; 69 (4): 467.
74. Vincenti F, Friman S, Scheuermann E, et al. Results of an international, randomized trial comparing glucose metabolism disorders and outcome with cyclosporine versus tacrolimus. Am J Transplant 2007; 7 (6): 1506.
75. Groth CG, Backman L, Morales JM, et al. Sirolimus (rapamycin)-based therapy in human renal transplantation: similar efficacy and different toxicity compared with cyclosporine. Sirolimus European Renal Transplant Study Group. Transplantation 1999; 67 (7): 1036.
76. Brattstrom C, Wilczek H, Tyden G, Bottiger Y, Sawe J, Groth CG. Hyperlipidemia in renal transplant recipients treated with sirolimus (rapamycin). Transplantation 1998; 65 (9): 1272.
77. Dittrich E, Schmaldienst S, Soleiman A, Horl WH, Pohanka E. Rapamycin-associated post-transplantation glomerulonephritis and its remission after reintroduction of calcineurin-inhibitor therapy. Transpl Int 2004; 17 (4): 215.
78. Senior PA, Zeman M, Paty BW, Ryan EA, Shapiro AM. Changes in renal function after clinical islet transplantation: four-year observational study. Am J Transplant 2007; 7 (1): 91.
79. Drachenberg CB, Klassen DK, Weir MR, et al. Islet cell damage associated with tacrolimus and cyclosporine: morphological features in pancreas allograft biopsies and clinical correlation. Transplantation 1999; 68 (3): 396.
80. Redmon JB, Olson LK, Armstrong MB, Greene MJ, Robertson RP. Effects of tacrolimus (FK506) on human insulin gene expression, insulin mRNA levels, and insulin secretion in HIT-T15 cells. J Clin Invest 1996; 98 (12): 2786.
81. Bell E, Cao X, Moibi JA, et al. Rapamycin has a deleterious effect on MIN-6 cells and rat and human islets. Diabetes 2003; 52 (11): 2731.
82. Zhang N, Su D, Qu S, et al. Sirolimus is associated with reduced islet engraftment and impaired beta-cell function. Diabetes 2006; 55 (9): 2429.
83. Desai NM, Goss JA, Deng S, et al. Elevated portal vein drug levels of sirolimus and tacrolimus in islet transplant recipients: local immunosuppression or islet toxicity? Transplantation 2003; 76 (11): 1623.
84. Meier-Kriesche HU, Stegall MD. Should we be performing pancreas transplants? Am J Transplant 2004; 4 (12): 1935.
85. Colton CK. Implantable biohybrid artificial organs. Cell Transplant 1995; 4 (4): 415.
86. de Vos P, Marchetti P. Encapsulation of pancreatic islets for transplantation in diabetes: the untouchable islets. Trends Mol Med 2002; 8 (8): 363.
87. Scharp DW, Mason NS, Sparks RE. Islet immuno-isolation: the use of hybrid artificial organs to prevent islet tissue rejection. World J Surg 1984; 8 (2): 221.

88. Gray DW. Encapsulated islet cells: the role of direct and indirect presentation and the relevance to xenotransplantation and autoimmune recurrence. Br Med Bull 1997; 53 (4): 777.
89. Emerich DF. Encapsulated CNTF-producing cells for Huntington's disease. Cell Transplant 1999; 8 (6): 581.
90. Lysaght MJ, Aebischer P. Encapsulated cells as therapy. Sci Am 1999; 280 (4): 76.
91. Hortelano G, Al-Hendy A, Ofosu FA, Chang PL. Delivery of human factor IX in mice by encapsulated recombinant myoblasts: a novel approach towards allogeneic gene therapy of hemophilia B. Blood 1996; 87 (12): 5095.
92. Prakash S, Chang TM. Microencapsulated genetically engineered live E. coli DH5 cells administered orally to maintain normal plasma urea level in uremic rats. Nat Med 1996; 2 (8): 883.
93. Yanay O, Barry SC, Flint LY, Brzezinski M, Barton RW, Osborne WR. Long-term erythropoietin gene expression from transduced cells in bioisolator devices. Hum Gene Ther 2003; 14 (17): 1587.
94. Geller RL, Loudovaris T, Neuenfeldt S, Johnson RC, Brauker JH. Use of an immunoisolation device for cell transplantation and tumor immunotherapy. Ann N Y Acad Sci 1997; 831: 438.
95. Loudovaris T, Jacobs S, Young S, Maryanov D, Brauker J, Johnson RC. Correction of diabetic nod mice with insulinomas implanted within Baxter immunoisolation devices. J Mol Med 1999; 77 (1): 219.
96. Martinson L, Pauley R, Brauker J. Protection of xenografts with immunoisolation membranes. Cell Transplant 1995; 3: 249.
97. Coleman DL, King RN, Andrade JD. The foreign body reaction: a chronic inflammatory response. J Biomed Mater Res 1974; 8 (5): 199.
98. Anderson JM. Inflammatory response to implants. ASAIO Trans 1988; 34 (2): 101.
99. Woodward SC. How fibroblasts and giant cells encapsulate implants: considerations in design of glucose sensors. Diabetes Care 1982; 5 (3): 278.
100. Tiedge M, Lortz S, Drinkgern J, Lenzen S. Relation between antioxidant enzyme gene expression and antioxidative defense status of insulin-producing cells. Diabetes 1997; 46 (11): 1733.
101. Jorns A, Tiedge M, Lenzen S, Munday R. Effect of superoxide dismutase, catalase, chelating agents, and free radical scavengers on the toxicity of alloxan to isolated pancreatic islets in vitro. Free Radic Biol Med 1999; 26 (9-10): 1300.
102. Enghofer M, Usadel KH, Beck O, Kusterer K. Superoxide dismutase reduces islet microvascular injury induced by streptozotocin in the rat. Am J Physiol 1997; 273 (2 Pt 1): E376.
103. Tiedge M, Lortz S, Munday R, Lenzen S. Protection against the co-operative toxicity of nitric oxide and oxygen free radicals by overexpression of antioxidant enzymes in bioengineered insulin-producing RINm5F cells. Diabetologia 1999; 42 (7): 849.
104. Giovagnoli S, Luca G, Casaburi I, et al. Long-term delivery of superoxide dismutase and catalase entrapped in poly(lactide-co-glycolide) microspheres: in vitro effects on isolated neonatal porcine pancreatic cell clusters. J Control Release 2005; 107 (1): 65.
105. Yamada K, Nonaka K, Hanafusa T, Miyazaki A, Toyoshima H, Tarui S. Preventive and therapeutic effects of large-dose nicotinamide injections on diabetes associated with insulitis. An observation in nonobese diabetic (NOD) mice. Diabetes 1982; 31 (9): 749.
106. Melo SS, Arantes MR, Meirelles MS, Jordao AA, Jr., Vannucchi H. Lipid peroxidation in nicotinamide-deficient and nicotinamide-supplemented rats with streptozotocin-induced diabetes. Acta Diabetol 2000; 37 (1): 33.
107. Korsgren O, Andersson A, Sandler S. Pretreatment of fetal porcine pancreas in culture with nicotinamide accelerates reversal of diabetes after transplantation to nude mice. Surgery 1993; 113 (2): 205.

108. Kaufman DB, Gores PF, Field MJ, et al. Effect of 15-deoxyspergualin on immediate function and long-term survival of transplanted islets in murine recipients of a marginal islet mass. Diabetes 1994; 43 (6): 778.
109. Ishizaki N, Zhu Y, Zhang S, et al. Comparison of various lazaroid compounds for protection against ischemic liver injury. Transplantation 1997; 63 (2): 202.
110. Noguchi N, Takahashi M, Tsuchiya J, Yamashita H, Komuro E, Niki E. Action of 21-aminosteroid U74006F as an antioxidant against lipid peroxidation. Biochem Pharmacol 1998; 55 (6): 785.
111. Cosenza CA, Cramer DV, Cunneen SA, Tuso PJ, Wang HK, Makowka L. Protective effect of the lazaroid U74006F in cold ischemia-reperfusion injury of the liver. Hepatology 1994; 19 (2): 418.
112. Xu HS, Stevenson WC, Pruett TL, Jones RS. Donor lazaroid pretreatment improves viability of livers harvested from non-heart-beating rats. Am J Surg 1996; 171 (1): 113.
113. Dupre J, Behme MT, McDonald TJ. Exendin-4 normalized postcibal glycemic excursions in type 1 diabetes. J Clin Endocrinol Metab 2004; 89 (7): 3469.
114. Chen YE, Drucker DJ. Tissue-specific expression of unique mRNAs that encode proglucagon-derived peptides or exendin 4 in the lizard. J Biol Chem 1997; 272 (7): 4108.
115. Eng J, Kleinman WA, Singh L, Singh G, Raufman JP. Isolation and characterization of exendin-4, an exendin-3 analogue, from Heloderma suspectum venom. Further evidence for an exendin receptor on dispersed acini from guinea pig pancreas. J Biol Chem 1992; 267 (11): 7402.
116. Kieffer TJ, McIntosh CH, Pederson RA. Degradation of glucose-dependent insulinotropic polypeptide and truncated glucagon-like peptide 1 in vitro and in vivo by dipeptidyl peptidase IV. Endocrinology 1995; 136 (8): 3585.
117. Urusova IA, Farilla L, Hui H, D'Amico E, Perfetti R. GLP-1 inhibition of pancreatic islet cell apoptosis. Trends Endocrinol Metab 2004; 15 (1): 27.
118. Movassat J, Beattie GM, Lopez AD, Hayek A. Exendin 4 up-regulates expression of PDX 1 and hastens differentiation and maturation of human fetal pancreatic cells. J Clin Endocrinol Metab 2002; 87 (10): 4775.
119. Xu G, Stoffers DA, Habener JF, Bonner-Weir S. Exendin-4 stimulates both beta-cell replication and neogenesis, resulting in increased beta-cell mass and improved glucose tolerance in diabetic rats. Diabetes 1999; 48 (12): 2270.
120. Gutniak M, Orskov C, Holst JJ, Ahren B, Efendic S. Antidiabetogenic effect of glucagon-like peptide-1 (7-36)amide in normal subjects and patients with diabetes mellitus. N Engl J Med 1992; 326 (20): 1316.
121. Nielsen LL, Young AA, Parkes DG. Pharmacology of exenatide (synthetic exendin-4): a potential therapeutic for improved glycemic control of type 2 diabetes. Regul Pept 2004; 117 (2): 77.
122. Nielsen LL, Baron AD. Pharmacology of exenatide (synthetic exendin-4) for the treatment of type 2 diabetes. Curr Opin Investig Drugs 2003; 4 (4): 401.
123. Fineman MS, Bicsak TA, Shen LZ, et al. Effect on glycemic control of exenatide (synthetic exendin-4) additive to existing metformin and/or sulfonylurea treatment in patients with type 2 diabetes. Diabetes Care 2003; 26 (8): 2370.
124. Basta G, Sarchielli P, Luca G, et al. Optimized parameters for microencapsulation of pancreatic islet cells: an in vitro study clueing on islet graft immunoprotection in type 1 diabetes mellitus. Transpl Immunol 2004; 13 (4): 289.
125. van Schilfgaarde R, de Vos P. Factors influencing the properties and performance of microcapsules for immunoprotection of pancreatic islets. J Mol Med 1999; 77 (1): 199.
126. Wang T, Lacik I, Brissova M, et al. An encapsulation system for the immunoisolation of pancreatic islets. Nat Biotechnol 1997; 15 (4): 358.
127. Anilkumar AV, Lacik I, Wang TG. A novel reactor for making uniform capsules. Biotechnol Bioeng 2001; 75 (5): 581.
128. Lim F, Sun AM. Microencapsulated islets as bioartificial endocrine pancreas. Science 1980; 210 (4472): 908.

129. Sun Y, Ma X, Zhou D, Vacek I, Sun AM. Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of microencapsulated porcine islets without immunosuppression. J Clin Invest 1996; 98 (6): 1417.
130. Fritschy WM, de Vos P, Groen H, et al. The capsular overgrowth on microencapsulated pancreatic islet grafts in streptozotocin and autoimmune diabetic rats. Transpl Int 1994; 7 (4): 264.
131. Zekorn TD, Horcher A, Mellert J, et al. Biocompatibility and immunology in the encapsulation of islets of Langerhans (bioartificial pancreas). Int J Artif Organs 1996; 19 (4): 251.
132. de Groot M, Schuurs TA, van Schilfgaarde R. Causes of limited survival of microencapsulated pancreatic islet grafts. J Surg Res 2004; 121 (1): 141.
133. Dufrane D, Steenberghe M, Goebbels RM, Saliez A, Guiot Y, Gianello P. The influence of implantation site on the biocompatibility and survival of alginate encapsulated pig islets in rats. Biomaterials 2006; 27 (17): 3201.
134. De Vos P, De Haan B, Pater J, Van Schilfgaarde R. Association between capsule diameter, adequacy of encapsulation, and survival of microencapsulated rat islet allografts. Transplantation 1996; 62 (7): 893.
135. Calafiore R, Basta G, Luca G, et al. Transplantation of pancreatic islets contained in minimal volume microcapsules in diabetic high mammalians. Ann N Y Acad Sci 1999; 875: 219.
136. Krol S, del Guerra S, Grupillo M, Diaspro A, Gliozzi A, Marchetti P. Multilayer nanoencapsulation. New approach for immune protection of human pancreatic islets. Nano Lett 2006; 6 (9): 1933.
137. Calafiore R, Basta G, Luca G, et al. Standard technical procedures for microencapsulation of human islets for graft into nonimmunosuppressed patients with type 1 diabetes mellitus. Transplant Proc 2006; 38 (4): 1156.
138. Calafiore R, Basta G, Luca G, et al. Microencapsulated pancreatic islet allografts into nonimmunosuppressed patients with type 1 diabetes: first two cases. Diabetes Care 2006; 29 (1): 137.
139. Thanos CG, Calafiore R, Basta G, et al. Formulating the alginate-polyornithine biocapsule for prolonged stability: Evaluation of composition and manufacturing technique. J Biomed Mater Res A 2007; 83 (1): 216.
140. Orive G, Hernandez RM, Gascon AR, et al. Cell encapsulation: promise and progress. Nat Med 2003; 9 (1): 104.
141. Kizilel S, Garfinkel M, Opara E. The bioartificial pancreas: progress and challenges. Diabetes Technol Ther 2005; 7 (6): 968.
142. Prochorov AV, Tretjak SI, Roudenok VV, Goranov VA. Long-term normalization of diabetes mellitus after xenotransplantation of fetal pancreatic islet cells into the blood stream without immunosuppresive therapy. Transplant Proc 2004; 36 (9): 2855.
143. Pakhomov O, Honiger J, Gouin E, Cariolet R, Reach G, Darquy S. Insulin treatment of mice recipients preserves beta-cell function in porcine islet transplantation. Cell Transplant 2002; 11 (7): 721.
144. Scharp DW, Swanson CJ, Olack BJ, et al. Protection of encapsulated human islets implanted without immunosuppression in patients with type I or type II diabetes and in nondiabetic control subjects. Diabetes 1994; 43 (9): 1167.
145. Brauker JH, Carr-Brendel VE, Martinson LA, Crudele J, Johnston WD, Johnson RC. Neovascularization of synthetic membranes directed by membrane microarchitecture. J Biomed Mater Res 1995; 29 (12): 1517.
146. Brauker J, Martinson LA, Young SK, Johnson RC. Local inflammatory response around diffusion chambers containing xenografts. Nonspecific destruction of tissues and decreased local vascularization. Transplantation 1996; 61 (12): 1671.
147. Loudovaris T, Mandel TE, Charlton B. CD4+ T cell mediated destruction of xenografts within cell-impermeable membranes in the absence of CD8+ T cells and B cells. Transplantation 1996; 61 (12): 1678.
148. Elliott RB, Escobar L, Calafiore R, et al. Transplantation of micro- and macroencapsulated piglet islets into mice and monkeys. Transplant Proc 2005; 37 (1): 466.

149. Tibell A, Rafael E, Wennberg L, et al. Survival of macroencapsulated allogeneic parathyroid tissue one year after transplantation in nonimmunosuppressed humans. Cell Transplant 2001; 10 (7): 591.
150. Padera RF, Colton CK. Time course of membrane microarchitecture-driven neovascularization. Biomaterials 1996; 17 (3): 277.
151. Trivedi N, Steil GM, Colton CK, Bonner-Weir S, Weir GC. Improved vascularization of planar membrane diffusion devices following continuous infusion of vascular endothelial growth factor. Cell Transplant 2000; 9 (1): 115.
152. Rafael E, Wernerson A, Arner P, Wu GS, Tibell A. In vivo evaluation of glucose permeability of an immunoisolation device intended for islet transplantation: a novel application of the microdialysis technique. Cell Transplant 1999; 8 (3): 317.
153. Rafael E, Wernerson A, Arner P, Tibell A. In vivo studies on insulin permeability of an immunoisolation device intended for islet transplantation using the microdialysis technique. Eur Surg Res 1999; 31 (3): 249.
154. Rafael E, Gazelius B, Wu GS, Tibell A. Longitudinal studies on the microcirculation around the TheraCyte immunoisolation device, using the laser Doppler technique. Cell Transplant 2000; 9 (1): 107.
155. Rafael E, Wernerson A, Wu GS, Tibell A. Improved survival of encapsulated islets and reduced fibrosis by preimplantation of the TheraCyte device. Acta Diabetol 1999; 36: 224.
156. Rafael E, Wu GS, Hultenby K, Tibell A, Wernerson A. Improved survival of macroencapsulated islets of Langerhans by preimplantation of the immunoisolating device: a morphometric study. Cell Transplant 2003; 12 (4): 407.
157. Sigrist S, Mechine-Neuville A, Mandes K, et al. Influence of VEGF on the viability of encapsulated pancreatic rat islets after transplantation in diabetic mice. Cell Transplant 2003; 12 (6): 627.
158. Krupetsky A, Parveen Z, Marusich E, Goodrich A, Dornburg R. Retroviral packaging cells encapsulated in TheraCyte immunoisolation devices enable long-term in vivo gene delivery. Front Biosci 2003; 8: a94.
159. Josephs SF, Loudovaris T, Dixit A, Young SK, Johnson RC. In vivo delivery of recombinant human growth hormone from genetically engineered human fibroblasts implanted within Baxter immunoisolation devices. J Mol Med 1999; 77 (1): 211.
160. Carr-Brendel VE, Geller RL, Thomas TJ, et al. Transplantation of cells in an immunoisolation device for gene therapy. Methods Mol Biol 1997; 63: 373.
161. Chou FF, Huang SC, Chen SS, Wang PW, Huang PH, Lu KY. Treatment of osteoporosis with TheraCyte-encapsulated parathyroid cells: a study in a rat model. Osteoporos Int 2006; 17 (6): 936.
162. Qi M, Gu Y, Sakata N, et al. PVA hydrogel sheet macroencapsulation for the bioartificial pancreas. Biomaterials 2004; 25 (27): 5885.
163. Keown PA. The highly sensitized patient: etiology, impact, and management. Transplant Proc 1987; 19 (1 Pt 1): 74.
164. Gill RG. Antigen presentation pathways for immunity to islet transplants. Relevance to immunoisolation. Ann N Y Acad Sci 1999; 875: 255.
165. Gill RG. Use of small animal models for screening immunoisolation approaches to cellular transplantation. Ann N Y Acad Sci 2001; 944: 35.
166. Gray DW. An overview of the immune system with specific reference to membrane encapsulation and islet transplantation. Ann N Y Acad Sci 2001; 944: 226.
167. Ono K, Lindsey ES. Improved technique of heart transplantation in rats. J Thorac Cardiovasc Surg 1969; 57 (2): 225.
168. Xu B, Broome U, Uzunel M, et al. Capillarization of hepatic sinusoid by liver endothelial cell-reactive autoantibodies in patients with cirrhosis and chronic hepatitis. Am J Pathol 2003; 163 (4): 1275.
169. Sterio DC. The unbiased estimation of number and sizes of arbitrary particles using the disector. J Microsc 1984; 134 (Pt 2): 127.
170. Weibel ER. Morphometry of the human lung: the state of the art after two decades. Bull Eur Physiopathol Respir 1979; 15 (5): 999.

171. Tatarkiewicz K, Hollister-Lock J, Quickel RR, Colton CK, Bonner-Weir S, Weir GC. Reversal of hyperglycemia in mice after subcutaneous transplantation of macroencapsulated islets. Transplantation 1999; 67 (5): 665.
172. Sharma A, Sorenby A, Wernerson A, Efendic S, Kumagai-Braesch M, Tibell A. Exendin-4 treatment improves metabolic control after rat islet transplantation to athymic mice with streptozotocin-induced diabetes. Diabetologia 2006; 49 (6): 1247.
173. Sorenby A, Rafael E, Tibell A, Wernerson A. Improved histological evaluation of vascularity around an immunoisolation device by correlating number of vascular profiles to glucose exchange. Cell Transplant 2004; 13 (6): 713.
174. De Vos P, Hillebrands JL, De Haan BJ, Strubbe JH, Van Schilfgaarde R. Efficacy of a prevascularized expanded polytetrafluoroethylene solid support system as a transplantation site for pancreatic islets. Transplantation 1997; 63 (6): 824.
175. Sorenby AK, Wu GS, Zhu S, Wernerson AM, Sumitran-Holgersson S, Tibell AB. Macroencapsulation protects against sensitization after allogeneic islet transplantation in rats. Transplantation 2006; 82 (3): 393.
176. King A, Lock J, Xu G, Bonner-Weir S, Weir GC. Islet transplantation outcomes in mice are better with fresh islets and exendin-4 treatment. Diabetologia 2005; 48 (10): 2074.
177. Farilla L, Bulotta A, Hirshberg B, et al. Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. Endocrinology 2003; 144 (12): 5149.
178. Hui H, Nourparvar A, Zhao X, Perfetti R. Glucagon-like peptide-1 inhibits apoptosis of insulin-secreting cells via a cyclic 5'-adenosine monophosphate-dependent protein kinase A- and a phosphatidylinositol 3-kinase-dependent pathway. Endocrinology 2003; 144 (4): 1444.
179. Sharma A, Kumagai-Braesch M, Yamamoto S, Sorenby A, Tibell A. A combination of islet pre-culture and recipient treatment with exendin-4 is required to improve the outcome of rat islet grafts in diabetic athymic mice. Submitted.
180. Shiroki R, Mohanakumar T, Scharp DW. Analysis of the serological and cellular sensitization induced by encapsulated human islets transplantation in type I and type II diabetes patients. Cell Transplant 1995; 4 (5): 535.
181. Siebers U, Zekorn T, Horcher A, et al. Microencapsulated transplantation of allogeneic islets into specifically presensitized recipients. Transplant Proc 1994; 26 (2): 787.
182. Orlowski T, Godlewska E, Moscicka M, Sitarek E. The influence of intraperitoneal transplantation of free and encapsulated Langerhans islets on the second set phenomenon. Artif Organs 2003; 27 (12): 1062.
183. Rose AG, Cooper DK, Human PA, Reichenspurner H, Reichart B. Histopathology of hyperacute rejection of the heart: experimental and clinical observations in allografts and xenografts. J Heart Lung Transplant 1991; 10 (2): 223.
184. Rose AG, Cooper DK. A histopathologic grading system of hyperacute (humoral, antibody-mediated) cardiac xenograft and allograft rejection. J Heart Lung Transplant 1996; 15 (8): 804.
185. Forbes RD, Kuramochi T, Guttmann RD, Klassen J, Knaack J. A controlled sequential morphologic study of hyperacute cardiac allograft rejection in the rat. Lab Invest 1975; 33 (3): 280.