CHAPTER ONE

GENERAL INTRODUCTION

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**Diabetes Mellitus** (DM) is the name of a clinical disorder characterized by an excess production of urine (diabetes means ‘to run through a siphon’) with a high concentration of sugar (mellitus means ‘sweet as honey’). Patients who suffer from DM have symptoms like excessive thirst, frequent urination, malnourishment, weight loss, and fatigue. DM is a chronic metabolic disease that is caused by an absolute or relative deficiency in insulin production that leads to increased concentrations of blood glucose. Compiled data from the World Health Organization (WHO) indicate that DM currently affects 150 million people worldwide, and that this number may well double in the course of the next two decades. Based on the etiology of the disease, two main types of diabetes can be distinguished. Type 2 DM is characterized by an ineffectiveness of the insulin produced or insulin resistance, which is caused by several factors. The risk for the development of type 2 DM is strongly familial, but the pathogenesis is mainly associated with increasing age, unhealthy diets, obesity and a sedentary life style, which explains why the mainstay of both treatment and prevention of type 2 DM is dieting and physical activity. Type 1 DM is characterized by an absolute deficiency in the production of insulin by the pancreas, which is the result of an autoimmune destruction of the insulin-producing cells in most cases. People who suffer from this autoimmunity have a genetic predisposition to the disease, which may be triggered by environmental factors (i.e., microbial, chemical, dietary). Without insulin treatment, type 1 DM patients are exposed to the risk of death from acute ketoacidosis. Type 1 DM is much less abundant than type 2 DM, and accounts for approximately 10% of all diabetes cases worldwide.

The current treatment of type 1 DM almost fully depends on the administration of insulin. Insulin is secreted from beta cells of the pancreatic islets, which were first described by Paul Langerhans as ‘vivid yellow specks scattered throughout the pancreas’ in 1869. Minkowski and von Mering made a key observation in 1889 when they discovered that removal of the pancreas led to the development of diabetes in dogs. It soon led to the conviction that a lack of islets of Langerhans results in diabetes. In 1922, Banting & Best published the first paper describing that pancreatic extracts could be used to correct hyperglycemia in depancreatectomized dogs (7). They initially called the newly isolated hormone ‘isletin’, but at the insistence of their coworker MacCleod, it became to be known as insulin (150). The discovery of insulin represents a landmark in the history of diabetes. In the same year, the first clinical test was performed on a 14-year old diabetic child, who showed a remarkable improvement of his diabetes after injection of the pancreatic extracts. These results were rapidly followed by the widespread introduction of exogenous insulin as a life-saving therapy into clinical practice. Since then, several lines of investigations have been initiated, which provided new insight into the normal
and deranged function of pancreatic islet cells, and which contributed the development of improved insulin therapy strategies (11). Investigations on the chemistry of insulin and major advances in molecular biology made it possible for human insulin to become the first protein to be commercially produced by recombinant DNA techniques in 1979 (5, 54). The quality of insulin preparations has been improved thanks to this DNA technology, which has also led to the development of new insulin analogs that enable more physiologic glucose control. In addition, improved insulin delivery systems, such as pens, pumps, and jets have been introduced during the years to provide convenience and to enhance patient compliance. Further improvement may be expected from new non-invasive insulin formulations, like oral and pulmonary insulin, which are currently tested with promising results (60).

Although type 1 DM can be well treated with the current insulin formulations, there are several drawbacks to insulin treatment. Life-long insulin therapy is associated with fluctuations of blood glucose levels, which lead to the development of diabetic complications located in the eyes, kidneys, nerves, and the microvessels in limbs. As a consequence, the expected lifespan of diabetic patients is shortened by an average of 15 years (103). Results from the DCCT (Diabetes Control and Complications Trial) in 1993 showed that the severity and the number of diabetic complications can be reduced, and that the progression of secondary lesions can be delayed by an intensive insulin therapy (1). However, intensive insulin therapy requires a complex treatment regimen with a large number of daily injections and is associated with the increased occurrence of hypoglycemic episodes. Furthermore, administration of the current insulin preparations with a pump can approach, but not equal the fine-tuned glucose regulation of the pancreas (86). Blood glucose fluctuations and the discomfort of daily injections are reasons to continue the search for a more permanent solution. One such solution may be to replace the dysfunctional pancreas by an insulin pump equipped with a permanent glucose sensor, which allows for the continuous glucose monitoring and maintains normoglycemia through feedback-controlled insulin release. Several types of sensors have been scrutinized for periods varying from several hours to several days (74, 75, 91). The current glucose sensors have a variable relation between the sensors signal and the blood glucose concentration during these observation periods, and they are therefore not available in clinical practice yet (72, 74, 75).

The only replacement therapy that currently can improve metabolic control compared to conventional and intensified insulin therapy is transplantation of insulin-producing tissue, which can be performed either by transplantation of the whole pancreas or by transplantation of the pancreatic islets of Langerhans only (Figure 1). In this chapter we will briefly mention the pros and cons of
transplantation of insulin-producing tissue as a treatment modality for diabetes. Graft rejection and donor shortage are limitations of transplantation that provide the rationale for the development of techniques like microencapsulation, which is a means to transplant pancreatic islets within immunoprotective microcapsules in order to prevent graft rejection. We will discuss the literature on causes of microencapsulated islet graft failure and possible solutions, in view of the potential of microencapsulation to improve the results of islet transplantation, and to establish a safe and simple cure for diabetes. The principal causes of microencapsulated islet graft failure provide the rationale for the investigations presented in this thesis.

**Figure 1.** *The current therapeutic strategies for the treatment of type 1 diabetes mellitus. Insulin injection therapy is the treatment of choice for the majority of type 1 diabetic patients.*

**Pancreas Transplantation**

From the early beginnings of pancreas transplantation in the sixties until the eighties, results were meager due to poor organ procurement and preservation methods, complications related to transplantation techniques, and high rejection rates. In the 1980s, better techniques for surgical management of pancreatic exocrine secretions combined with careful candidate selection and innovations in immunosuppressive therapy resulted in major improvements in patient and graft survival (139). At the end of the eighties the one-year pancreas graft survival rates approximated 75% (59). Further improvements during the 1990s led to the current one-year patient and pancreas graft survival rates of approximately 98% and 85%, respectively (59, 90). These survival rates relate to diabetic recipients with end-stage renal failure, who have received a simultaneous pancreas and kidney graft. Successful pancreas transplantation provides improved blood glucose
regulation compared to insulin therapy and has beneficial effects on secondary diabetic lesions. Transplantation also delays the progression of complications like neuropathy and nephropathy (68, 95, 104). In addition, nephropathic lesions can even gradually disappear after pancreas transplantation (46). Whether the effect of pancreas transplantation is also beneficial on retinopathy and angiopathy can not yet be determined because of the advanced nature of these complications at the moment of transplantation.

Despite benefits, pancreas transplantation faces two drawbacks. The first and most important drawback is the requirement of immunosuppressive medication to establish graft acceptance, since they are associated with deleterious side effects. Immunocompromised patients are more susceptible for viral, fungal and bacterial infection and have an increased risk for the development of malignancies. The increased risk for the latter has been calculated to be four to up to 500-fold (111, 149). Furthermore, many of the current immunosuppressive agents cause general toxicity and organ damage (including the graft), depending on the type and dosage of immunosuppression (92, 100). Because of these adverse side effects, the majority of pancreas transplants (approximately 83% during the last decade) are performed in combination with a kidney transplant (simultaneous or pancreas after kidney) for patients with diabetic nephropathy (63). The risk of adding a pancreas transplant is acceptable under these circumstances since these patients already receive immunosuppression for their kidney transplant, which is the therapy of choice for end-stage renal disease. Pancreas transplantation alone is being performed with an increasing frequency and with increasing success (current one-year graft survival is 76% (59)). This treatment is performed in a small number of type 1 diabetic patients without nephropathy, but with recurrent episodes of hypoglycemic unawareness, in order to restore normoglycemia.

The second drawback that concerns pancreas transplantation is related to the risks of surgery. Pancreas implantation requires a major operative procedure, which carries the risk of surgical complications, complications due to leakage of exocrine tissue, and graft thrombosis (70, 142). Notably, many of these post-transplant complications are the consequence of the exocrine part of the pancreas, which takes up more than 98% of the volume of the graft but does not produce any insulin. Transplantation of only the endocrine pancreas, i.e. the insulin producing islets of Langerhans, requires not more than a small procedure. This greatly reduces the peri-operative surgical risks and eliminates post-operative complications due to exocrine secretions. Moreover, the endocrine islet tissue has the advantage that it may be modulated prior to transplantation to further reduce the risk of rejection. These benefits are the principle rationale for islet transplantation.
Before islets of Langerhans can be transplanted they have to be isolated from the pancreas. The procedure for the liberation of islets is elaborate, and it requires major skill to obtain a mass of islets that is pure, vital and functional. In rodents, the first successful islet isolations with collagenase were performed in 1965 by Moskalewski (102). Lacy and Kostianowksi improved this enzymatic dissociation by intraductal infusion of the collagenase solution prior to digestion (80). The first successful transplantation of islets was performed a few years later and recorded in 1972 by Ballinger and Lacy (6). Many experimental studies in rodents followed and it was repeatedly demonstrated that islet transplantation could establish normoglycemia in diabetic rats and mice. Unfortunately it became obvious that islet isolation is much more difficult with pancreases derived from large animals and humans. It took almost two decades of development of islet isolation techniques before the first successful human islet transplantation could be performed.

The first well-documented and successful clinical islet transplantation was performed by Scharp et al. in 1990. A type 1 diabetic patient, who had previously received a successful kidney transplantation, received human islets via the portal vein, and subsequently remained off insulin with increased C-peptide levels for more than two weeks (128). In spite of this important success, the survival rate of islet grafts remained low for several consecutive years. The international Islet Transplant Registry organized by the transplantation center in Giessen, Germany, reported that less than 12% of the islet allografts from 1990 until 2000 remained insulin-free for one year (14), by far inferior to the whole pancreas graft survival rates in that same period. In the year 2000, a major breakthrough was reported by Shapiro et al. from Edmonton, Canada, who were the first to demonstrate that islet graft survival can be as successful as whole pancreas graft survival (130). An improved glucocorticoid-free immunosuppressive regimen, the use of multiple donors, excellent islet isolation and purification methods, and immediate transplantation without xenoproteins were keys to the successful Edmonton protocol. Intraportal transplantation of a mean of 11,500 islets/kg led to full insulin independence for one year in seven subsequent patients (130). Other centers have initiated similar transplant programs since, and the efficacy of the Edmonton protocol has been recapitulated by the same group (125) and recently also by others (93).

Despite these promising results, two major obstacles for the widespread use of islet transplantation as a common treatment modality for diabetes type 1 still have to be overcome. The first obstacle is the obligatory use of
immunosuppressive drugs, which has deleterious side effects like the increased risk of infection and malignancies. As with whole pancreas transplantation, these side effects restrict the application of islet transplantation to patients for whom the risks associated with immunosuppression outweigh the risk for the development of diabetic complications. The second obstacle is the necessity of multiple donors. While transplant centers have to cope with a worldwide organ shortage, at least two donors are necessary for successful islet transplantation (130). Even if the number of patients was not restricted by immunosuppressive side effects, only 0.1% of the type 1 diabetic population could be transplanted with the currently limited supply of organ donors (131). This emphasizes the need to increase the efficiency of organ use and justifies the search for alternative donor sources.

Several improvements may help to eliminate the necessity of multiple donors. One way is to improve islet isolation and purification methods in order to increase the islet yield from one pancreas. The human pancreas contains one to two million islets and currently the mean yield for clinical transplantation is approximately 350,000 islet-equivalents per pancreas, which means that islet isolation efficiency is only 18-35% (131). Another way to prevent the need for multiple donors is to improve the efficiency of organ use by further promoting graft acceptance and survival. Since only 25-50% of the islet tissue appears to survive implantation, full graft acceptance may eliminate the need of retransplantation (131). Improving prevention of tissue rejection can be achieved by further improvement of immunosuppressive regimens, which would also decrease the risk of deleterious side effects. Apart from modulating the immune system of the recipient, alteration of the graft may help to prevent rejection. Genetic engineering opens up the perspective to specifically alter gene expression of islets in order to decrease their immunogenicity or to increase their resistance to rejection. Another alternative strategy that does not involve direct modification of the recipient or the graft and that may provide a solution to both the necessity of immunosuppression and to the problem of organ shortage is the encapsulation technique. Encapsulation is a means to prevent rejection by separation of the graft from the host by a mechanical barrier, which enables transplantation without immunosuppression and opens up the perspective to use non-human donor sources. It can be argued that islet transplantation cannot become a standard treatment for type 1 DM until graft acceptance can be established without deleterious side effects for the recipient and until a plentiful source of islets can be identified. The use of alternative donor sources and the encapsulation technique as a possible solution for both rejection and organ shortage are subjects that will be discussed in the following two sections.
The discrepancy between supply and demand of organs for transplantation is an impediment for the clinical application of islet transplantation. Means to solve the problem of organ shortage could include the use of animal tissue, the use of cell lines, and possibly the generation of new islets from embryonic or adult stem cells. Embryonic stem cells originate from the inner cell mass of the early blastocyst or fetal gonadal tissue. They are considered to be pluripotent, i.e. able to differentiate in almost all cells that normally arise from the three embryonic germ layers (2, 9). Their therapeutic potential was demonstrated by Soria et al., who generated insulin-secreting cells from embryonic stem cells, which normalized blood sugar levels after transplantation in diabetic mice (138). Ethical concerns surrounding the use of embryonic cells have prompted to search for alternative, i.e. adult sources (2, 89). Adult stem cells are not pluripotent, i.e. capable of producing a limited range of differentiated cell lineages appropriate to their location (2). For the generation of insulin producing cells, pancreatic duct cells are suitable as progenitor cells (12). It has been demonstrated that mouse islets generated from duct cells reversed hyperglycemia after implantation into NOD mice (119), and that human islets can be successfully generated from human pancreatic duct cells in vitro (13). The principal advantage of adult stem cells is that recipients can be transplanted with their own newly generated islets. A drawback however, is the chance for recurrence of the disease as a consequence of autoimmunity. Encapsulation may provide a means to protect a stem cell-derived graft without the necessity of immunosuppression. But first, more research is necessary to prove whether the use of stem cells can meet up to its expectations. Transplantation of stem cell derived islet cells to humans is only applicable when enough mature cells with a stable phenotype and without a hyperproliferative nature can be generated (9).

The generation of insulin-producing cell lines has been studied extensively because of their major advantage that they can be grown in theoretically unlimited quantity at relatively low cost (40, 98, 105). Two methods that have been adopted for the generation of cell lines in the early eighties are the X-ray radiation of β-cells to induce insulinoma (RINm5F) (19, 50) and the transformation of β-cells with simian virus (SV40) (127). However, inappropriate glucose responsiveness and uncontrolled proliferation of β-cells made most early cell lines unsuitable for transplantation. Improved glucose sensing was achieved by the generation of hybrid cell lines by electrofusion (96, 97). But, because of the immortalized state of the cells they remained unsuitable for transplantation. Recently, the transgenic expression of the SV40 T antigen oncprotein in murine β-cells under control of the tetracycline operon has led to the generation of β-
cell lines with a well-differentiated and stable phenotype (42, 47, 101). These beta cells can be easily expanded in vitro and their proliferation is arrested upon harvesting thanks to the conditional gene expression system. Growth arrest is followed by an increase in their insulin production and storage up to levels comparable to normal mouse islets. Transplantation of such cells into the peritoneum of chemically induced diabetic mice successfully reversed diabetes for periods of months (101). The most important obstacle for the application of human cell lines is the risk for the development of tumors after transplantation (41, 123). Additional safety strategies are required to reduce the potential risk of uncontrolled growth, like the introduction of suicide genes to allow cell elimination in case of tumor growth. Furthermore, additional protection is required to protect the β-cells from recurring autoimmunity. This protection may well be offered by cell encapsulation.

The use of animals as an alternative donor source for humans has been a subject for many studies. The pig has been considered a potentially ideal donor source for several reasons including the large number of donors available, the possibility of genetic modification and the relative compatibility of pig and human physiology (114). Islet isolation in adult pigs has been generally characterized by difficulties leading to low yields and poor viability, which explains why porcine islet transplantation in diabetic rodents has had varying success rates during the nineties (37, 76, 99, 120, 158). Many groups have therefore searched for alternatives for adult pigs like fetal and newborn islet-like cell clusters. The first transplant procedures in man were performed with fetal porcine islet tissue, however, this did not reduce the insulin requirements of the diabetic recipients (3, 146). Currently, a sufficient number of purified porcine islets with good in vitro function can be isolated (77, 78). The experience of islet xenotransplantation in men is at present restricted to the unpublished communications of the Mexican group of R. Valdes, who transplanted twelve adolescents in an effort to cure their diabetes. The implants that consisted of both porcine islets and Sertoli cells allowed one child in the trial to stop taking insulin injections completely, and five others to take less insulin than before. The presentation of this trial has met with substantial criticism and evoked controversy (4, 17). During the nineties, there was an increasing concern with xenotransplantation as a possibility to transfer potentially hazardous viruses into man. Since then, the evidence that transplanted pig tissue does not cause infection with endogenous porcine retroviruses has accumulated (44, 66, 94, 109, 143). Nevertheless, the potential risk of introducing an unknown virus that may cause epidemic infections similar to other viruses that were initially derived from animals (e.g. HIV, Hepatitis B, influenza, and SV 40) has prevented the therapeutic use of islet xenografts in most countries.
Besides pigs, other animals have been studied for their potential to provide an unlimited source of donor islets. One unique model that has been utilized for discordant islet transplantation by the Canadian group of J. R. Wright is based on the application of Brockman bodies, which are the islet equivalents from the teleost fish Tilapia (156, 157, 159). Brockman bodies have proven to induce normoglycemia in mammalian animal models (159, 160). Piscine islets are potentially ideal xenogeneic donors because fish have minimal production costs and because fish islets can easily be isolated. Since fish have little phylogenetic similarity with man, the risk of zoonosis is less with fish than with pig, which is a strong argument in favor of xenotransplantation of piscine islets over porcine islets. The main obstacle for the use of piscine islets has been the lack of homology between fish and human insulin, which is likely to lead to insulin resistance after transplantation in humans. Therefore Wright et al have cloned the human insulin gene with the future intent of transfection of Tilapia fish stem cells with the insulin gene (156). If this modification can be performed without affecting normal function remains to be determined.

**IMMUNOPROTECTION BY ENCAPSULATION**

With encapsulation, islets are enclosed in a matrix surrounded with a semi-permeable membrane, which allows for the passage of small molecules like insulin and glucose, but does not allow the entrance of the much larger cells and antibodies of the immune system. Such a physical barrier can thus prevent allograft rejection, which depends on recognition of MHC by host lymphocytes. Furthermore it can prevent antibody-mediated cytotoxicity, which plays a role in both the autoimmune destruction of beta cells and in allo- as well as xenograft rejection (99, 154). Protection by encapsulation can thus enable transplantation of islet tissue in the absence of immunosuppression. Since encapsulation may prevent xenograft rejection, it also opens up the perspective to transplant animal tissue. This provides a solution to the problem of organ shortage, since animals can be bred for transplantation purposes in large numbers. Dilemmas with regard to ethics and the risk for viral infections have restricted the use of animal tissue for human transplantation purposes so far and may eventually prevent a common application of xenotransplantation in future. These matters of debate have however not prevented the search for a successful encapsulation system. This research is driven by the potential benefit of the technique to contribute to a safe and simple cure not only for diabetes, but also for a variety of other endocrine diseases, which may be treated by substitution with appropriate (non-) human cells.
The idea of using encapsulation to prevent the immune system to be in contact with cells is approximately 50 years old (115). Many different kinds of encapsulation systems have been studied since and they are generally divided into three categories (81). Devices of the first category are characterized as intravascular macrocapsules, which are usually perfusion chambers that are directly connected to the blood circulation. Devices of the second category are not intra- but extravascular macrocapsules, which are usually diffusion chambers in the shape of a tube or disc that can be implanted intraperitoneally or subcutaneously. The third category is extravascular and does not involve macro-, but microcapsules, which -dependent on the size and the number of capsules- can be implanted to several different sites of the body. The most commonly used microcapsules are composed of alginate-poly-L-lysine alginate (APA) and were originally described by Lim and Sun in 1980 (87). Cells are enclosed in an alginate core, which is covered by poly-L-lysine (PLL), a poly-amino acid that gives the capsules semi-permeable properties (FIGURE 2). The PLL layer can be modified, which makes it possible to achieve many different grades of permeability (55, 124). PLL is also important for capsule stability and it is absolutely required for the integrity of Ca\textsuperscript{2+}-alginate capsules. A second layer that consists of alginate is applied for coverage of the unbound PLL groups.

Algin is a component of the extracellular matrix of brown algae and consists of the polysaccharides beta-D-manuronic acid (M) and 1,4-linked alpha-L-guluronic acid (G). Raw alginate can be purified and sterilized to a biocompatible

**FIGURE 2.** Microencapsulated pancreatic rat islets and the concept of microencapsulation. The alginate-poly-L-lysine microcapsule prevents the entrance of immune cells and antibodies, while it allows the passage of insulin and glucose.
material, *i.e.* its composition is inert and does not evoke an inflammatory response. Dissolved alginate has a high viscosity, which is suitable for the formation of small droplets. These droplets solidify to become hydrogel beads in solutions with divalent cations like Ca$^{2+}$ and Ba$^{2+}$, which bind to the polysaccharides G and M. The G/M ratio determines several main properties. Beads made from high G alginate are more stable and therefore more resistant to mechanical stress. Beads made of high M alginate bind more effectively with PLL, which has two advantages. First, the efficient binding of high M alginate with PLL can be used to decrease the capsule permeability, thereby improving the immunoprotective properties of capsules (26, 145). Second, better PLL binding means less non-bound PLL on the outside of the capsules, thereby reducing the risk of inducing fibrosis by positively charged PLL groups that are not well covered by the second alginate layer (116, 140). Alternatives for alginate are polyethylene glycol (24), polyacrylates (129), agarose (71), chitosan (162), and also multicomponent capsules have been applied (151), but until now with limited success.

**ALGINATE-POLY-L-lysine microencapsulation**

Microencapsulation is a subject of study for a variety of endocrine diseases, which may be treated by substitution with the appropriate cells (22, 82). Successful function of encapsulated hepatocytes after transplantation in animals has been documented (25, 61). Encapsulated parathyroid tissue has been transplanted with success in animals, and recently even in man (64, 65). Possibly, encapsulation can also be used for the treatment of neuro-degenerative diseases like Parkinson and Huntington (45, 88). Here, we focus on transplantation of encapsulated pancreatic islets, which has been performed in animal species like rats (33, 106, 148), mice (153), dogs (85, 136), and monkeys (141). Encapsulated islets are always implanted in the peritoneal cavity. This is the only implantation site that is large enough to accommodate an encapsulated islet graft, which consists of a few to several thousands of microcapsules. In all studies, normoglycemia was achieved within a few days after implantation and persisted for a substantial period of time. In the absence of immunosuppression, the graft survival of encapsulated islets was distinguishably prolonged compared to non-encapsulated islets, but the duration was unfortunately limited to periods varying from several months in rats (33) until up to two years in dogs (136). In 1994, Soon Shiong *et al.* reported insulin independence in a type 1 diabetic patient after encapsulated islet transplantation (137). The transplantation of 10,000 human islets/kg was performed in the presence of a low-dose of cyclosporin and the graft was replenished with 5,000 human islets/kg six months after the first implantation.
GENERAL INTRODUCTION

Basal C-peptide secretion increased, concomitant with the drop in insulin requirement, from less than 0.1 ng/ml pretransplant to 1.0 ng/ml at the eight month, which confirms sustained insulin secretion from the encapsulated islets. The patient subsequently returned to exogenous insulin therapy and with another supplemental dose of 5,000 human islets/kg at 33 months ongoing islet function with tight glycemic control was reported for 58 months (135). This report is the only well-documented study on transplantation of encapsulated islets in man. Although this case and the animal studies illustrate the potential applicability of the microencapsulation technique, graft survival is too limited for encapsulated islets to become a widespread treatment in clinical practice at present. One could of course aim for repeated transplantation during the life span of a patient. This is, however, not feasible for an increasing number of diabetics, while transplant centers have to cope with a general donor shortage and alternative donor sources are not available yet.

CAUSES OF MICROENCAPSULATED ISLET GRAFT FAILURE

A better insight into the causes of encapsulated islet graft failure may help to find a way to improve graft survival. One important observation is that encapsulated autograft and allograft survival rates are similar, which implies that graft failure is not caused by rejection due to allograft recognition (30). If graft failure cannot be explained by allograft rejection, other factors must be involved. In search of these factors encapsulated islet graft failure was analyzed in our laboratory. We showed that there is a gradual decrease in islet function, a gradual increase in central necrosis, a continuous increased replication of islet cells and a non-progressive overgrowth of a portion of the encapsulated islet graft (33). Three important aspects of the encapsulated islet graft technique may be associated to these phenomena. The first is related to the biocompatibility of the graft. A number of capsules lack biocompatibility, which gives an explanation for the occurrence of overgrowth. The second is related to the immunoprotective properties of the microcapsules. Immunoprotection is incomplete because capsules may allow for the passage of small pro-inflammatory factors, which lead to cell death and dysfunction. The third factor is related to a large distance between the encapsulated islets and the blood supply. An important consequence of the large diffusion distance is the limited supply of oxygen, which leads to hypoxia and causes necrosis, islet dysfunction, and may be responsible for the increase in islet cell replication. The lack of biocompatibility, the limited immunoprotection and hypoxia are the issues discussed in further detail in the next sections.
Pericapsular overgrowth of microcapsules due to lack of biocompatibility is responsible for loss of part of the graft. Overgrowth on capsules is established within the first few weeks after transplantation and does not increase thereafter (16, 33, 113). Only a small portion of approximately 10% of a retrieved encapsulated islet graft is affected by overgrowth with fibroblasts and macrophages (31, 33). However, a much higher percentage of approximately 40% of the number of initially implanted islets is lost due to overgrowth (33). Biocompatibility of encapsulated islets depends on the composition of the alginate, the purity of the alginate and the integrity of the microcapsules. Purification (i.e., the removal of contaminants like endotoxins and polyphenols) and sterilization of alginate with an intermediate G composition results into optimal biocompatibility, leaving lack of integrity of the graft as the main cause of cellular overgrowth (30, 35). Lack of integrity is characterized by breakage due to capsule instability and by physical irregularities on the capsule surface (Figure 3). Irregularities like tails and craters lead to overgrowth of a portion of the capsules, which usually remains well below 5% of empty capsules retrieved from the peritoneum (28, 30). Physical irregularities are mainly the consequence of inadequately encapsulated islets (29, 67). The occurrence of inadequately encapsulated islets strongly depends on the capsule diameter, i.e., small capsules contain more protruding islets (27, 29). Protrusion of islets due to inadequate encapsulation in 750 µm capsules is estimated to be responsible for approximately 10% of the overgrowth of an encapsulated islet graft (27, 29, 31).

Physical irregularities give an explanation for the occurrence of overgrowth on a number of encapsulated islets, but it does not explain why 40% of the number of initially implanted islets are lost as a consequence of overgrowth. Apparently, even perfectly smooth islet containing capsules are overgrown by macrophages, which suggests that besides alginate composition, purity and capsule integrity, other causative mechanisms are involved in the occurrence of overgrowth. These mechanisms are directly related to the islets themselves (84, 133), and can collectively be defined as chemotaxis (Figure 3). Two chemotactic pathways may be involved in the attraction and activation of macrophages locally. One chemotactic pathway is the passive shedding of antigens by islets. This mode of chemotaxis is more apparent for xenografts than for allografts, since virtually every protein shed by a xenograft is different from the host (53, 57, 133). Antigen release, especially the release of alpha 1, 3 – galactose, attracts and activates macrophages, which in turn release cytokines like IL-1β, TNF-α, and IFN-γ, but also nitric oxide and oxygen radicals (57, 108). These macrophage-derived factors are probably small enough to pass the semi-permeable membrane.
of microcapsules and may well affect encapsulated islet graft function and vitality. Recently, alpha 1, 3 – galactosyltransferase deficient pigs were produced, which is an important step towards the reduction of xenograft rejection, and may significantly contribute to the realization of pig-to-human transplantation (112). The second chemotactic pathway involved in the attraction of macrophages is not characterized by the passive leakage of waste products, but characterized by the active production of chemoattractant factors by islets. Such factors are named chemokines and they are typically induced by primary pro-inflammatory mediators such as interleukin-1 and tumor necrosis factor (56). One candidate chemokine that is expressed in pancreatic beta cells and also involved the attraction of macrophages after islet transplantation is MCP-1 (Chemo Attractant Protein – 1) (18, 110). MCP-1 is a small molecule (12 kD) that may well pass the semi-permeable membrane of microcapsules and thus stimulate the attraction of macrophages with by islets in microcapsules. Chemotaxis may thus be held responsible for the occurrence of overgrowth that cannot be explained by physical imperfections, nor by rejection.

Lack of biocompatibility due to irregularities on the capsule surface may be solved by technical improvements of capsule integrity. Greater stability of microcapsules can be achieved by using Ba\textsuperscript{2+} instead of Ca\textsuperscript{2+} during the encapsulation procedure (163). In addition to the greater mechanical stability, Ba\textsuperscript{2+}-capsules have another advantage in relation to biocompatibility. Ba\textsuperscript{2+}-

\textbf{Figure 3.} \textit{Cellular overgrowth of microcapsules is caused by physical irregularities (i.e. tails, craters, and protruding islets), and by islet-derived factors (‘chemotaxis’).}
capsules do not have a PLL layer, which implies that imperfect capsules, either caused by tails, craters, inadequately encapsulated islets or broken capsules, do not necessarily evoke an inflammatory response due to incomplete PLL coverage. Indeed, successful transplantation with low occurrence of overgrowth of islet containing Ba\textsuperscript{2+}-beads has repeatedly been reported (39, 73, 107, 164). However, Ba\textsuperscript{2+}-capsules also have a disadvantage. In contrast to capsules with PLL, the permeability of Ba\textsuperscript{2+}-beads cannot easily be modified. So, the improved biocompatibility of Ba\textsuperscript{2+}-capsules is associated with reduced possibilities to vary the pore size of the capsules, which makes it difficult to find an optimal balance between immunoprotection and functional performance, as will be discussed in the following section.

Decreasing the permeability of the microcapsules can prevent the passage of small deleterious molecules. However, decreasing the pore size of microcapsules may not only prevent the passage of cytokines like IL-1\textbeta (17.5 kD) and TNF-\alpha (51 kD), but is also likely to interfere with the insulin (6 kD) secretory response of encapsulated islets. Improving the immunoprotective properties can be achieved by finding the optimal balance between protection and function. If full protection cannot be achieved, it should be considered to combine encapsulation with other means of protection in order to prevent loss of islet function and vitality by cytotoxicity. Encapsulation offers the possibility to co-encapsulate islets with other cell types that may provide improved protection and can support islet function. Coencapsulation with autologous erythrocytes was found to be an effective and easy way of providing protection against macrophage-mediated lysis (155). Also coencapsulation with Sertoli cells lead to significantly prolonged islet graft survival times (15, 160). Sertoli cells

\begin{tikzpicture}
\node (islet) at (0,0) {Islet};
\node (antibody) at (-1,-1) {Antibody};
\node (immune) at (-1,-2) {Immune cell};
\node (insulin) at (1,0) {Insulin};
\node (glucose) at (1,-1) {glucose};
\node (toxic) at (0,-2) {Toxic factors!};
\draw[->] (islet) -- (antibody);
\draw[->] (islet) -- (immune);
\draw[->] (islet) -- (insulin);
\draw[->] (islet) -- (glucose);
\end{tikzpicture}

\textbf{Figure 4.} Microcapsules have limited immunoprotective properties. The capsule membrane not only allows the passage of insulin and glucose, but also of small toxic factors like cytokines and nitric oxide.
are cells derived from testis that protect cells from rejection by release of immunosuppressive factors. Also, genetic engineering opens up the perspective to specifically modulate islets with genes that increase cell resistance to deleterious molecules and with genes that can affect the function of immune effector cells outside the capsules. Transfection with the anti cell-death protein Bcl-2 prevents cytokine and NO induced cell death (23, 117, 126). Adenoviral transfection with genes encoding for an inhibitor of the pro-inflammatory cytokine TNF-α (TNFi) (38), or an IL-1 receptor antagonist (52) leads to limited beta-cell damage by leukocytes, and to protection against Il-1beta induced NO formation, respectively. During the recent years a whole scale of other genes encoding for proteins like heme oxygenase (HO-1) (113), manganese superoxide dismutase (MnSOD) (10), tissue inhibitor of metalloproteinase (TIMP) (62), Hepatocyte Growth Factor (HGF) (49), Insulin-like Growth Factor I (IGF-I) (51) and A-20 (58) have been used for genetic modification. Overexpression of such genes has beneficial effects with regard to the prevention of the process of autoimmunity and graft rejection, and may thus improve the resistance against cytotoxicity. Perhaps such an improvement is sufficient to offer full immunoprotection when it is offered in combination with encapsulation.

HYPOXIA

A third factor that contributes to the encapsulated islet graft failure is hypoxia. Islets within the pancreas are provided with a glomerular-like network of capillaries, which is destroyed by islet isolation. The presence of microcapsules prevents revascularisation of islets, which normally occurs within the first few weeks after implantation. Because revascularisation cannot occur, and since the supply of oxygen through the peritoneum is by passive diffusion only, instead of direct delivery from the blood stream, encapsulated islets suffer from irreversible and chronic hypoxic stress. Not only the oxygen supply, but also the insulin delivery is hampered by passive diffusion through the peritoneum. Results from our laboratory show that limited diffusion leads to reduced insulin secretory responses (34, 48). As a consequence, successful reversal of diabetes in rats required a 2-4 times higher islet mass for an encapsulated islet graft in the peritoneum compared to a non-encapsulated islet graft under the kidney capsule (134). Although it is generally assumed that hypoxia contributes to encapsulated islet graft failure, it is unclear to what extent. Despite hypoxia, encapsulated islets establish and maintain normoglycemia for periods of several months. This graft survival makes it reasonable to assume that hypoxic stress is either limited in severity or restricted to a portion of the graft only. The specific relation between hypoxia and encapsulated islet graft failure remains to be elucidated.
A possible solution to the problem of hypoxia is to increase the resistance of islet tissue to hypoxia and thus prolong graft survival. An increase in resistance of islets to hypoxia may be induced prior to transplantation by means of ischemic pre-conditioning (20, 147) or by heat shock (8). Another option is to stimulate the expression of Bcl-2, Bcl-xL as well as ICE-like proteases, which were reported to effectively retard chemical hypoxia-induced necrotic cell death (132). Such treatments merely provide temporary salvation. This can be of assistance to bridge a short period of hypoxia, but it is not enough to realize long-term resistance to hypoxia as required for encapsulated islet transplantation. A more permanent solution would be to use the natural resistance to hypoxia of Brockman bodies (157). These islet equivalents can be isolated from Tilapia fish that are adapted to live in stagnant hypoxic water. Additional advantages of Brockman bodies are minimal production costs and the possible decreased risk of zoonosis (156).

Another more definite solution to the problem of limited diffusion would be to implant the encapsulated islet graft to an implantation site that permit close contact between bloodstream and the islet tissue. The most successful transplantation site for non-encapsulated islets is the liver. Therefore, Leblond et al. studied the possibility to transplant small (~315 µm) APA microcapsules into the liver (83). They show that intra-hepatic implantation of small empty capsules is feasible and safe, but whether implantation remains successful after inclusion of islets remains to be determined. Small capsules are associated with a high percentage of surface irregularities due to incomplete encapsulation, which in turn induces pericapsular overgrowth and leads to graft failure (27, 134). Robitaille et al. studied the possibility to use the epididymal fat pads as an implantation site for microcapsules, but its application is hampered by a pericapsular reaction (121, 122). An alternative approach is to create well-vascularized implantation site in the abdominal cavity or under the skin. In our laboratory, a prevascularized expanded polytetrafluorethylene (PTFE) solid support in the peritoneal cavity was tested, which was found to be an efficacious transplantation site for non-encapsulated islets in rats, and which is potentially suitable for encapsulated islets (32). Wang et al. used a prevascularised subcutaneous site for the implantation of macroencapsulated islets and reported normalization of blood glucose levels for ~100 days (152). A major drawback of the subcutaneous site is, however, that the superficial presence of the transplant is associated with a high risk of mechanical stress and consequently the risk for damage to the graft.
Islet transplantation is a cure for diabetes with limitations caused by risks associated with immunosuppression, and by donor organ shortage. Encapsulation provides a means to transplant islets without immunosuppressive agents and may enable the performance of xenotransplantation. We presented our view on the principal causes of microencapsulated islet graft failure, which are related to a lack of biocompatibility, limited immunoprotection and hypoxia. These principal causes provide the rationale for the investigations presented in this thesis, which will be outlined in the following chapter.
REFERENCES


88. Lindner M.D. and Emerich D.F. Therapeutic potential of a polymer-encapsulated L-DOPA and dopamine-producing cell line in rodent and


