CHAPTER SEVEN

SUMMARY AND GENERAL DISCUSSION
Diabetes Mellitus type 1 is a metabolic disease that is characterized by an absolute shortage of insulin. Transplantation of insulin producing pancreatic tissue is a possible cure for type 1 diabetic patients who currently depend completely on insulin injections to maintain normoglycemia. Successful transplantation of the whole pancreas offers improved blood glucose regulation compared to both normal and intensified insulin treatment and can thus prevent all, and can even cure some, diabetic complications (12,14). However, pancreas transplantation is limited to a small number of patients because it requires major surgery and because graft acceptance demands immunosuppression. The latter is associated with side effects that in most cases do not outweigh the discomfort of insulin injection and the risk for diabetic complications. Since only 1-2 % of the pancreas consists of the insulin producing islets of Langerhans, transplantation of isolated pancreatic islets has been proposed as an insulin replacement therapy, in order to decrease the risk of peri-operative complications. A further advantage of this approach is that the immunogenicity of isolated islets can be reduced in vitro prior to transplantation. One method that can establish such a modification is encapsulation, a technique that provides islet tissue with a shield, which protects the islets against an attack of immune cells and antibodies. Encapsulation thus provides protection against rejection by a physical semi-permeable barrier, and may thus allow transplantation with minimal or no immunosuppressive therapy. The most common type of encapsulation is alginate-poly-L-lysine alginate (APA) microencapsulation, as originally described by Lim and Sun in 1980 (13). APA microcapsules can not only prevent the use of immunosuppression, but may even provide a solution to the problem of donor shortage by enabling the use of animal tissue for transplantation purposes.

Encapsulated islet transplantation has been performed in several diabetic animals like mice (8), rats (5), dogs (21), and monkeys (23). Transplantation of an encapsulated islet graft in human has been restricted to one diabetic patient only (22). In all cases, encapsulated islets were implanted into the peritoneal cavity and normoglycemia was established with no or low immunosuppression. Although this illustrates the principal applicability of the encapsulation technique, the islet graft survival rates are generally restricted to periods of several months. Prolongation of graft survival is necessary in order to start clinical trials. Experimental studies in our laboratory showed that encapsulated islet graft failure is characterized by a gradual loss of function, which is associated with a gradual increase in necrosis, a continuous increased replication rate of islet cells, and a non-progressive overgrowth of a portion of the encapsulated islets (5). On the basis of these and previous (3,6) observations from our laboratory, three main causes of encapsulated islet graft failure can be identified. These are; first, the limited immunoprotective properties of the microcapsules; second, a lack of
SUMMARY AND GENERAL DISCUSSION

The mechanisms of encapsulated islet graft failure are described in Chapter 1 and this provides the rationale for the studies presented in this thesis, which are outlined in Chapter 2.

Immunoprotection is limited because microcapsules allow the passage of small molecules, which are not only insulin and glucose, but most probably also free radicals and cytokines like IL-1β and TNF-α. Improvement of immunoprotection can be achieved simply by reducing the capsules’ permeability in order to prevent the entrance these cytokines. However, decreasing the permeability for cytokines may also interfere with the insulin secretion capacity of encapsulated islets. In search of an optimal balance between protection and function, we studied diffusion of the macrophage-derived cytokines IL-1β and TNF-α, and insulin in microcapsules with different grades of permeability. Our data are presented in Chapter 3, and they show that microcapsules can protect islets against the deleterious effects of cytokines, while encapsulated islet function remains largely unaffected. Islets encapsulated in beads exposed for 15 minutes to poly-l-lysine (PLL) maintain high insulin secretory response, while this permeability grade greatly reduces the passage of cytokines with approximately 99% compared to beads without a PLL layer. Capsules with 15 min PLL provide improved protection compared to 5 and 10 min PLL capsules. However, this improved protection is also associated with a one-third reduction in insulin secretion compared to 5 and 10 min PLL capsules. It is therefore likely that the islet mass of a graft with 15 min PLL capsules has to be increased in order to compensate for the functional loss. Notably, 10 min PLL may well provide enough protection against the deleterious effects of cytokines, as appears from a subsequent study, which is presented in Chapter 5.

Besides the limited immunoprotective properties two additional factors contribute to encapsulated islet graft failure. These are macrophage overgrowth as a consequence of a lack of biocompatibility and hypoxia as a result of insufficient blood supply. We wanted to determine whether the deleterious effects of macrophages on overgrown capsules spread out to non-overgrown encapsulated islets, and how encapsulated islets respond to hypoxic stress, in order to increase the insight in encapsulated islet graft failure. Islet function, islet vitality, islet cell replication, and gene expression were analyzed during both coculture with overgrown capsules and hypoxic culture, as presented in Chapter 5 and Chapter 6, respectively. Gene expression analyses requires RT-PCR and since microcapsules interfere with both cDNA synthesis and PCR amplification it was necessary to develop a method to remove the capsules from the islets. We developed a novel procedure, which allows for the relative harmless removal of islets prior to analysis. In Chapter 4 we show that our method does not affect
islet vitality, that it has only minor effects on islet function and morphology, and that it allows for mRNA quantification without interfering with islet gene expression. The novel method was required for the molecular analyses presented in CHAPTER 5 and CHAPTER 6, and it enabled us to find significant differences in gene expression levels between control and experimental groups.

Lack of biocompatibility leads to pericapsular overgrowth, which destroys a considerable portion of the islet graft, i.e. approximately 40% of the number of initially transplanted islets. Pericapsular overgrowth consists of macrophages that produce a whole scale of factors that are toxic to islets. To determine if the deleterious effects of macrophages on overgrown microcapsules spread out to non-overgrown encapsulated islets, we set up an \textit{ex vivo} model in which freshly encapsulated islets are cocultured with overgrown capsules. CHAPTER 5 describes the coculture experiments, which led to two important findings. First, the close presence of overgrown capsules affects neighboring non-overgrown encapsulated islets. Apparently macrophages can not only directly contribute to graft failure by destroying islets within overgrown capsules, but also indirectly by affecting function and vitality of neighboring non-overgrown encapsulated islets. Second, our data suggest that nitric oxide (NO) produced by macrophages rather than IL-1\textbeta\hspace{1em} or TNF-\alpha is the detrimental factor. Although a coculture experiment with an NO-inhibiting agent in order to verify the toxic effect of NO was not executed, the suggestion that NO is the causative factor of macrophage induced dysfunction and cell death in this study is supported by the results of Wiegand \textit{et al.} (25). They also showed that NO secreted by activated macrophages destroys islets despite encapsulation, and that both inhibition of NO-formation and scavenging of NO can protect encapsulated islets from destruction.

The observation that microcapsules can protect against macrophage-derived cytokines in CHAPTER 5 is in line with the results on capsules with different grades of permeability as presented in CHAPTER 3. Apparently, the immunoprotective properties of 10 min PLL capsules as applied during the coculture study are sufficient to prevent the passage of macrophage-derived cytokines. It is however not sufficient to prevent the passage of small radical molecules like NO. These results emphasize the need to eliminate overgrowth, which has two beneficial effects. It can not only increase the number of available islets, but it can apparently even improve function and vitality of the non-overgrown part of the graft. Since it is difficult to avoid overgrowth completely in a macrophage-rich peritoneal cavity, additional means of protection are necessary. Our study indicates that the additional protection should aim for protection against NO-toxicity.

Insufficient blood supply leads to oxygen deprivation, which contributes to graft failure. In CHAPTER 6 we studied encapsulated islets during hypoxic culture,
in order to specifically determine the relation between oxygen deprivation and graft failure. Our results show that severe hypoxia is associated with total loss of islet function and near-total loss of islet vitality. An important finding in this study is the hypoxia-induced increase of Monocyte Chemoattractant Protein 1 (MCP-1) mRNA expression. MCP-1 has been reported to attract macrophages after transplantation of non-encapsulated islets (16). Since MCP-1 (11 kD) is small enough to pass the semi-permeable membrane, this chemotactic factor may well be one of the islet-derived factors that contributes to the occurrence of pericapsular overgrowth. Like others, we found that islets constitutively express MCP-1 (2,17). Here, we show that MCP-1 protein is able to pass the semi-permeable membrane and that its mRNA expression is enhanced by hypoxic stress. The hypoxia-induced increase in MCP-1 mRNA expression in islets is in line with results from other groups who found hypoxia to increase MCP-1 mRNA expression in melanoma cells and in fibroblasts (9,11). In our study, the increase of MCP-1 during hypoxia was only found on an mRNA level and not recovered on a protein level. The apparent discrepancy between mRNA and protein levels during hypoxia can be explained in two ways. A first explanation is that MCP-1 protein levels are low because of the lower number of vital cells in encapsulated islets after hypoxic culture. The number of cells does not affect the gene expression level, which is corrected for the amount of material by means of an internal standard. A second explanation is that the amount of MCP-1 protein is lower compared to mRNA levels because the protein is degraded by proteolytic activity, a process that is associated with necrosis, which in turn is high in response to hypoxia. Additionally, we cannot exclude the possibility of post-transcriptional downregulation of MCP-1 expression, although this is not expected since mRNA and protein levels were found to correlate well in mouse islets (2). The increased MCP-1 mRNA during hypoxic culture led to the suggestion that hypoxia further contributes to the occurrence of overgrowth. The increase in MCP-1 expression is more likely to occur in large islets, since they suffer more from hypoxia than small islets. This mechanism may well contribute to loss of 40% of the number of initially transplanted islets due to overgrowth, a percentage that cannot be explained by chemical or physical irregularities of microcapsules only.

Increased cell proliferation is a phenomenon that has been observed in encapsulated islets after transplantation. What causes this hyperproliferation is not known, and we hypothesized that the increased replication rate is either cytokine-mediated or related to hypoxia. To gain more insight into this phenomenon we studied cell proliferation during coculture (CHAPTER 5) and during hypoxia (CHAPTER 6). Unfortunately, our data do not provide a conclusive answer to the cause of hyperproliferation. With coculture, the replication rates were
decreased rather than increased, which can be attributed to NO that is known to suppress cell proliferation (7,10,18). With hypoxia, the replication rates were not significantly affected. These results imply that there must be another reason for the observed hyperproliferation after transplantation. The islet cell replication rates found of the control groups in vitro varied between 2% and 6%. This resembles the replication rates found after transplantation (4-6%), which is tenfold higher compared to the replication rate found in native pancreatic islets (0.65%) (5). The elevated replication rates in vitro are in accordance with previous findings in our laboratory, which showed that free and encapsulated islets have proliferation rates of 6.1% and 6.2%, respectively (5). The high replication in vitro may be explained by the relative high glucose concentration in the culture medium (8.3 mM). Sjoholm et al. demonstrated that islet cell proliferation correlated well with glucose concentration levels. Rat islets have a replication rate of 3.0% if cultured with 3.3 mM glucose and 5.8% if cultured with 11.1 mM glucose (20). In contrast to in vitro, hyperglycemia was not the cause of the hyperproliferation in vivo, since the increased replication was also found in an encapsulated islet graft transplanted in normoglycemic non-diabetic animals (5).

In our experimental studies in vitro, islet dysfunction and cell death occurred within a few days, whereas encapsulated islets in vivo survive for periods of several months and graft failure can be characterized as a gradual process. The discrepancy between in vitro and in vivo results may be explained by a difference between the oxygen tension during the culture experiments and the oxygen tension in the peritoneum. Although there is some debate on the actual oxygen tension in the peritoneum, it probably varies between 26 and 77 mmHg, which is similar to 3.5 and 10% O₂ in terms of air percentages, respectively. Coculture experiments were performed at normoxia, i.e. 20% O₂, which is higher than the oxygen tension in the peritoneum. Since macrophage function is reduced by low oxygen tension (15,19), the effect of overgrowth on neighboring capsules in vivo is probably less severe than as observed here in vitro. Hypoxia experiments were performed at a very low oxygen tension, i.e. 1% O₂. Consequently, islets, especially the inner core of large islets, most probably suffered from anoxia rather than hypoxia in our experiments. The observed loss of function and vitality caused by hypoxia is thus probably less outspoken in vivo than as observed here in vitro.

So far, we discussed factors that restrict the success of the microencapsulation technique. A variety of strategies may give a future perspective to the application of immunoprotective capsules in clinical practice. One such strategy may aim at the improvement of the immunoprotective properties of the capsules. Although the immunoprotection by the semi-permeable layer of microcapsules is limited, our permeability and coculture experiments show that
the passage of macrophage-derived cytokines can be inhibited. These studies provide support for the use of amino acid treatment of alginate beads. The one factor that remains small enough to enter the capsules with deleterious consequences is nitric oxide. An elegant strategy to establish additional protection in order to prevent NO-toxicity is to coencapsulate islets with cells that scavenge NO, like erythrocytes. Improved graft survival has been reported not only after coencapsulation of islets with erythrocytes (25), but also after coencapsulation with Sertoli cells (1,29). Sertoli cells are derived from the testis and have immunoprotective properties. Besides NO-toxicity, hypoxia contributes to encapsulated islet graft failure. The problem of hypoxia may be solved by transplantation to sites that permit closer contact with the blood circulation system. Such a site may well be artificially created and can be situated subcutaneously or intra-peritoneally (4,24). Another definite solution to the problem of hypoxia would be to use the natural resistance of Brockmann bodies. These are islet equivalents from the Tilapia fish, that live in stagnant water and whose metabolism is adapted to hypoxic conditions (26-28). If xenotransplantation is considered, piscine islets are also potentially ideal xenogeneic donors because of several additional reasons. Fish have minimal production costs, fish islets are easily isolated and 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 the porcine islets.

In conclusion, three major factors contribute to the limited graft survival of encapsulated islets. These are limited immunoprotective properties of the capsules, a lack of biocompatibility of the encapsulated islets, and an insufficient blood supply for the islets in microcapsules. To overcome these causes of failure, a combination of improvements is required. Our studies suggest that additional protection against nitric oxide may improve graft survival and that a solution for the oxygen deprivation may not only improve encapsulated islet function and vitality, but may also reduce the occurrence of overgrowth. Which combination of strategies in combination with encapsulation can fulfill the promise of providing a better and safer cure for diabetes alternative to insulin remains to be determined.
23. Sun Y., Ma X., Zhou D., Vacek I., and Sun A.M. Normalization of diabetes in spontaneously diabetic cynomologus monkeys by xenografts of
REFERENCES


