Journal of Surgical Research **121,** 141–150 (2004)
doi:10.1016/j.jss.2004.02.018

RESEARCH REVIEW

Causes of Limited Survival of Microencapsulated Pancreatic Islet Grafts

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Submitted for publication November 4, 2003

Successful transplantation of pancreatic tissue has been demonstrated to be an efficacious method of restoring glycemic control in type 1 diabetic patients. To establish graft acceptance patients require lifelong immunosuppression, which in turn is associated with severe deleterious side effects. Microencapsulation is a technique that enables the transplantation of pancreatic islets in the absence of immunosuppression by protecting the islet tissue through a mechanical barrier. This protection may even allow for the transplantation of animal tissue, which opens the perspective of using animal donors as a means to solve the problem of organ shortage. Microencapsulation is not yet applied in clinical practice, mainly because encapsulated islet graft survival is limited. In the present review we discuss the principal causes of microencapsulated islet graft failure, which are related to a lack of biocompatibility, limited immunoprotective properties, and hypoxia. Next to the causes of encapsulated islet graft failure we discuss possible improvements in the encapsulation technique and additional methods that could prolong encapsulated islet graft survival. Strategies that may well support encapsulated islet grafts include co-encapsulation of islets with Sertoli cells, the genetic modification of islet cells, the creation of an artificial implantation site, and the use of alternative donor sources. We conclude that encapsulation in combination with one or more of these additional strategies may well lead to a simple and safe transplantation therapy as a cure for diabetes. **© 2004 Elsevier Inc. All rights reserved.**

Key Words: **transplantation; pancreatic islets; microencapsulation; biocompatibility; permeability; macrophages; cytokines; NO; hypoxia.**

INTRODUCTION

Diabetes mellitus type 1 accounts for approximately 10% of all diabetic cases worldwide and is characterized by an absolute insulin deficiency. Insulin injection therapy as a treatment for type 1 diabetic patients is lifesaving, but it cannot fully prevent the development of complications of the eyes, kidneys, nerves, and the cardiovascular system including the microvessels in the limbs. The only replacement therapy that currently improves metabolic control other than conventional and intensive insulin therapy is transplantation of insulin-producing tissue. Transplantation can be performed either by implantation of the pancreatic organ or by implantation of only the pancreatic islets of Langerhans. Results of pancreas transplantation have steadily improved with time from a 1-year pancreas graft survival of 75% at the end of the eighties to the current patient and graft survival rates of approximately 98 and 85%, respectively [1, 2]. Results of islet transplantation were far less favorable during that period. The international Islet Transplant Registry reported that fewer than 12% of the islet allografts from 1990 to 2000 remained insulin-free for 1 year [3]. But at the beginning of this century Shapiro *et al*. demonstrated that islet transplantation can be as successful as pancreas transplantation [4]. The success of their Edmonton protocol has renewed a worldwide interest in pancreatic islet transplantation, which has two principal advantages when compared to pancreas transplantation. First, it does not require major surgery, but only a small implantation procedure with which an islet mass is delivered to the liver by intraportal infu-

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0022-4804/04 $30.00
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sion. Second, islet tissue has the advantage that it may be modulated prior to implantation to reduce the risk of rejection.

A major obstacle for both pancreas transplantation and pancreatic islet transplantation is the requirement of immunosuppressive drugs to establish graft acceptance. Immunosuppression is associated with deleterious side effects, such as increased susceptibility to viral, fungal, and bacterial infections, and increased risk (4- to up to 500-fold) for the development of malignancies [5, 6]. For this reason, transplantation of either a pancreas or pancreatic islet tissue has been restricted to patients for whom the adverse effects of immunosuppression outweigh the risks associated with further development of diabetic complications. As a practical consequence, transplantation of pancreas or islets is mainly restricted to diabetic recipients of a renal transplant on the basis of severe diabetic nephropathy and end stage renal failure, since they already receive immunosuppression for their kidney graft [7]. Pancreas transplantation alone is being performed with an increasing frequency and with increasing success in type 1 diabetic patients without nephropathy, but with recurrent episodes of hypoglycemic unawareness, to restore normoglycemia [1].

Another obstacle to the widespread application of pancreas or islet transplantation is the worldwide shortage of organ donors. Even without deleterious immunosuppressive protocols, only 0.1% of the type 1 diabetic population could be transplanted with the currently limited supply of donor organs [8]. The use of donor organs is especially inefficient with islet transplantation, since successful islet transplantation requires multiple (two to four) donors per recipient [9]. It can therefore be argued that islet transplantation cannot become a standard treatment modality for people with type 1 diabetes until graft acceptance can be established without deleterious side effects for the recipient and until a plentiful source of islets can be identified.

One strategy that may provide a solution both to the problems associated with immunosuppression and to the problem of organ shortage is immunoprotection by encapsulation. This technique aims to protect tissue or cells against immune cell- and antibody-mediated rejection by separation of the transplanted tissue from the host by enveloping the graft in a semipermeable capsule as a mechanical barrier. Immunoprotection by encapsulation enables transplantation without immunosuppressive drugs and opens up the perspective of using animal donor sources. Despite some promising results in animal studies, graft survival of immunoprotected grafts is still too short to introduce this technology into clinical practice.

In this review we discuss the principal causes that limit the success of immunoprotection by encapsulation of pancreatic islets, with specific focus on microencapsulation.

IMMUNOPROTECTION BY ENCAPSULATION

With immunoprotection by encapsulation, islets are enclosed in a matrix surrounded by a semipermeable membrane, which allows for the passage of small molecules like insulin and glucose, but not for the entry of the much larger cells and antibodies of the immune system (Fig. 1). Such a physical barrier can thus prevent allograft rejection, which depends on recognition of the MHC by host lymphocytes. Furthermore it can prevent antibody-mediated cytotoxicity, which plays a role in the autoimmune destruction of β cells, as well as in allo- and xenograft rejection [10, 11]. Immunoprotection by encapsulation can thus enable transplantation of islet tissue in the absence of immunosuppression. Since immunoprotection may prevent xenograft rejection, it also opens up the perspective of transplanting animal tissue. Dilemmas with regard to ethics and the risk of viral infections have restricted the use of animal tissue for human transplantation purposes thus far and may eventually prevent a common application of xenotransplantation in the future. These matters of debate, however, have not prevented the search for a successful encapsulation system. This research is driven by the potential contribution of the technique 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 from being in contact with cells is approximately 50 years old [12]. Many different kinds of encapsulation systems have been studied since and they are generally divided into three categories [13]. Devices of the first category are characterized as intravascular macrocapsules, which are usually perifusion 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 disk that can be implanted intraperitoneally or subcutaneously. The third category is extravascular and involves not macro- but microcapsules, which—depending on the size and the number of capsules—can be implanted in several different sites in 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 [14]. Cells are enclosed in an alginate core, which is covered by poly-L-lysine (PLL), a polyamino acid that gives the microcapsules semipermeable properties (Fig. 1). The PLL layer can be modified, which makes it possible to achieve many different grades of permeability [15, 16]. PLL is also important for microcapsule stability and it is absolutely required

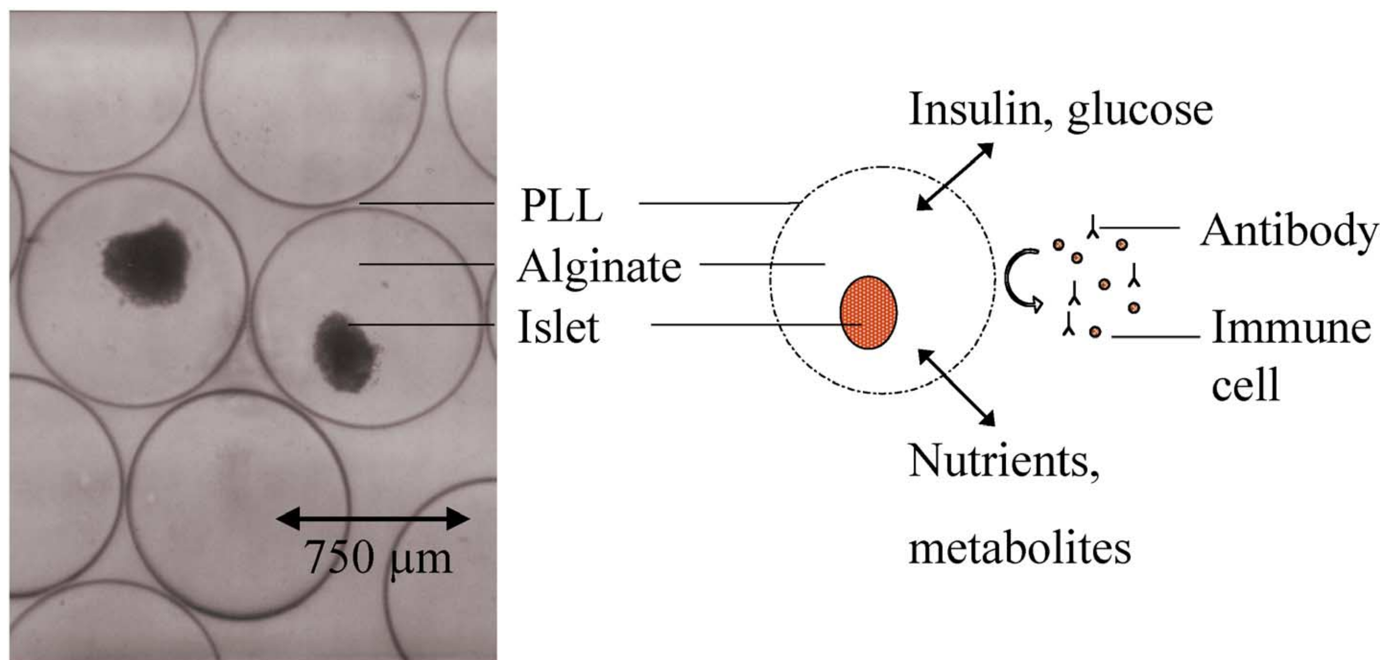

FIG. 1. Encapsulated pancreatic rat islets and the concept of microencapsulation. The poly-L-lysine (PLL) layer of microcapsules prevents the entrance of immune cells and antibodies, while it allows the passage of insulin, glucose, and nutrients. (Color version of figure is available online.)

for the integrity of Ca^{2+}–alginate capsules. A second layer that consists of alginate is applied for coverage of the unbound PLL groups.

Alginate is a component of the extracellular matrix of brown algae and consists of the polysaccharides β-D-manuronic acid (M) and 1,4-linked α-L-guluronic acid (G). Raw alginate can be purified and sterilized to a biocompatible 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, such as 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 alginates 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 microcapsules [17, 18]. Second, better PLL binding means less nonbound 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 [19, 20]. Alternatives for alginate are polyethylene glycol [21], polyacrylates [22], agarose [23], and chitosan [24], and multicomponent capsules have also been applied [25], 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 [26, 27]. Successful function of encapsulated hepatocytes after transplantation in animals has been documented [28, 29]. Microencapsulated parathyroid tissue has been transplanted with success in animals and recently even in humans [30, 31]. Possibly, encapsulation can also be used for the treatment of neurodegenerative diseases, such as Parkinson's and Huntington's diseases [32, 33]. Here, we focus on transplantation of microencapsulated pancreatic islets, which has been performed in rats [34–36], mice [37], dogs [38, 39], and monkeys [40]. Microencapsulated islets are always implanted in the peritoneal cavity. This is the only implantation site that is large enough to accommodate a microencapsulated islet graft, which consists of several thousand 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 that of non-encapsulated islets, but the duration of euglycemia was unfortunately limited to periods varying from several months in rats [36] until up to 6 months in dogs [38]. In 1994, Soon Shiong *et al.* reported insulin independence in a type 1 diabetic patient after microencapsulated islet transplantation

[41]. 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 5000 human islets/kg 6 months after the first implantation. 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 8th month, which confirms sustained insulin secretion from the encapsulated islets. The patient subsequently returned to exogenous insulin therapy and with another supplemental dose of 5000 human islets/kg at 33 months ongoing islet function with tight glycemic control was reported for 58 months [42]. This report is the only well-documented study of transplantation of microencapsulated islets in humans. Although this case and the animal studies illustrate the potential applicability of the microencapsulation technique, graft survival is too limited for use of microencapsulated islets to become a widespread treatment in clinical practice at present. On the other hand, microencapsulated islet transplantation therapy could aim for repeated transplantation during the life span of a patient. This, however, is 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 microencapsulated islet graft failure may help in finding a way to improve graft survival. One important observation is that microencapsulated autograft and allograft survival rates are similar, which implies that graft failure is not caused by rejection due to allograft recognition [43]. 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 nonprogressive overgrowth of a portion of the microencapsulated islet graft [36]. Three important aspects of the microencapsulated islet graft technique may be associated with these phenomena. The first is related to the biocompatibility of the graft. A number of microcapsules lack biocompatibility, which explains the occurrence of overgrowth. The second is related to the immunoprotective properties of the microcapsules. Immunoprotection is incomplete because capsules may allow the passage of small proinflammatory factors, which lead to cell death and dysfunction. The third factor is related to the great distance between the encapsulated islets and the blood supply. An important consequence of the great diffusion distance is the limited supply of oxygen, which leads to hypoxia, causes islet dysfunction and necrosis, and may be responsible for the increase in islet cell replication. Lack of biocompatibility, limited immunoprotection, and hypoxia are issues discussed in further detail in the next sections.

BIOCOMPATIBILITY

Pericapsular overgrowth of microcapsules due to lack of biocompatibility is responsible for the loss of part of a graft. Overgrowth on microcapsules is established within the first few weeks after transplantation and does not increase thereafter [36, 44, 45]. Only a small portion of approximately 10% of a retrieved encapsulated islet graft is affected by overgrowth with fibroblasts and macrophages [36, 46]. However, a much higher percentage of approximately 40% of the number of initially implanted islets is lost due to overgrowth [36]. 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 such as endotoxins and polyphenols) and sterilization of alginate with an intermediate G composition results in optimal biocompatibility, leaving lack of integrity of the graft as the main cause of cellular overgrowth [43, 47]. Lack of integrity is characterized by breakage due to capsule instability and by physical irregularities on the capsule surface (Fig. 2). Irregularities such as 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 [43, 48]. Physical irregularities are mainly the consequence of inadequately encapsulated islets [49, 50]. The occurrence of inadequately encapsulated islets strongly depends on the microcapsule diameter, i.e., small capsules contain more protruding islets [50, 51]. Protrusion of islets due to inadequate encapsulation in 750-μm microcapsules is estimated to be responsible for approximately 10% of the overgrowth of an encapsulated islet graft [46, 50, 51].

Physical irregularities give an explanation for the occurrence of overgrowth on a number of encapsulated islets, but do not explain why 40% of the initially implanted islets are lost as a consequence of overgrowth. Apparently, even perfectly smooth islet containing microcapsules 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 [52, 53] and can collectively be defined as chemotaxis (Fig. 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. Local attraction of macrophages by shedding is more apparent for xenografts than for allografts, since virtually every protein shed

FIG. 2. Cellular overgrowth of microcapsules is caused by physical irregularities (i.e., tails, craters, and protruding islets) and by islet-derived factors ("chemotaxis"). (Color version of figure is available online.)

by a xenograft is different from the host [52, 54, 55]. Xenogenic antigen release, especially the release of α-1,3-galactose, attracts and activates macrophages, which in turn release cytokines such as Il-1β, TNF-α, and IFN-γ, but also nitric oxide and oxygen radicals [54, 56]. These macrophage-derived factors are small enough to pass the semipermeable membrane of microcapsules and may well affect encapsulated islet graft function and vitality. Recently, α-1,3-galactosyltransferase-deficient pigs have been produced, which is an important step toward the reduction of xenograft rejection and may significantly contribute to the realization of pig-to-human transplantation [57]. The second chemotactic pathway involved in the attraction of macro-

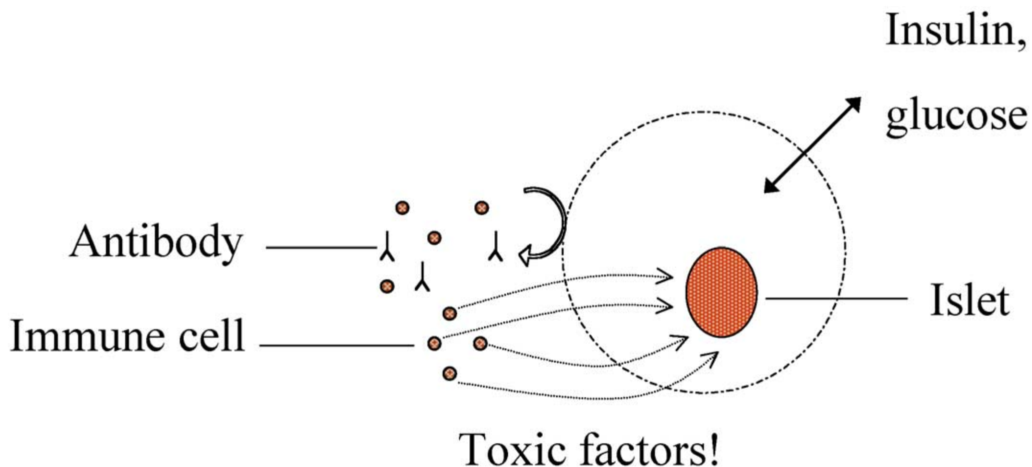

FIG. 3. Microcapsules have limited immunoprotective properties. The capsule membrane allows not only the passage of insulin and glucose, but also of small toxic factors such as cytokines and nitric oxide. (Color version of figure is available online.)

phages is not characterized by the passive leakage of waste products, but by the active production of chemoattractant factors by islets. Such factors are called chemokines and they are typically induced by primary pro-inflammatory mediators such as interleukin-1 and tumor necrosis factor [58]. One candidate chemokine that is expressed in pancreatic β cells and also involved the attraction of macrophages after non-encapsulated islet transplantation is MCP-1 (monocyte chemoattractant protein 1) [59, 60]. MCP-1 is a small molecule (12 kDa) that can pass through the semipermeable membrane of microcapsules and may thus stimulate the attraction of macrophages by islets within microcapsules [61]. Chemotaxis may thus be responsible for the occurrence of overgrowth that cannot be explained by physical imperfections, nor by rejection.

Further reduction in the occurrence of overgrowth can improve encapsulated islet graft survival, not only increasing the functional volume of the graft, but also reducing the deleterious effects of overgrowth on neighboring non-overgrown encapsulated islets. In one of our studies of the causes of encapsulated islet graft failure we showed that the close proximity of overgrown microcapsules leads to islet dysfunction and necrosis in neighboring non-overgrown encapsulated islets [62]. Overgrowth due to irregularities on the capsule surface may be solved by technical improvements in capsule integrity. One such improvement may be to use Ba^{2+} instead of Ca^{2+} during the solidification step of the microencapsulation procedure. Ba^{2+} capsules have a high mechanical stability without PLL, a major advantage compared to the Ca^{2+} capsules, which require PLL to retain capsule integrity [63]. Consequently, Ba^{2+} capsules with imperfections, caused by tails, craters, inadequately encapsulated islets, or broken capsules, do not necessarily evoke an inflammatory response due to incomplete PLL coverage. Successful transplantation with low occurrence of overgrowth of islet containing Ba^{2+} beads has repeatedly been reported [64–67]. However, the ability of Ca^{2+} microcapsules with PLL to protect against deleterious effects of cytokines provides support for the use of polyamino acid treatment of alginate capsules [18]. Further reduction of overgrowth can be achieved by the removal of microcapsules with surface irregularities prior to implantation. A method such as the lectin-binding assay that has been developed in our laboratory may be an appropriate tool for identifying incomplete microencapsulated islets prior to transplantation [50].

IMMUNOPROTECTIVE PROPERTIES

A second factor that contributes to encapsulated islet graft failure is the limited immunoprotection of microcapsules. The semipermeable PLL layer effectively prevents the passage of large cells and antibodies of the immune system. However, small molecules such as cytokines or radicals may still enter microcapsules (Fig. 3) [68, 69]. Nitric oxide (NO) and cytokines such as Il-1β (17.5 kDa), TNF-α (51 kDa), and IFN-γ (81 kDa) are examples of factors that have been shown to exert deleterious effects on β cell function and vitality [70–74]. Activated, but not resident, macrophages are the most important source of these factors [75]. *In vivo* experiments support the role of macrophages as contributors to encapsulated islet graft failure. Depletion of peritoneal macrophages by 15-deoxyspergualin or by clodronate liposomes distinctly improved encapsulated islet graft survival [56, 76]. Results from our laboratory suggest that NO rather than cytokines exerts the deleterious effects of macrophages [62]. Apparently the semipermeable PLL membrane can prevent the passage of cytokines such as Il-1β and TNF-α [18]. These observations are in line with those from a study by Wiegand *et al*., who showed that both inhibition of NO formation and scavenging of NO can protect encapsulated islets from destruction by activated macrophages [75]. Since NO is too small to prevent its passage across the capsule membrane without affecting insulin secretion, additional means of protection against NO toxicity are required. Encapsulation offers the possibility of co-encapsulating islets with other cell types that may provide improved protection and can support islet function. Co-encapsulation with autologous erythrocytes was found to be an effective and easy way of providing protection against macrophage-mediated lysis [75]. Also, co-encapsulation with Sertoli cells, which release immunosuppressive factors, made possible significantly prolonged islet graft survival times [77, 78]. This approach is an alternative for combining encapsulation with transient local immunosuppressive medication to suppress NO production by macrophages to fully protect the graft from immune destruction.

An alternative approach to providing additional protection is by means of genetic engineering, which opens up the perspective of specifically modulating 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 of islet cells with the anti-celldeath protein Bcl-2 prevents cytokine- and NO-induced cell death [79–81]. Therefore genetic engineering in combination with encapsulation may well offer sufficient immunoprotection.

HYPOXIA

A third factor that contributes to 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 revascularization of islets, which normally occurs within the first few weeks after

non-encapsulated islet transplantation into the portal vein. Encapsulated islets suffer from irreversible and chronic hypoxic stress, because revascularization cannot occur and since the supply of oxygen through the peritoneum is by passive diffusion only, instead of direct delivery from the blood stream. Not only oxygen supply but also insulin delivery is hampered by passive diffusion through the peritoneum. Results from our laboratory show that limited diffusion leads to reduced insulin secretory responses [82, 83]. As a consequence, successful reversal of diabetes in rats requires a two to four times higher islet mass for an encapsulated islet graft in the peritoneum than that for a non-encapsulated islet graft under the kidney capsule [84]. 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 only a portion of the graft. Results from our laboratory showed that encapsulated islets respond to hypoxia by increasing their MCP-1 mRNA expression [61]. Analysis of mRNA expression was enabled by a novel method that effectively removes capsules from islets by means of trypsin and EDTA [85]. High MCP-1 expression can be understood as a signal of islets to promote angiogenesis to resolve a lack of oxygen [86]. In a transplantation setting, however, the chemotactic activity of MCP-1 may contribute to graft failure by attracting cytokine-producing macrophages [60]. This increase in MCP-1 is more likely to occur in large islets, since they suffer more from hypoxia than small islets. Strategies to eliminate hypoxia are thus important, not only to improve islet function and vitality, but also to reduce the attraction of macrophages by encapsulated islets.

A possible solution to the problem of hypoxia is to modify the resistance of islet tissue to hypoxia and thus prolong graft survival. An increase in the resistance of islets to hypoxia can be induced prior to transplantation by means of ischemic preconditioning [87, 88] or by heat shock [89]. Another option is to stimulate the expression of Bcl-2, Bcl-xL, as well as ICE-like proteases, which have been reported to effectively retard chemical hypoxia-induced necrotic cell death [90]. Such treatments merely provide temporary salvation. They can be of assistance in bridging a short period of hypoxia, but are not sufficient to realize long-term resistance to hypoxia, as required for an optimal mode of encapsulated islet transplantation in an unmodified peritoneum. A more permanent solution would be to use the natural resistance to hypoxia of Brockman bodies [91]. These islet equivalents can be isolated from tilapia fish that are adapted to living in stagnant hypoxic water. Additional advantages of Brockman bodies are minimal production costs and the possible decreased risk of zoonosis [92].

Another definitive solution to the problem of limited diffusion could be the implantation of the encapsulated islet graft into a transplant site that permits close contact between the bloodstream and islet tissue. The most successful transplantation site for non-encapsulated islets is the liver. Therefore, Leblond *et al*. studied the possibility of transplanting small (~315 μm) APA microcapsules into the liver [93]. They showed that intrahepatic implantation of small empty capsules is feasible and safe, but whether implantation remains successful after the 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 [51, 84]. Robitaille *et al*. studied the possibility of using the epididymal fat pads as an implantation site for microcapsules, but its application is hampered by a pericapsular reaction [94, 95]. An alternative approach is to create a well-vascularized implantation site in the abdominal cavity or under the skin. In our laboratory, a prevascularized expanded polytetrafluorethylene 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 [96]. Wang *et al*. used a prevascularized subcutaneous site for implantation of macroencapsulated islets and reported normalization of blood glucose levels for approximately 100 days [97]. 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 of damage to the graft.

FINAL REMARKS

Islet transplantation is a cure for diabetes with limitations caused by risks associated with immunosuppression and donor organ shortage. Microencapsulation provides a means to transplant islets without immunosuppressive agents and may enable the performance of xenotransplantation. We have presented our view of the principal causes of microencapsulated islet graft failure, which are related to a lack of biocompatibility, limited immunoprotection, and hypoxia. A variety of strategies, such as genetic engineering, co-encapsulation, the use of alternative donor sources, improvement in oxygen supply, or the establishment of hypoxia resistance, may give a future perspective to the application of immunoprotective capsules in clinical practice. It remains to be determined which combination of strategies with encapsulation can fulfill the promise of establishing a simple and safe transplantation therapy as a cure for diabetes.

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