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Engraftment and Growth of Transplanted Pancreatic Islets

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ABSTRACT

Transplantation of pancreatic islets may provide a cure for type 1 diabetes. How–ever, this treatment can currently be offered only to very few patients. To improve transplantation success we need to understand better the mechanisms of how the implanted islets survive, grow and/or maintain adequate function. We herein report on our studies to evaluate the factors responsible for the engraftment, *i.e.* revascularization, reinnervation *etc.*, of transplanted islets and relate these factors to the metabolism and growth of the islets. Graft metabolism can be monitored by microdialysis probes that allow for the measurement of minute amounts of islet metabolites and hormonal products. Growth of the endocrine cells can be stimulated both *in vitro* before implantation and *in vivo* post-transplantation. Another problem is rejection of transplanted islets, which may be overcome by the microencapsulation of islets. The knowledge gained by the present studies will enable us to elucidate the optimal treatment of islets to ensure a maximal survival of the transplanted islets, and may be applied also to clinical islet transplantation.

INTRODUCTION

Transplantation of insulin-producing β-cells, either as a whole pancreas graft or as isolated islets of Langerhans, is the only currently treatment available that can lead to insulin independence in patients with type 1 diabetes. The results after whole pancreas implantation are good, with a 1-year graft survival of 85–90% (54), whereas many of the islet transplants fail within a time span of a few weeks or months. Most islet transplants (>90%) have failed within 1 year (34). The reasons for this appalling degree of functional failures after islet transplantation compared with those of the whole organ are largely unknown, although chronic rejection or recurrence of autoimmune disease have been suggested to be contributing factors (20,39,97). Another important issue may be the presence of hyperglycaemia after transplantation, since this has been shown to be detrimental for islet graft survival and function (36,45,48, 63).

Since human islet transplantation has been only moderately successful, it is encouraging that experimental islet transplantation in rodents and larger animals has a high degree of success (*cf.* 51). In humans, the morbidity and surgical complications associated with islet transplantation are almost negligible (34), whilst whole pancreas transplants are technically much more difficult to perform, and more serious complications can arise (54). Substituting whole-organ grafting with islet transplantations would, therefore, be desirable, provided that an improvement of the results could be achieved. It is noteworthy that state-of-the-art islet implantations, *i.e.* those fulfilling strict criteria for preparation of donor material, have a much higher success rate, approaching 40% 1-year graft survival, than that reported in the Islet Transplant Registry (see 39). These findings reinforce the notion that islet implantations can become a treatment of choice, at least for some diabetic patients, provided that, among other factors, a sufficient amount of viable donor material can be obtained.

During the last two decades, research within the fields of experimental and clinical islet transplantation has been performed at the Department of Medical Cell Biology at Uppsala University. Our research has involved islets from both foetal and adult donors, from humans as well as rodents, and has been focussed mainly on three subjects of major importance for the survival and functional maturation of the graft, *viz.* engraftment, growth/differentiation and immunoisolation with microcapsules.

ENGRAFTMENT OF TRANSPLANTED ISLETS

In contrast to whole-organ transplantations where direct vascular anastomoses are performed, implantations of cells or cell aggregates necessitate the adaptation of the implant to the surrounding tissues in the recipient. An adequate supply of nourishment and oxygen must be secured, as well as incorporation of the transplant in the "milieu interieur" of the new organism. This engraftment process is crucial, since it dictates the extent to which transplanted β-cells survive. It should be noted that most experiments referred to below have been performed in syngeneic animal models. The influence of immunosuppressive drugs and rejection episodes is excluded from these evaluations, which should be kept in mind when interpreting the findings. One indication of the importance of these factors is the much higher success rate of autotransplantations of islets when compared with allotransplantations in humans (34).

Nutritive blood flow can be re-established by connecting the graft to the vascular system of the recipient by angiogenesis, *i.e.* the formation of new blood vessels from pre-existing cells in the adjacent microvasculature (27). Whether vasculogenesis, *i.e.* the formation of blood vessels from immature precursor cells, contributes to islet graft revascularization is at present unknown. It cannot be ruled out, however, that such blood vessel formation is of some importance, especially in foetal grafts.

Re-establishing revascularization obviously makes the graft easily influenced by

humoral control mechanisms exerted by the recipient. In contrast, a total exogenous denervation occurs after all types of transplantation. There is some debate as to whether a functionally important reinnervation can occur after transplantation. Although some ingrowth of sympathetic nerves along the adventitia of afferent blood vessels may occur in transplanted whole organs (96), this is less likely to occur after implantation of cells or cell aggregates, disregarding nerve cell transplants. Furthermore, denervation may alter the sensitivity of the graft to transmitter substances released by adjacent neurons, *e.g.* denervation hypersensitivity (19,23). In addition to revascularization and reinnervation, the formation of a new connective tissue stroma, as well as the adaptation of grafted single cells to their new environment is important for the functional outcome of cellular transplantations. With the exception of bone marrow transplantations, very little is known about these aspects of engraftment.

Revascularization of islet grafts

The normal morphology of the pancreatic islet is highly complex, with at least four different endocrine cell types arranged around a sinusoidal network of fenestrated capillaries. The capillaries are fundamentally important, not only for delivery of oxygen and nutrients to the endocrine cells, but also to provide humoral signals from other cells in the body, as well as disposing of the secreted hormones. The islet cells have been suggested to be organised so that the arterial blood first reaches the β-cells, then the α- and δ-cells, respectively (10,73). However, other theories have also been proposed (*cf.* 11). This centrifugal direction of the blood flow has been claimed to be essential for normal islet function, since high local hormone concentrations can either stimulate or suppress the secretion of the "downstream" endocrine cells (73). The effluent blood vessels consist of both veins and of an insulo-acinar portal system, comprising small vessels connecting the islet capillaries with capillaries in the exocrine parenchyma. The extent of this portal system is species-dependent, and in rodents it seems as if large islets mainly possess venules, that empty directly into intra-lobular veins (10). The regulation of islet blood perfusion comprises a complex interaction between nervous, metabolic, hormonal and autocrine signals (see 35), allowing for remarkable versatility in islet blood flow in response to exogenous stimuli.

Islet transplantation severely disturbs the complex anatomical architecture. Not only are the vascular and nervous connections interrupted, but the pre-transplant culture period leads to the de-differentiation or death of both endothelial cells and most nervous elements (18,66). Angiogenesis leading to the formation of a new graft vasculature occurs rapidly, and 1 week after transplantation there is a glomerular-like capillary network similar to that in native islets after transplantation of either adult or foetal islets (5,44,61,72,74,90). The newly formed vessels acquire morphological features characteristic of fenestrated islet endothelium, which is probably independent of the implantation organ, *i.e.* the origin of the newly formed microvessels (32,44,57,90). To what extent revascularization varies between different implantation sites is, however, essentially unknown. It is also unknown to

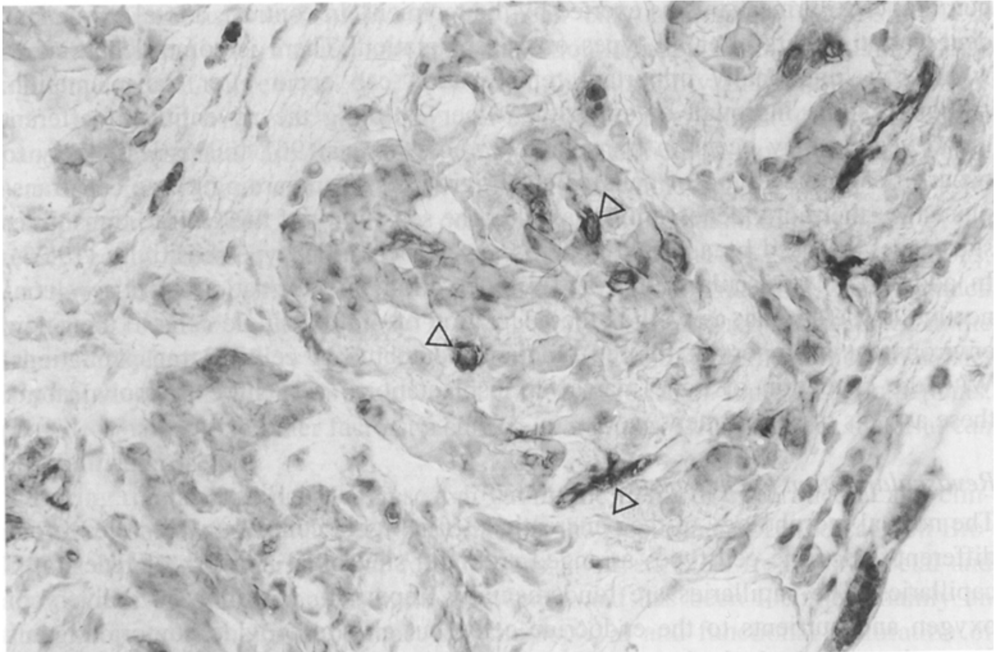

Figure 1. Syngeneic islet graft under the renal capsule 4 weeks after implantation. The endothelium is stained with BS-1 (arrows). Magnification ?X.

what extent, if any, vasculogenesis, *i.e.* formation of blood vessels from immature progenitor cells, participates in the formation of new blood vessels in grafts. It can be envisaged that this process is of some importance especially when grafting foetal islets, which are likely to contain more immature precursor cells. One indication of this is our recent finding (44) that so called intussusceptive capillary growth of new capillaries is seen after implantation of foetal islet like cell-clusters (ICC). This phenomenon, i.e. the growth of tissue pillars into pre-existing microvessels rather than a growth of blood vessels *per se*, has been documented mainly in foetal organs (12).

Evaluating the degree of revascularization of transplants has been a problem because of the difficulty in selectively staining for microvascular endothelial cells in rodents. In some studies staining for von Willebrand factor has been applied (21,59,60), but this necessitates the use of cryostat sections. Enzyme histochemical stainings for alkaline phosphatases have been used, but they are tedious to perform and interpreting findings is difficult, since the islet d-cells are also stained (84). We have evaluated the possibility of using other markers, and have found that the lectin Bandeiraea simplicifolia (BS-1; 53) provides reliable stainings of islet microvascular endothelium (Figure 1). This allow appropriate morphometrical investigations of the degree of revascularization at different times after implantation at various grafting sites to be performed.

Due to methodological problems the blood perfusion of transplanted islets has been difficult to evaluate. Using the laser-Doppler technique, a blood perfusion similar to, or even higher, than that of the adjacent renal cortex has been recorded in foetal porcine ICC implanted under the kidney capsule (49,74). Using a microsphere technique 4-weeks transplantation adult rat islets autotransplanted at this site were found to have a blood perfusion of the same order of magnitude as native islets in the pancreas (37). In contrast, after syngeneic rat islet transplantations under the renal capsule, a graft blood flow of 10–50% of that seen in native islets has been observed >1 month post-transplantation (16,17). Similar results have also been noted after implantation of human islets into athymic mice (unpublished observation). Since the autotransplanted animals were subjected to a partial pancreatectomy before the measurements, the increased functional demand on the grafts in these animals may have interfered with the results. All these results suggest, irrespective of the actual flow values obtained, *pro primo* that graft blood perfusion is regulated separately from that of the implantation organ, and *pro secundo* that it is only to a small extent responsive to normal regulatory mechanisms. However, a maturation of this occurs with time (37,49).

We have recently described an interesting feature of native islet capillaries, namely their low capillary blood pressure, which is approximately 50% of that seen in adjacent exocrine capillaries, *viz.* 3 mm Hg (Figure 2; 15). The newly formed capillaries of transplanted pancreatic islets acquire a blood pressure similar to that of the implantation organ, both when implanted into the liver, spleen or under the renal capsule (14, unpublished observation). This suggests that transplanted islets can be exposed to a capillary hypertension, depending on the choice of the implantation organ. Experimental data suggests that a chronic increase in capillary pressure can be of pathophysiological significance for diabetes-induced functional impairment of several organs, e.g. retina and kidneys, by causing damage to the blood vessels (100). We are investigating to what extent this also occurs in transplanted islets.

The partial pressure of oxygen is much lower in islets grafted under the renal capsule than in native islets (16), and remains so for up to 9 months post-transplantation (17). Moreover, this also seems to be the case for islets implanted into the spleen or liver (unpublished observation). Some of the newly formed blood vessels, however, seem to be incomplete, and consist of non-endothelialized, blood filled cavities (57). These structures have been referred to as "blood lakes" (see 51), but their true nature is still obscure. Because they lack a distinct wall structure, it is unlikely that they represent a blood vessel type amenable to regulation. Their presence may explain in part the impaired blood flow regulation through islet grafts. However, the incomplete blood vessels may also reflect the low graft oxygen tension *per se*. Indeed, it has previously been demonstrated that prolonged hypoxia alters endothelial barrier function (2). Whether the intra-graft non-endothelialized structures may later develop into normal microvessels is unknown, but their number decreases with time after implantation (57). This creates the exciting possibility that this process may be affected by *e.g.* treatment with angiogenesis-stimulating factors, which would then lead to an earlier maturation of the graft vasculature, and

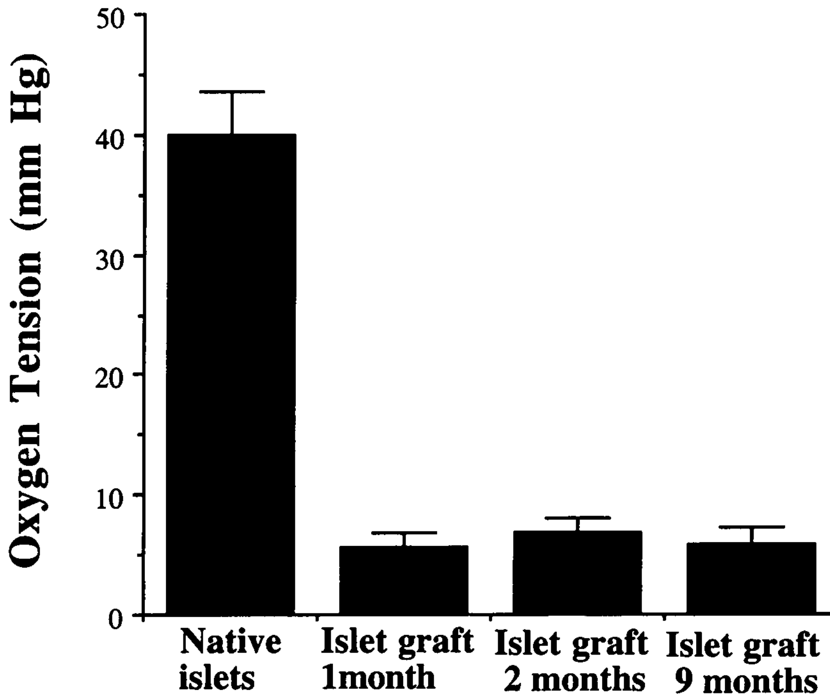

Figure 2: Partial pressures of oxygen in native and transplanted islets at different times after implantation. Values are means ± SEM of 7–10 experiments. Modified from Carlsson et al (2000).

thereby an increased delivery of oxygen to the tissues. Previous positive results after local administration of angiogenesis stimulators (33,83), together with the observation that vascular endothelial growth factor expression is increased in isolated islets (29,92), suggest that this area is important for our understanding of the engraftment process.

Reinnervation of islet grafts

Studies on the reinnervation of transplanted islets have mainly focussed on demonstrating nerve fibres in the grafted islets with immunohistochemical techniques. The nerve fibres associated with islet grafts were found to be mainly sympathetic, but both cholinergic and peptidergic nerves were also seen. The nerve fibres developed slowly over time, with a maximum observed 4 months post-transplantation (30, 42,46,67,70), and a full maturation occurring in foetal grafts not earlier than >12 months posttransplantation (49). Also an earlier development of intrinsic nerves within the first post-implantation weeks has been observed (67,68). A unique property of islet grafts is that the presence of β-cells within the grafts is a prerequisite for the occurrence of reinnervation (64). In comparison, there was no reinnervation when grafts consisting only of α- and δ-cells were implanted, sug-

gesting that signals from b-cells are necessary for reinnervation to occur (64). The nature of this signal(s) is not yet known. It should be noted that revascularization of islet grafts takes place irrespective of the cellular composition of islet transplants (8).

Somewhat surprisingly, recent studies have demonstrated that neuronal cells survive both the isolation and implantation procedure, and are able to re-establish connections with extrinsic nerves as well as develop an intrinsic nervous system (67,68). An even larger number of neurons survive after transplantation of foetal tissues, and also in these grafts a neuronal network develops (see 1). In view of these findings it is obvious that transplanted islets become reinnervated, but the long-term functional importance remains unknown.

It was initially suggested that transplantation might confer a denervation hypersensitivity to the implanted islets (69). Recent studies, however, have shown that transplantation instead induces an impaired sensitivity to both noradrenaline and cholinergic substances (78,79,80). These experiments have been limited to adult islets implanted under the renal capsule of syngeneic mice. However, experiments with human islets implanted into nude, athymic mice have demonstrated similar phenomena (Shi, Täljedal and Andersson; unpublished observations). The extent to which the species, choice of implantation site or the age of the donor influences graft neuronal transmitter receptor numbers and subtypes remains unknown. It seems nevertheless clear that the initial denervation can change the sensitivity to neurotransmitter substances, and thereby also influence graft function.

Metabolism of islet grafts

The disturbances in the normal cellular architecture of the transplanted islets most likely impose a considerable risk for metabolic disturbances, leading to an impaired endocrine function. The metabolism of grafted islets has been studied previously (22,36,43,47), but the importance of the initial hypoxia induced by lack of blood microvessels has been difficult to ascertain, mainly due to methodological problems. We, and others, have recently performed studies with microelectrodes which are implanted into native or grafted islets (16,17,77) to characterise, at the cellular level, the oxygen tension and metabolite concentrations under different functional conditions. In a similar way, it is possible to position a microdialysis probe (cf. 52) within an islet transplant for prolonged time periods. The graft function can thereby be monitored at regular time intervals. Preliminary experiments with this microdialysis technique have been successful, and we have obtained information on the metabolism of grafted islets in relation to their blood perfusion. It may also be that the presence of other relevant substances of suitable molecular sizes, *e.g.* hormones and some cytokines can be assessed with this technique. However, other challenges remain, such as the extent to which a prevailing hyperglycaemia after islet implantation affects engraftment, and thereby also the release of insulin (often referred to as "glucose toxicity"; 36). In this context, we have previously shown that human islets are more susceptible than rodent islets to a prolonged period of hyperglycaemia (36).

GROWTH OF TRANSPLANTED PANCREATIC ISLETS

One of the major obstacles for clinical islet transplantation is the lack of donors. Thus, increasing the number of β-cells recovered from each donor by refining procurement and islet isolation techniques is important (see 18). Our studies of the engraftment process aim to optimise the number of endocrine cells surviving the implantation procedure. Another possibility to increase the amount of material accessible for transplantation is to stimulate the growth and/or differentiation of β-cells *ex vivo* or *in vivo* (3,7,9,18,65,85), or to genetically engineer insulin producing cells for transplantation (99).

We have developed techniques to isolate foetal porcine islet-like cell clusters (ICC; 50), and have also studied the differentiation and function of these grafts in different animal models (*e.g.* 47,76) and after transplantations into humans (31). The time required for graft function to occur is consistently much longer than after implantation of adult islets, *viz.* 5–6 weeks vs. 2–3 days. However, the growth potential is pronounced, and the differentiation process can be affected both *ex vivo* before implantation and *in vivo* after transplantation (76).

Our research has been extensively focussed on the growth of transplanted islets, and effects of genetic and environmental modifications have been carefully analysed in different mouse strains (*e.g.* 4,6,24,58,86,87,88,98). These studies have demonstrated that mature b-cells retain an ability to proliferate, albeit at a low pace, with approximately 0.1–0.2% of them proceeding through the cell cycle at a given moment. Of interest is that this can be affected by the post-transplantation environment, which opens up the possibilities to influence the size and composition of the grafts. Much of this research has been carried out with human islets, and such experiments have been performed in collaboration with the Central Unit of β-cell Transplant at Vrije Universiteit, Brussels, Belgium. The human islets have been implanted either into athymic nude mice, or into immune-competent rodents using an immunosuppressive protocol with repeated injections of antilymphocyte serum (for details, see 87). Our experiments have demonstrated that human islets have a low, but significant, proliferative activity both *in vitro* and *in vivo* after transplantation (Figure 3). After implantation of human islets into lean (-/?) or obese (*ob/o*b) C57Bl/6 mice, an animal model associated with a pronounced stimulation of β-cell growth (6), both duct cell and β-cell proliferation was stimulated by the obese-hyperglycaemic environment (88,89). Interestingly, the replication of transplanted human duct cells was most stimulated, in that a fourfold increase of the labelling index of such cells was observed in the *ob/ob* mice (88). Similar findings were made in recipient mice undergoing nephrectomy of the contralateral kidney after subcapsular human islet implantation (87). The proliferative capacity of the human β-cells decreased, whereas that of the duct cells increased, with the age of the islet donors (87,88). Ongoing experiments evaluate the possibility of stimulating growth of transplanted and endogenous islets with growth hormone (*cf.* 65). Preliminary results show that the expression of Nkx 6.1, *i.e.* a transcription factor which during differentiation becomes restricted to β-cells (25), increases in the native islets, sug-

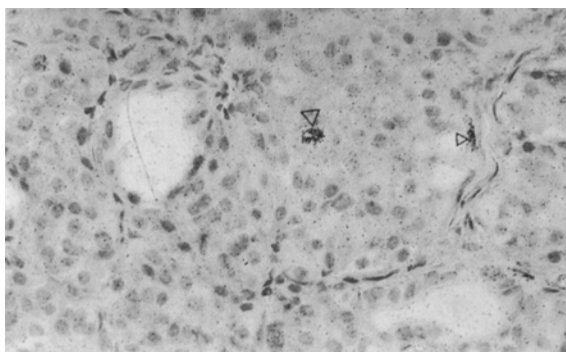

Figure 3. Autoradiographically labelled nuclei of a β-cell (large arrow) and fibroblast (small arrow) of grafted human islets 2 weeks after implantation. 100X.

gesting that a differentiation occurs (unpublished observation). Furthermore, a specific growth of the endocrine cells within grafted islets seems to occur in this experimental model (unpublished observation).

Differentiation of β-cells is of considerable interest when transplanting foetal pancreatic tissues. There have been new insights into the molecular mechanisms of pancreatic embryonic development over the last years (for a review, see 25). We have recently initiated studies on preadipocyte factor-1/Δ-like (Pref-1) mRNA, since this protein is expressed both during development and growth of the endocrine pancreas (13). With the aim of identifying growth hormone regulated genes in islets, we previously cloned Pref-1 from rat neonatal islets (13). Pref-1 mRNA was upregulated 3–4 fold after 48 h culture with human growth hormone and prolactin. Pref-1 is abundant during embryonic growth, but restricted to islets, adrenals and the pituitary in adults. During pregnancy high mRNA contents are found in islets of the mother as well as of the foetal pancreas, with both declining after birth. In neonatal islets Pref-1 becomes restricted to a subpopulation of β-cells. These data, together with a high homology of Pref-1 to the Drosophila protein Δ, led to the speculation that Pref-1, in analogy with Δ, participates in cell-cell interaction by binding to a Notch like receptor. The signalling properties of this receptor could help to maintain the embryonic pancreas in a proliferative, but less differentiated state. These studies on Pref-1 may eventually provide new methods to increase the yield of pancreatic islets and β-cells (cf. 13).

MICROENCAPSULATION OF ISLETS

Immunosuppressive therapy is a prerequisite for the success of islet transplantation, not only to inhibit rejection but also to prevent recurrence of the autoimmune disease. Therefore, implantations have been confined to patients receiving such therapy for other reasons, usually kidney transplantation (34). The side-effects associated with currently available immunosuppressive drugs outweigh the potential benefits of islet transplantation to other patient categories (56).

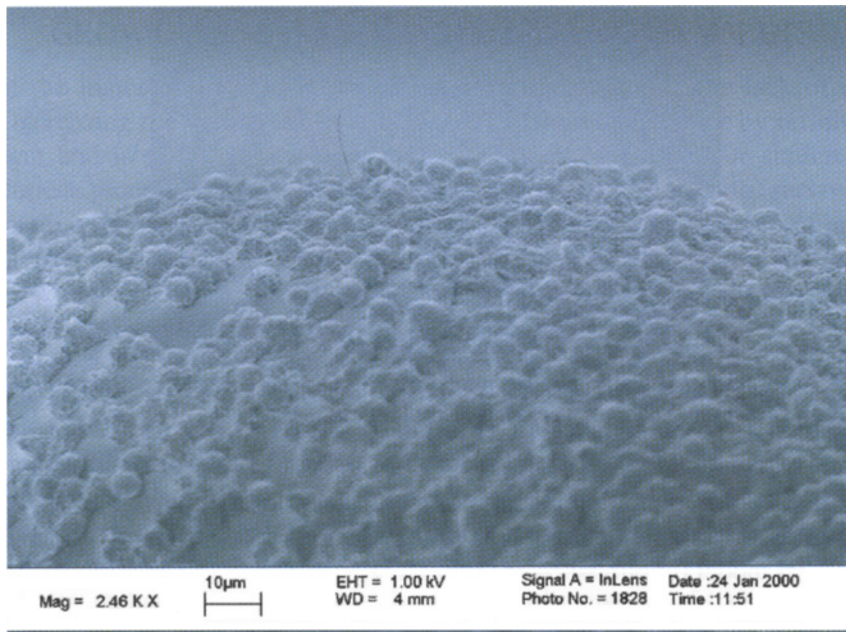

Figure 4. Scanning electron micrograph of a microcapsule with macrophages on its surface. Bar 10 µm.

An alternative approach would be to prevent access of the immune cells to the grafted islets by protecting them with semi-permeable membranes, without compromising the diffusion of nutrients, oxygen and released insulin. Such membrane capsules would have the additional benefit of protecting the islets from recurrence of disease (81). Capsules tested experimentally have varied in chemical composition, size and form (55,62,71). Most laboratories now favour the use of microcapsules, *i.e.* there is usually only one islet within each capsule. Microcapsules have a size of approximately 500–600 µm, which means that these grafts can be transplanted only at few implantation sites, preferentially into the peritoneal cavity. The capsules currently used in experimental research usually consist of alginate/polylysine/alginate which provides adequate biocompatibility and an immune barrier that does not compromise the function of the islet (28,41,75).

Disregarding anecdotal cases reporting cure of type 1 diabetes in humans after implantation of encapsulated islets (82), most studies have been unsuccessful manly due to fibrotic overgrowth of the capsules and/or cell death within the capsules (41,95). The current research within this field aims to investigate the relationship between the biocompatibility and capsule composition. Previous (91,93) and ongoing studies show quite explicitly that the polylysine layer is responsible for the growth of fibroblasts and macrophages on the capsule surface (Figure 4). We have also investigated the degree to which suppressive cytokines released from the surrounding macrophages and other immune cells penetrate into the gel-like matrix of

the capsules. In combination interleukin-1β and tumour necrosis factor-α impaired islet function when added to cultured, encapsulated islets (40). These findings highlight the importance of continuing the search for an optimal membrane composition for islet implantation.

CONCLUDING REMARKS

Despite the slow increase in success rate of clinical islet transplantation, we consider this treatment for type 1 diabetes to be of major importance for the future. The reason for this optimism is the profound increase in our understanding of the growth, normal cell biology and physiology of implanted pancreatic islets gathered during recent years. This has led to improved handling of the implants *ex vivo* before implantation, as well as *in vivo* post-implantation to minimise graft losses in the experimental situation, and hopefully soon also in the clinical setting. Since the 1-year survival of islet grafts has increased from a few per cent to approximately 40% during the last few years when applying stringent selection criteria for recipients and donors, we consider it likely that results will improve further. Eventually, it can be anticipated that islet implantations will have a 1-year graft function similar to what is seen after whole-pancreas transplantations. The possibility to encapsulate islets will be also of major importance in this context, since this will eliminate the need for immune-suppressive therapy, meaning that patients with type 1 diabetes can be treated much earlier during the course of their disease.

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