Survival and protein adsorption on immunoisolated islet grafts

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Introduction

During recent years, islet transplantation has been shown to be an efficacious method for the treatment of Diabetes Mellitus. Unfortunately, the technology of islet transplantation is only applied on a minor scale due to the necessity to apply lifelong immunosuppression. This obstacle can be overcome by immunoisolation of the islets. Immunoisolation applies to all technologies that use semipermeable mechanical barriers to protect grafts against antibodies and immune cells but allows for diffusion of nutrients, waste products and, the therapeutic molecules. The most intensively studied immunoisolation technology for pancreatic islets is microencapsulation in alginate-based capsules. During the past two decades, many demonstrated the principle applicability of the alginate-encapsulation technique for treatment of endocrine disorders such as Diabetes Mellitus. Both allografts and xenografts have shown prolonged survival after implantation in chemical and autoimmune diabetic animal models.
Important advances have been made in encapsulation research during recent years. Application of well-defined, purified alginates and a well controlled production process can avoid inflammatory reactions against the capsule membranes. In spite of these advances survival of graft is still limited to periods up to 6 months which is too short to merit clinical application. This limited graft survival is due to loss of up to 60% of the endocrine islet volume (De Vos P et al. 1997) in the immediate period after transplantation. Recently it has been shown that this loss is caused by an up to now unrecognized inflammatory response in the immediate period after implantation. Due to the mandatory surgery, capsules are exposed to both blood and peritoneal fluid containing bioactive molecules. These bioactive molecules adsorb on the capsule surface and induce deleterious immunological reactions or it changes the chemistry of the capsule surface and thereby change the functional properties of the capsules. This inflammatory response can be enhanced and prolonged by imperfections on the capsules such as protruding cells or incompletely covered PLL molecules.
To overcome this aspecific adsorption of proteins we have performed two separate studies. First we have designed a novel encapsulation system to overcome enhancement of the inflammatory response by protruding cells or incompletely covered PLL molecules. Next we studied, by applying microFTIR, the protein adsorption properties of alginates in order to select alginates with minimal adsorption properties.

Materials and Methods

Male inbred AO-rats weighing 300-350 gram served as donors. Male BL-6 mice weighing 25 to 31 gram were used as recipients of encapsulated islet xenografts. Diabetes was induced by injection of 190 mg/kg of streptozotocin via peritoneal injection. Glucose concentration in blood was determined with glucose test bandelettes. Only animals with severe weight loss, polyuria, polydipsia and blood glucose levels exceeding 20 mM over a period of at least ten days were used as recipients. After transplantation glucose levels were measured once a week for one year. Animals with two blood glucose measurements above 12,5 mM were considered diabetic and sacrificed.
Islets were isolated as previously described (Wolters et al. 1990). After culture, islets were washed three to five times with RPMI containing 10% FCS and and were subsequently washed three times with Ca^{2+}-free KRH containing 135 mM NaCl.

Encapsulation (conventional method)
After washing the islets were mixed with 3,3 % intermediate-G alginate (Kelco) in a ratio of 2000 islets per ml alginate. The 3,3 % alginate solution was converted into droplets using an air-driven droplet generator as previously described. Subsequently, the alginate droplets were transformed to alginate beads by gelling in a 100 mM $CaCl_2$ solution for at least 5 min. Subsequently, the Ca-alginate beads were suspended for 3 minutes in KRH containing 2.5 mM $CaCl_2$. A poly-L-lysine (PLL) membrane was formed by suspending the alginate beads in 0.1% PLL solution for 5 or 10 minutes. Non-bound PLL was removed by three successive washings during 3 min with Ca^{2+}-free KRH. The outer alginate-layer was subsequently applied by 5 minutes incubation in ten times diluted alginate solution. Finally, the capsules had a diameter of 750-800 μm.

Islet encapsulation (double layer method)
After washing the islets were mixed with 2,7 % intermediate-G alginate (Kelco) in a ratio of 1000 islets with 100 μl Alginate. The alginate solution was converted into droplets using an electrostatic-bead generator. Polylysine-alginate encapsulation was performed as described elsewhere (Halle et al, 1994). Briefly, the alginate droplets were transformed to alginate beads by gelling in a 100 mM $CaCl_2$ solution for at least 5 min. After gelation the beads had a diameter of 275-325 μm. Subsequently, the Ca-alginate beads were suspended for 1 min in KRH. A PLL membrane was formed by suspending the alginate beads in 0.05% PLL solution for 7 minutes. Non-bound PLL was removed by three successive washings during 3 min with Ca^{2+}-free KRH. The outer alginate-layer was subsequently applied by 5 minutes incubation in ten times diluted alginate solution. Finally, the capsules had a diameter of 325-350 μm.
The inner capsules were suspended in a 4 ml 1.9 % high-G (> 45% G) alginate (Kelco) solution for a second envelopment in beads. The alginate containing capsules were converted into droplets using an air-driven droplet generator. After gelation the beads had a diameter of 750-800 μm.

Protein adsorption on the chemistry and characteristics of alginate beads
For protein adsorption studies *in vitro*, human serum (HS) isolated from whole blood was donated by healthy volunteers. Human peritoneal fluid (PF) was obtained from a male donor within 12 hours of his decease. Samples of 100 microcapsules were transferred to a polypropylene test tube. The supernatant (i.e. KRH) was removed by aspiration. Serum (or PF) was diluted 1:1 in KRH and 1 ml of the diluted serum (or PF) was added to each test tube. Samples were incubated in a warm water bath at 37°C with gentle agitation for 1 hour. Afterwards, the serum was removed and the microcapsules were rinsed 5 times with KRH containing 2.5 mM $CaCl_2$ before spectra were collected. A Jasco MFT-2000 apparatus (Tokyo, Japan), supported by Jasco FT software, was used for the mFTIR analysis. The spectra were collected at room temperature in the 4000-600 cm^{-1} wavenumber range in transmittance mode.
Alginate beads with and without incubation in PF or HS were placed on a hydraulic pressed KBr disk. The beads were analyzed on the top of a KBr disk at room temperature. Since the core of the beads were too concentrated for the mFTIR analysis in transmittance mode, the sample was analyzed by setting the microscope aperture on the marginal side of the bead. In all cases, the background was previously measured at a microscopic aperture size below the sample size.

Statistical analysis
Results are expressed as mean ± SEM. Statistical comparisons were made with the Kaplan Meier logrank test. A *p* value of less than 0.05 was considered statistically significant.

Results and Discussion
Previous studies have shown that protruding islet cells and unbound proinflammatory PLL at the capsule surface after conventional encapsulation enhance inflammatory responses in the immediate period after implantation (De Vos et al. 1996, De Vos et al 2002).

In order to avoid protrusion of cells and PLL at the surface we designed a new encapsulation system. This system is composed of a double capsule system with an immunoisolating inner membrane and a outer alginate layer to provide biocompatibility (figure 1).

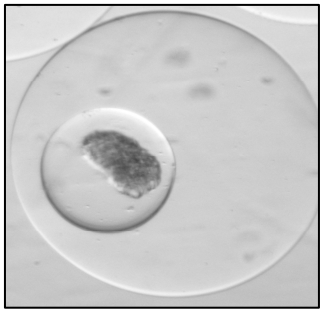

Figure 1. Double encapsulated islet in capsule of intermediate-G and poly-L-lysine membrane within high-G bead.

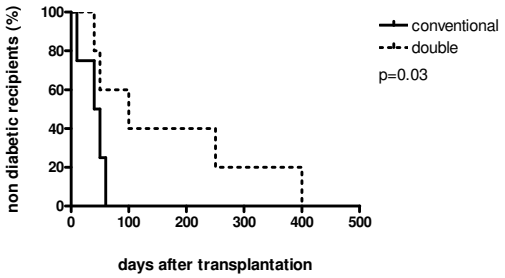

Figure 2. Kaplan meiers plot of encapsulated islet from AO rats transplanted in diabetic Bl-6 mice (xeno) with the conventional method and the novel double encapsulation.

The efficacy of this new concept was studied in an xenomodel of AO encapsulated rat islets. Streptozotocin diabetic BL6 mice served as donor. We compared the graft survival of the double layer encapsulated islets with the conventional system in a xenotransplantation. For each transplanted animal we used one rat donor from which we isolated a total of 800-1000 islets, which corresponds to 2.2-2.8 µl of islet tissue. Islets were cultured before encapsulation in order to reduce contamination of the grafts with exocrine tissue, ducts or damaged cells. The capsule size encapsulated islets ranged from 750 to 825 µm.

All transplanted animals (n=9; 4 conventional and 5 double encapsulated) became normoglycemic within 1 week (Figure 2). The longest survival time of the conventional encapsulated graft was 49 days (with a median of 39) in contrast to 400 days (with a median of 84) of the double encapsulated islet graft (which returned to hyperglycemia at 1 year after retrieval of the capsules). This demonstrates the efficacy of the new approach.

The new method was associated with much less fibrotic overgrowth than with the conventional system. Only 1 percent were found to be affected by inflammatory cells with the new double layer system while it was 27 percent with the conventional system. However, it was still not zero which was the rationally to study the protein adsorption capacity of the alginate as such.

Protein adsorption and chemistry of alginate beads

In the above mentioned in vivo study we applied intermediate-G alginate for the inner immunoisolating capsules since intermediate-G alginate are well known to bind PLL in an efficacious way. The outer capsule was produced from high-G alginates since high-G alginate is providing a high mechanical stability and durability. Since we still found some overgrowth on a portion of the novel double layer system we decided to compare the protein adsorption capacity of intermediate and high-G alginates by applying mFTIR. This was done by studying the protein adsorption from human serum and human peritoneal fluid.

mFTIR spectrum of intermediate-G and high-G alginate beads shows a broad absorption band at around 3370 cm^{-1} for the –OH groups, two peaks at 1605 and 1417 cm^{-1}, related to the – COO^- groups and an absorption band between 1200 and 1000 cm^{-1}, corresponding to the vibration of C – O bond (Figure 3a and b). Incubation of the intermediate-G beads with human serum resulted in the appearance of two peaks in the mFTIR spectrum at 1446 and 1403 cm^{-1}(fig. 3c). Incubation of the intermediate-G beads with human peritoneal fluid showed no difference in mFTIR spectrum (fig. 3e).

High-G alginate beads also showed a difference in spectrum due to incubation with human serum. The incubation resulted in the appearance of two peaks in the mFTIR spectrum at 1618 and 1211 cm^{-1} (fig. 3d). Surprisingly the beads also showed a difference in spectrum after incubation with human peritoneal fluid. The incubation resulted in the broadening of the shoulder in the mFTIR spectrum around 1200 cm^{-1} (fig. 3f).

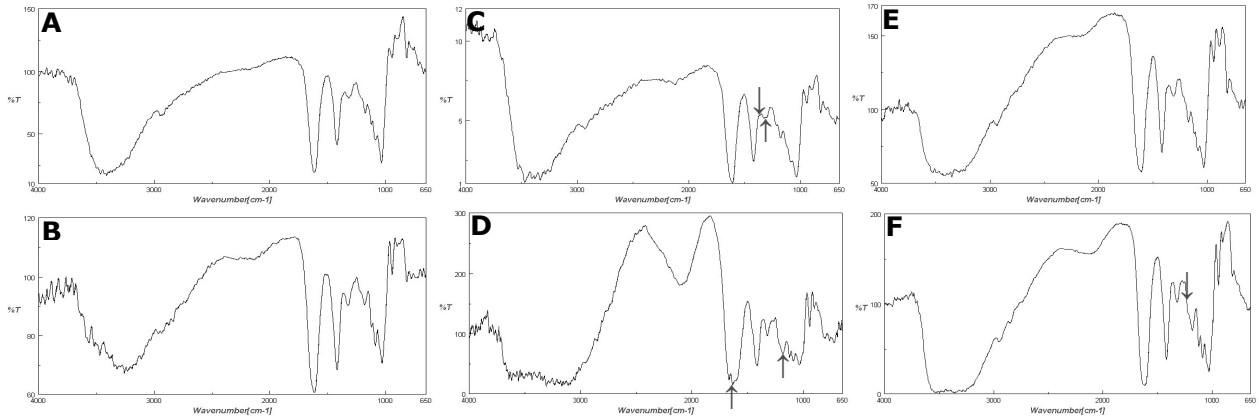

Figure 3. mFTIR spectrum of Keltone alginate beads (a), after incubation in humane serum (c) and after peritoneal fluid incubation (e). mFTIR spectrum of Manugel alginate beads (b) after incubation in humane serum (d) and after peritoneal fluid incubation (f).

Conclusions

In the current study we present a novel encapsulation method which avoids protruding cells and unbound proinflammatory PLL. The novel encapsulation method is associated with longer graft survival times when compared to the conventional system. With conventional capsules the median was 39 days while it was 91 days with the double layer system. The beneficial effects of the new capsule type can also be depicted from the fibrotic overgrowth rates in this xenograft model. This was 27 percent with conventional capsules while it was only 1 percent with the double layer system. Since the novel system still showed some fibrotic overgrowth we decided to study protein adsorption of beads prepared of intermediate-G and high-G alginates. The results show that high-G alginates should be avoided on the surface of the capsule since this alginate showed quite some adsorption and functional changes after incubation in human serum and peritoneal fluid. In future studies in vivo studies the outer high-G alginate will be substituted by intermediate-G alginate.

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