

# Sustained function of alginate-encapsulated human islet cell implants in the peritoneal cavity of mice leading to a pilot study in a type 1 diabetic patient

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## Abstract

**Aims/hypothesis** Alginate-encapsulated human islet cell grafts have not been able to correct diabetes in humans, whereas free grafts have. This study examined in immunodeficient mice whether alginate-encapsulated graft function was inferior to that of free grafts of the same size and composition. **Methods** Cultured human islet cells were equally distributed over free and alginate-encapsulated grafts before implantation in, respectively, the kidney capsule and the peritoneal cavity of non-obese diabetic mice with severe combined immunodeficiency and alloxan-induced diabetes. Implants were followed for in vivo function and retrieved for analysis

of cellular composition (all) and insulin secretory responsiveness (capsules).

**Results** Free implants with low beta cell purity ( $19\pm 1\%$ ) were non-functional and underwent 90% beta cell loss. At medium purity ( $50\pm 1\%$ ), they were functional at post-transplant week 1, evolving to normoglycaemia (4/8) or to C-peptide negativity (4/8) depending on the degree of beta cell-specific losses. Encapsulated implants immediately and sustainably corrected diabetes, irrespective of beta cell purity (16/16). Most capsules were retrievable as single units, enriched in endocrine cells that exhibited rapid secretory responses to glucose and glucagon. Single capsules with similar properties were also retrieved from a type 1 diabetic recipient at post-transplant month 3. However, the vast majority were clustered and contained debris, explaining the poor rise in plasma C-peptide.

**Conclusions/interpretation** In immunodeficient mice, i.p. implanted alginate-encapsulated human islet cells exhibited a better outcome than free implants under the kidney capsule. They did not show primary non-function at low beta cell purity and avoided beta cell-specific losses by rapidly establishing normoglycaemia. Retrieved capsules presented secretory responses to glucose, which was also observed in a type 1 diabetic recipient.

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**Keywords** Cell therapy · Encapsulation · Islet transplantation

## Abbreviations

BW	Body weight
CK	Creatinine kinase
NOD/SCID	Non-obese diabetic/severe combined immunodeficiency

## Introduction

Intraportal islet cell transplantation can correct hyperglycaemia in type 1 diabetic patients, but most recipients exhibit a progressive reduction in implant function over the subsequent 5 years [1]. This decline might be attributed to various causes including inflammatory and immune responses to the donor tissue [2–4]. In order to protect grafts against this reactivity, encapsulation methods have been developed for implants in extra-hepatic sites [5, 6]. Alginate microcapsules have been the longest in use and technical advances have been made in improving their biocompatibility, stability and permeability, and in producing variants with selected properties [7–9]. They have allowed successful allotransplants of islet tissue in the peritoneal cavity of rodents [10–12] and of large animals [13, 14], often without the need for continuous immune suppression [11–14].

Clinical studies with alginate-encapsulated human islet cells have not yet reproduced these promising data in man [15–17]. In the small series undertaken so far in type 1 diabetic recipients it is unclear whether the poor outcome was caused by an initially insufficient beta cell mass or by compromised survival of implants in the peritoneal cavity. Outcome of human islet cell implants can differ with their site, and is probably influenced by variability in their cellular composition whereby a large proportion of non-endocrine cells might interfere with function [18].

In the present study we compared the metabolic outcome of free and alginate-encapsulated human beta cell preparations with the same cellular composition following implantation in, respectively, the kidney subcapsular space and the peritoneal cavity of diabetic immunodeficient mice. Alginate-encapsulated implants exhibited a better metabolic effect and appeared to be composed of beta cells with potent glucose responsiveness. These data led us to a pilot study in a type 1 diabetic patient to examine whether functional beta cells could also be retrieved from the peritoneum 3 months after injection of an alginate-encapsulated human islet cell graft.

## Methods

*Preparation of free and alginate-encapsulated human beta cell grafts for transplantation in diabetic NOD/SCID mice* Cultured human beta cell preparations were provided by our beta cell bank, which receives donor organs from

the Eurotransplant International Foundation (Leiden, the Netherlands). Isolates that could not be used for clinical transplantation were made available according to Eurotransplant guidelines and under approval of our institutional ethics committee. Six preparations were used for transplantation in mice. After 3–15 days' culture, they were characterised for their cellular composition including beta cell number, purity and insulin content [19]. The available human cell isolates were combined and divided over preparations for free and alginate-encapsulated grafts aiming at injection of  $2 \times 10^6$  beta cells per mouse. For encapsulation,  $15 \times 10^6$  to  $20 \times 10^6$  cells/ml were suspended in 1.8% ultrapure alginate (68% glucuronic acid, 32% mannuronic acid; Pronova UP-LVG [Nova Matrix, Sandvika, Norway]; 0.3 mol/l mannitol [Sigma-Aldrich, Steinheim, Germany]; pH 7.2–7.4) before generation of micro-droplets in barium–calcium solution (50 mmol/l calcium chloride and 1 mmol/l barium chloride in 10 mmol/l MOPS [J. T. Baker, Deventer, the Netherlands]; 0.15 mol/l mannitol and 0.05% Tween-20 [Merck, Darmstadt, Germany]; pH 7.2–7.4) [9]. Free and alginate-embedded preparations were cultured overnight before transplantation in 7- to 9-week-old male non-obese diabetic/severe combined immunodeficient (NOD/SCID) mice (NOD.CB17-*Prkdc*<sup>scid</sup>/J; Charles River, L'Arbresle, France). Diabetes was induced by tail vein injection of alloxan (50 mg/kg; Sigma-Aldrich) 36 h before transplantation under general anaesthesia (ketamine/xylazine). At transplantation, all animals had glycaemia  $>25$  mmol/l. Each free graft was divided into two fractions of similar size for insertion in the left and right renal subcapsular space [20]; the alginate-encapsulated equivalent was injected i.p. A sample was taken from each graft for pre-transplant analysis. The 'Principles of laboratory animal care' were followed, as well as specific national laws where applicable. The clinical graft was prepared from four donors and alginate-encapsulated using the same procedure.

*Function of human beta cell implants* The term 'graft' is used for cell preparations before implantation, and 'implant' for the tissue after injection. Function of implants was assessed through plasma human C-peptide levels and blood glucose concentrations. Human C-peptide was measured by time-resolved fluorescence immunoassay [21] with a lower detection limit of 0.03 nmol/l [22]; a minimum level of 0.17 nmol/l was set as the criterion for a functioning implant [23]. A 2 h fasting morning glycaemia  $<8.5$  mmol/l was taken as an index of metabolic correction, being  $>20$  mmol/l in all diabetic controls. Since the pancreatic insulin content of all alloxan-treated animals was under 5% of values in non-diabetic controls, glycaemic correction was attributed to a metabolically adequate implant.

*Cellular composition of human beta cell implants* Implants were harvested at post-transplant week 5. Retrieved

capsules were counted and processed for immunocytochemistry and insulin assay [24]. In recipients of free grafts, the right kidney was snap-frozen in liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until extraction in acetic acid for the insulin assay [24]. The left kidney was fixed in 4% (wt/vol.) phosphate-buffered formalin, embedded in paraffin and serially sectioned for histological (haematoxylin–eosin–safran staining) and immunohistochemical analysis. Cell types were identified by rabbit anti-glucagon (1:1,000 to 1:5,000), guinea pig anti-insulin (1:1,000 to 1:10,000) (both antibodies raised by C. van Schravendijk at our centre), rabbit anti-synaptophysin (1:5,000; Acris, Herford, Germany) for endocrine cells, and mouse anti-creatinine kinase (CK)19 (1:20; Dako, Glostrup, Denmark) for duct cells. The endocrine and duct cell masses were determined as cell volumes according to Cavalieri's principle. Sections sampled throughout the graft were stained and photographed on the Pathway BioImager 855 or 435 for determining positive surface areas (IP Lab software; BD Biosciences, San Jose, CA, USA), which, together with the distance between sections, allowed calculation of total cell masses [25]. In recipients of encapsulated grafts, capsules were retrieved by peritoneal lavage and examined under a stereomicroscope for integrity and adherence of fibrosis and cells. Selected numbers were taken for electron microscopy and immunocytochemical analysis (fixation in 2.5% [wt/vol.] glutaraldehyde) [26] and for overnight culture before perfusion at different glucose concentrations [27, 28]. Dead cells were identified by ruptured plasma membranes. Their percentage was determined in free and encapsulated preparations at the time of transplantation and explantation.

*Transplantation of an alginate-encapsulated human beta cell graft in a type 1 diabetic patient* An alginate-encapsulated human beta cell preparation was injected i.p. into a 61-year-old female type 1 diabetic patient (68 kg body weight [BW]) who was under immune-suppressive therapy for an intraportal islet cell graft that she had received 5 years earlier. For 9 months, her plasma C-peptide levels had been 0.03–0.07 nmol/l, which was under the 0.17 nmol/l limit that was set for a functional intraportal implant [23]. She had been informed that the present protocol was intended for patients with a failing islet cell graft but still on immune therapy, and consented to participate in the study.

The protocol was approved by the institutional ethics committee and registered on ClinicalTrials.gov (NCT01379729). The encapsulation procedure was conducted in our beta cell bank, which is accredited for preparing clinical grafts. The graft, containing  $2.4 \times 10^6$  beta cells/kg BW (approximately 300,000 islet equivalent [19]), was injected in the peritoneal cavity through laparoscopy under general anaesthesia. Immune therapy consisted of basiliximab (20 mg i.v. on day of transplantation and post-transplant day 4) and continued

maintenance dosages of tacrolimus (trough levels 8–10 ng/ml) and mycophenolate mofetil (1.5 g/day). At post-transplant month 3, a second laparoscopy was performed to inspect the peritoneal cavity for adhesions and for retrieval of implanted capsules. Free-floating capsules were obtained by peritoneal lavage and processed as described. Capsules that adhered to the peritoneum were biopsied with surrounding tissue and processed for light microscopic analysis.

*Statistical analysis* Results are expressed as means  $\pm$  SEM. Statistical analysis was carried out using Prism 5 (GraphPad Software, San Diego, CA, USA). Differences between experimental groups were calculated by unpaired two-tailed *t* test or paired two-tailed *t* test when appropriate. Comparison of multiple groups was performed by one-way ANOVA using Tukey's post hoc test.

## Results

*Comparison of metabolic effect of free and alginate-encapsulated human beta cell implants* We compared the metabolic effect of free and alginate-encapsulated human beta cell grafts with similar composition (Tables 1 and 2), following implantation in, respectively, the renal subcapsular space and the peritoneal cavity.

Free grafts reduced glycaemia on post-transplant day 1, but all 12 recipients were again hyperglycaemic at post-transplant week 1 ( $>20$  mmol/l), four being non-functional (plasma human C-peptide  $<0.17$  nmol/l) and eight presenting an average level of  $0.93 \pm 0.09$  nmol/l (Table 1). The subgroup with non-functional grafts had received the same number of beta cells as the eight that were C-peptide-positive, but at lower purity and with a lower cellular insulin content (Table 1); they remained non-functional during follow-up. Of the eight with function at post-transplant week 1, four progressed to normoglycaemia during the subsequent 3 weeks, with progressively increasing human C-peptide levels; the four others remained hyperglycaemic with declining C-peptide levels (Fig. 1).

Alginate-encapsulated grafts resulted in functional implants and normoglycaemia in 16/16 recipients from post-transplant week 1 until the end of the study (Table 2, Fig. 1). Four had received a graft with similar composition to that in free grafts which resulted in non-function.

*Correlation between metabolic outcome of free human beta cell grafts and composition and functional state of implants at post-transplant week 5* Free implants were classified according to their metabolic outcome, i.e. primary non-function from post-transplant week 1 ( $n=4$ ), transient function with sustained hyperglycaemia ( $n=4$ ), and metabolic correction (sustained function and normoglycaemia,  $n=4$ ).

**Table 1** Influence of graft composition on primary (non-) function of human islet cell implants in diabetic NOD/SCID mice

Variable	Free graft			Encapsulated graft
Plasma human C-peptide at post-transplant week 1 (nmol/l)	0.03–0.16	0.6–1.56	0.03–1.56	>0.17
<i>n</i>	4/12	8/12	12/12	16/16
Cellular composition				
Beta cells (%)	19±1	50±1**	39±4	42±4
Alpha cells (%)	4±1	14±1**	11±2	12±1
Duct cells (%)	68±1	33±2**	45±5	41±4
Beta cells				
Number (×10 <sup>6</sup> /kg BW)	82±4	82±4	82±3	95±6
Insulin content (µg/graft)	20.8±0.6	41.4±5.6*	34.6±4.8	33.8±3.4

Data represent means±SEM

Statistical analysis of differences between subgroups of free grafts was calculated using one-way ANOVA with Tukey's post hoc test

\* $p < 0.05$ , \*\* $p < 0.001$  (there was no difference in respective percentages of dead cells: <10% in all [sub]groups)

Their respective composition at post-transplant week 5 was compared with each other as well as with that of the endocrine pancreas in normal mice. In each of the three subgroups, implants presented clusters of endocrine cells between duct-like structures that were lined with CK19-positive cells. There was virtually no inflammation. There was no difference in their respective CK19-positive duct cell mass (measured as volume; Table 3). In the primary non-function subgroup, the mass was comparable with the endocrine cell mass (insulin- and glucagon-positive volumes). This subgroup had received grafts with a significantly higher proportion of duct cells (68% vs 33% in the two others;  $p < 0.001$ ). The subgroups with transient function and with metabolic correction contained, respectively, a five- and tenfold higher beta cell mass, whereas their alpha cell mass was only 2.5-fold higher. The differences in metabolic outcome at post-transplant week 5 are thus correlated to the beta cell mass at this time point.

When expressing beta cell mass in the implants as a percentage of that in the normal adult mouse pancreas, it represented 6% in the primary non-function group, 33% in the transient function group and 64% in the metabolic correction group (Table 3). Since each subgroup had received a similar number of beta cells, it is concluded that the two hyperglycaemic groups lost, respectively, ten- and twofold more donor beta cells compared with the

normoglycaemic group. Such a loss was not seen for the alpha cells, which represented 50% in the primary non-function group and at least 100% in the two other groups.

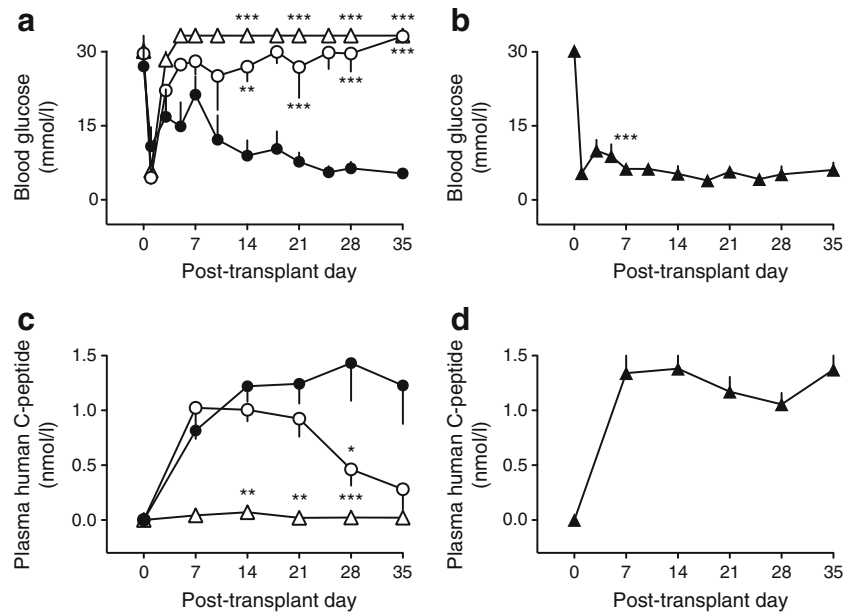
The insulin content of implants (Table 3) in the subgroups with primary non-function (1 µg) or transient function (2.7 µg) was 15- to 20-fold lower than that in their initial grafts (20–40 µg; Table 1). This decline resulted in part from a loss of beta cells, as indicated by a comparison of their beta cell mass with that of the corresponding duct cell mass and that in the subgroup with metabolic correction (Table 3). In addition, the remaining cells degranulated as derived from the insulin content per beta cell volume and as expected after prolonged hyperglycaemia. The insulin content of transiently functioning implants corresponded to 9% of that in the normal mouse pancreas but was 60% in implants with sustained metabolic correction (Table 3). Since the latter (17.9 µg) was also 50% lower than in the initial grafts (34.6 µg; Table 1), it may be concluded that a substantial beta cell loss also occurred in this subgroup.

*Correlation between metabolic outcome of alginate-encapsulated human beta cell grafts and composition and functional state of implants at post-transplant week 5* At post-transplant week 5, most alginate capsules were free floating in the abdomen. They were retrieved by peritoneal

**Table 2** Metabolic outcome of free and alginate-encapsulated human islet cell implants in diabetic NOD/SCID mice

Graft	No. of recipients with plasma human C-peptide >0.17 nmol/l				No. of recipients with blood glucose <8.5 mmol/l			
	Post-transplant week				Post-transplant week			
	1	5	20	30	1	5	20	30
Free	8/12	4/12	–	–	0/12	4/12	–	–
Encapsulated	16/16	16/16	6/6	3/3	12/16	16/16	6/6	3/3

**Fig. 1** Blood glucose (a, b) and plasma human C-peptide (c, d) levels expressed as mean ± SEM. (a, c) Recipients of free graft under kidney capsule: white triangles, subgroup with persistent C-peptide <0.17 nmol/l (primary non-function, n=4); white circles, subgroup with persistent hyperglycaemia and progressively decreasing plasma C-peptide levels (transient function, n=4); black circles, subgroup with normalisation of glycaemia (metabolic correction, n=4). (b, d) Recipients of encapsulated graft (n=16) in peritoneal cavity. Recipients of all subgroups are compared with kidney capsule–metabolic correction recipients. One-way ANOVA with Tukey’s post hoc test: \*p<0.05, \*\*p<0.01, \*\*\*p<0.001



lavage and examined for their structure, cellular composition and functional properties. The capsules showed no fibrosis or cellular overgrowth; more than 95% were intact and round-shaped (Fig. 2a,b). They contained mostly living cells (15% dead cells), the majority in the form of endocrine cell clusters with well-granulated cells and intact ultrastructure, the minority in the form of non-granulated cells (Fig. 2c,d). The percentages of beta and alpha cells were significantly higher than those in the initial graft (Table 4), and thus also the percentage of endocrine cells.

Encapsulated implants were examined for their glucose-inducible insulin release following overnight culture. Capsules were loaded on a perfusion column and exposed to four different glucose concentrations with and without glucagon [27, 28]. Insulin release was expressed as a percentage of the cellular hormone content at the start. Release above basal (2.5 mmol/l) was induced by 5 mmol/l glucose and dose-dependently increased by 10 and 20 mmol/l, with further amplification by the addition of glucagon (Fig. 2e).

**Table 3** Size and composition of human islet cell implants retrieved from NOD/SCID mice at post-transplant week 5

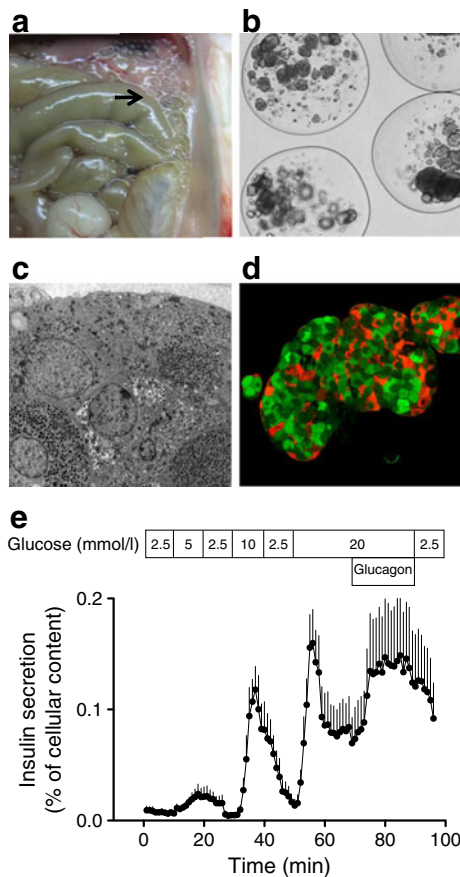
	Free graft			Normal mouse pancreas (n=6)
	Primary non-function (n=4)	Transient function (n=4)	Metabolic correction (n=4)	
<b>Cell mass in implant (µl)</b>				
Beta cells	0.05±0.03***	0.28±0.09*	0.54±0.06	0.85±0.06*
Alpha cells	0.10±0.05	0.27±0.05	0.26±0.04	0.20±0.01
Alpha + beta cells	0.15±0.07***	0.55±0.14	0.80±0.04	1.05±0.07
Duct cells	0.12±0.06	0.09±0.02	0.10±0.01	NA
<b>Insulin content (µg)</b>				
Implant	1.0±0.1***	2.7±0.4	17.9±2.1	NA
Pancreas	1.6±1.5	0.3±0.2	0.7±0.3	30.0±5.8**

Data represent means ± SEM

Statistical analysis of differences between groups was calculated using an unpaired two-tailed *t* test and expressed as differences from the subgroup with metabolic correction

\*p<0.05, \*\*p<0.01, \*\*\*p<0.001

NA, not applicable



**Fig. 2** Analysis of encapsulated human beta cell implant in NOD/SCID mice at post-transplant week 5. Free-floating capsules (*arrow*) in the peritoneal cavity (**a**) were retrieved as single units without fibrosis or cellular overgrowth (**b**). They contained well-granulated endocrine islet cells (**c**) that stained positively for insulin (green) and glucagon (red) (**d**). Their insulin release was examined in perfusion at 10 min episodes of varying glucose concentration with and without 10 nmol/l glucagon. The rate of insulin release is expressed as a function of the cellular insulin content before perfusion (**e**). Data represent means  $\pm$  SEM from five independent experiments

*Long-term survival and function of alginate-encapsulated human beta cell implants in NOD/SCID mice and in a type 1 diabetic patient* Six NOD/SCID mouse recipients of alginate-encapsulated human beta cells grafts were followed for 20–30 weeks. All maintained plasma human C-peptide levels above 0.5 nmol/l with non-fasting blood glucose <8.5 mmol/l. When the capsules were retrieved at the end of this study, they were microscopically comparable with those at post-transplant week 5.

On the basis of these observations, an encapsulated human beta cell preparation was injected into the peritoneal cavity of a type 1 diabetic patient under maintenance immune-suppressive therapy for a prior intraportal beta cell graft. The cellular composition of this preparation was comparable with that used in mice, but the number of implanted beta cells was more than 30-fold lower when expressed per kg BW ( $2.4 \times 10^6$  vs  $95 \times 10^6$

**Table 4** Cellular composition of human islet cell implants at post-transplant week 5 with that of corresponding grafts

Composition	Free graft			Encapsulated graft with metabolic correction (n=16)
	Primary non-function (n=4)	Transient function (n=4)	Metabolic correction (n=4)	
<b>Graft</b>				
Beta cells (%)	19 $\pm$ 1	47 $\pm$ 1	52 $\pm$ 2	42 $\pm$ 4
Alpha cells (%)	4 $\pm$ 1	14 $\pm$ 1	15 $\pm$ 1	12 $\pm$ 1
Duct cells (%)	68 $\pm$ 1	32 $\pm$ 4	32 $\pm$ 3	41 $\pm$ 4
<b>Implant</b>				
Beta cells (%)	18 $\pm$ 4	42 $\pm$ 5	60 $\pm$ 4	54 $\pm$ 5 <sup>***</sup>
Alpha cells (%)	36 $\pm$ 1 <sup>***</sup>	45 $\pm$ 4 <sup>**</sup>	29 $\pm$ 5	18 $\pm$ 2 <sup>**</sup>
Duct cells (%)	46 $\pm$ 4 <sup>*</sup>	14 $\pm$ 2 <sup>*</sup>	11 $\pm$ 1 <sup>*</sup>	20 $\pm$ 6 <sup>***</sup>

Data represent means  $\pm$  SEM

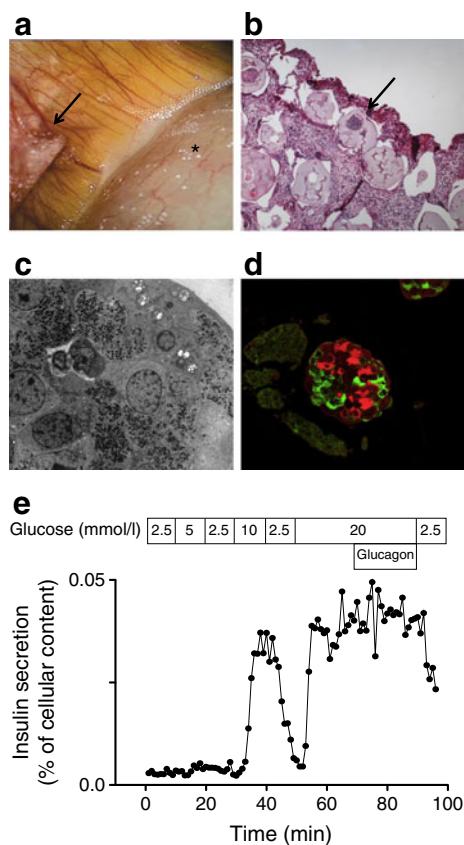
Statistical analysis of differences between percentages in grafts and implants was calculated using a two-tailed paired *t* test

\**p*<0.05, \*\**p*<0.01, \*\*\**p*<0.001

beta cells/kg BW). Plasma C-peptide levels increased above pre-transplant levels for 12 weeks (from <0.07 nmol/l before transplant to 0.13–0.6 nmol/l after transplant) but only reached the 0.17 nmol/l threshold of a functional implant during the first week [23]. During the 3 month follow-up the daily insulin dose and diabetes auto-antibody status remained unchanged (insulin autoantibodies <0.6%, insulinoma-associated antigen 2 antibodies <0.44%, GAD antibodies <2.6%, islet cell antibodies 200% Juvenile Diabetes Research Foundation units) [29]; no HLA class I or class II antibodies were induced.

Upon laparoscopy at post-transplant month 3, no macroscopic abnormalities were observed and no signs of adhesion between abdominal organs. Clusters of capsules were noticed at several sites, including the greater omentum and the pelvic floor (Fig. 3a). They were embedded in vascularised fibrous tissue containing scattered macrophages and giant cells but few lymphocytes and polynuclear cells; their content mainly consisted of debris; the small number with living cell aggregates was located at the surface of the clusters (Fig. 3b).

We also noticed numerous single capsules loosely attached to the peritoneum and easily retrievable by lavage (Fig. 3a). Their surface was free of fibrosis and attached cells; in phase contrast, they appeared similar to those retrieved from immune-incompetent mice. Several contained living cell aggregates which, on electron microscopy, presented intact well-granulated endocrine cells (Fig. 3c). The ultrastructural and immunocytochemical analysis was indicative of endocrine cell enrichment compared with the initial graft (62% beta and 16% alpha cells vs 42% and 15% at the start) (Fig. 3d). Significantly fewer non-granulated duct cells were counted (12% vs 45% at the start), possibly



**Fig. 3** Analysis of encapsulated human beta cell implant in a type 1 diabetic patient at post-transplant week 12. During laparoscopy, a small fraction of single capsules (arrow) was found free floating in the peritoneal cavity; the largest number was clumped together (asterisk) on the peritoneal wall (a). Aggregated capsules contained mostly cellular debris; some at the periphery presented islet cells (arrow) (b). Free-floating capsules contained well-granulated endocrine islet cells (c) that stained positively for insulin (green) and glucagon (red) (d). Their insulin release was examined in perfusion at 10 min episodes of varying glucose concentrations with and without 10 nmol/l glucagon. The rate of insulin release is expressed as a function of the cellular insulin content before perfusion (e)

because of their low survival in the capsules (44% dead cells vs 6% at the start). When free-floating capsules were perfused, a rapid and potent insulin secretory response was induced by 10 mmol/l glucose. The amplitude was maintained at 20 mmol/l glucose but not amplified, nor after the addition of glucagon, which is at variance with capsules retrieved from mice (Fig. 3e). Expression of insulin release as a percentage of cellular insulin content at the start indicated that basal release was low and that glucose-stimulated release represented a smaller fraction than in capsules retrieved from mice. Further studies on retrieved beta cells are needed to identify whether this lower discharge is caused by a low percentage of glucose-activated beta cells and/or by an insufficient stimulus–secretion coupling in responsive cells as a result of an inadequate microenvironment.

## Discussion

Immunodeficient rodents can serve as a model to predict, or at least test, the metabolic efficacy of human beta cell grafts used in clinical trials [30–32]. This study investigated the influence of alginate encapsulation on their outcome in diabetic NOD/SCID mice and examined influences of their cellular composition. Encapsulated implants in the peritoneal cavity induced an immediate and sustained correction of blood glucose in all recipients, whereas this was only the case for one-third of free implants under the kidney capsule, with a delay of more than 1 week. Subcapsular implants underwent a substantial beta cell loss particularly in grafts with a larger duct cell mass. This difference has not been previously observed. Our data are difficult to compare with those of other studies given the differences in graft characteristics and implant sites [33, 34]. Outcome can vary with the use of fresh or cultured preparations, with differences in cellular composition and in initial beta cell number and purity [35]. These influences should be identified, as they guide the preparation of clinical grafts and the interpretation of their outcome.

Use of cultured human beta cell grafts allows their characterisation and standardisation in size, viability and cellular composition. Our preparations consist of over 90% beta, alpha and duct cells. The high percentage of duct cells (40% on average) raises questions about their influence on graft outcome in patients [35]. In rat islet cell grafts, a high proportion of pancreatic non-endocrine cells were found to interfere with their function as intraportal implants [18]. When investigated in vitro, human duct cells exhibited inflammatory and immune properties [36–41]. The cells reacted more vigorously to cytokines than did associated endocrine cells as judged by the expression of MHC class II [36], CD40 [38, 41] or nitric oxide synthase, and by the synthesis and release of other cytokines [37, 39]. They were also found to exert a potent procoagulant activity, related to their higher expression of tissue factor [40]. Indirect support also comes from diabetic nude rats in which intraportally injected human islet cell implants caused an inflammatory reactivity that was followed by failure to correct hyperglycaemia [21]. There was less inflammation in omental implants, but sustained metabolic correction was only seen for grafts with a lower percentage of duct cells [21]. The present data in mice also indicate this correlation for free grafts under the kidney capsule. In this model, the failure of grafts with a larger duct cell mass may not only be attributable to a higher inflammatory activity but may also be caused by more severe hypoxia in their compact subcapsular implants. A comparative prospective study in man has not yet been conducted, as it faces the difficulty of preparing human islet cell grafts with low percentages of duct cells. In a retrospective analysis of type 1 diabetic islet

graft recipients, a weak correlation was seen between the number of duct cells injected and the acute insulin secretory response at post-transplant year 2, but this was not further examined against other variables [42].

Only a subgroup of free graft recipients (4/12) exhibited a metabolic correction, as in other animal studies with human islet cell grafts [43–45]. The effect was not achieved within the first week, probably reflecting the time needed to vascularise implants in an extravascular space [46], and probably not facilitated in compact subcapsular implants. Revascularisation may have been too slow or inadequate in the eight other recipients, leading to necrosis and, hence, undetectable (primary non-function) or metabolically insufficient plasma human C-peptide levels that progressed to negativity (transient function). At post-transplant week 5, these failing implants contained a beta cell mass that was disproportionately low when compared with the corresponding alpha or duct cell mass. This was also the case with respect to the beta cell mass in normalised animals that had received the same number of beta cells at the start. Such beta cell preferential loss might result from their higher susceptibility to hypoxia during impaired or delayed revascularisation. It was highest in the primary non-function subgroup, possibly related to their higher proportion of duct cells and the higher cell mass that had to be implanted in order to inject similar numbers of beta cells. It might also have been caused by the chronic hyperglycaemic state [47], which could also explain the progressive beta cell loss in the transient function subgroup. Beta cells that survived in these diabetic animals were degranulated, which, together with the beta cell loss, reduced the insulin content of the failing implants to 5% of that in the initial grafts and to 9% of the values in a normal mouse pancreas. These processes did not occur, or to a much lower extent, in implants of the subgroup with sustained metabolic correction: their beta cell mass and insulin content represented 60% of the values in the normal mouse pancreas, sufficient to maintain high plasma human C-peptide levels.

Alginate-encapsulated implants in the peritoneal cavity corrected hyperglycaemia immediately, which is indicative of diffusion—from the first post-transplant days—of sufficient insulin from the free-floating capsules. In all recipients, metabolic correction was maintained until the end of the study, irrespective of the percentage of duct cells in the initial grafts. Higher proportions of duct cells thus interfere less with human beta cell functions in this condition where the graft is dispersed and free floating in alginate microcapsules without direct contact with host tissue. The majority of injected capsules were retrieved by peritoneal lavage and were found to be virtually free of fibrosis. They contained up to 80% viable cells with marked endocrine cell enrichment compared with the initial grafts. This enrichment was also noticed in free implants with metabolic correction and may result from a higher loss of duct cells than beta cells in implants with

adequate perfusion at an early stage. The ability to retrieve encapsulated human beta cell implants from the recipients led us to investigate directly their functional properties during the phase where they achieve a sustained metabolic correction. Their perfusion demonstrated rapid, potent and dose-dependent insulin responses to glucose, together with a marked amplifying effect of glucagon, which are the hallmarks of physiologically responsive beta cells [48].

On the basis of the observations in mice we conducted a pilot study in a type 1 diabetic patient to assess whether similar properties were identified in her alginate-encapsulated human beta cell implant. Previous studies have reported on the techniques, the safety and the outcome of alginate-encapsulated human [15, 17] and porcine [49] islet cell grafts in the peritoneal cavity of diabetic patients. They have documented the feasibility of this type of implantation but not yet its metabolic success. We selected a patient who was on immune suppression for a prior intraportal beta cell implant which had recently lost function. The patient gave her consent for a peritoneal implant under prolonged immune therapy, i.e. until loss of function. This pilot study thus did not assess whether encapsulation provided any immune protection but investigated whether encapsulated implants maintain surviving and functioning beta cells. As in the paper of Tuch et al [17], plasma C-peptide levels were only indicative of graft function during the first post-transplant week. Up to month 3, they remained higher than pre-transplantation levels, always marginally low, but reflecting the presence of functional beta cells within implanted capsules as was demonstrated by analysis of retrieved capsules. These were sampled from a free-floating fraction and found to contain endocrine islet tissue with intact and well-granulated alpha and beta cells. Perfusion showed the presence of glucose-sensitive beta cells, with a low basal release, a potent response to 10 mmol/l glucose and a sustained release at 20 mmol/l glucose whether glucagon was present or not. However, in contrast to the study in mice, the majority of capsules were found to be aggregated in large clumps, sticking to the abdominal wall, surrounded by fibrotic tissue and containing mostly debris. An inflammatory reaction to the biomaterial may have initiated aggregation. The persistent hyperglycaemia may have impaired beta cell survival, leading to beta cell loss, as in free implants in mice, and subsequently to aggregation of debris-containing capsules. Aggregation of capsules may also have occurred by their clustering at adhesion or sedimentation points in the abdominal cavity, such as the pelvic floor, leading to hypoxia and debris. It is a likely cause of their metabolic failure in man, rather than the inability of human islet endocrine cells to survive and function in alginate capsules. Methods that avoid aggregation should therefore improve metabolic outcome. Their development needs to combine expertise in biomaterials and beta cell biology with the availability of preclinical models for translation to clinical trials.



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