Insulin secretion dynamics of free and alginate-encapsulated insulinoma cells

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Abstract This study investigates the effect of alginate/poly-L-lysine/alginate (APA) encapsulation on the insulin secretion dynamics exhibited by an encapsulated cell system. Experiments were performed with the aid of a home-built perfusion apparatus providing a 1 min temporal resolution. Insulin profiles were measured from: (i) murine insulinoma β TC3 cells encapsulated in calcium alginate/poly-L-lysine/alginate (APA) beads generated with high guluronic (G) or high mannuoric (M) content alginate, and (ii) murine insulinoma β TC-tet cells encapsulated in high M APA beads and propagated in the presence and absence of tetracycline. Results show that encapsulation in APA beads did not affect the insulin secretion profile shortly post-encapsulation. However, remodeling of the beads due to cell proliferation affected the insulin secretion

profiles; and inhibiting remodeling by suppressing cell growth preserved the secretion profile. The implications of these findings regarding the in vivo function of encapsulated insulin secreting cells are discussed.

Keywords Alginate · Encapsulation · β TC insulinomas · Secretion dynamics · Perfusion system

Introduction

Insulin-dependent diabetes is a serious pathological condition affecting well over 2 million people in the United States, and the number of cases, both of the type 1 juvenile diabetes and the type-2 adult onset diabetes, are increasing. A tissue engineered pancreatic substitute consisting of glucose-responsive, insulin-secreting cells enclosed in a semi-permeable membrane for partial immune protection of the implant from the immune system of the host, constitutes a promising approach for providing a more physiologic, less invasive, and potentially less costly treatment of the disease relative to insulin injections. To overcome the limited availability of human islets, as well as the difficulties which exist with animal islets in regard to their large-scale isolation, immune acceptance, and potential transmission of pathogenic viruses to the host, our group and others have been

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investigating the use of continuous, insulin-secreting cell lines in encapsulation devices.

In the context of pancreatic substitutes, alginates have been used to encapsulate mammalian islets and insulin secreting cell lines in a variety of geometries with microencapsulation in spherical beads being the most prevalent (Yang et al. 1994; Uludag et al. 2000). Although the physical properties of alginates have been investigated (Thu et al. 1996a, b, 2000) and there is an abundance of literature demonstrating the efficacy of alginate encapsulated cells to restore normoglycemia in diabetic animals (Trivedi et al. 2001; de Vos et al. 2002), the biochemical consequences of alginate encapsulation on insulin secreting cells have not been thoroughly characterized. In earlier studies, we demonstrated that encapsulation of the murine insulinoma line β TC3 in calcium alginate/poly-L-lysine/alginate (APA) beads did not significantly affect cellular viability or bioenergetic status shortly after encapsulation and that insulin secretion from the encapsulated cells retained glucose responsiveness (Sambanis et al. 1994). However, in longer-term cultures, alginate composition has a profound effect on the growth characteristics of encapsulated β TC3 and β TC-tet mouse insulinomas (Constantinidis et al. 1999; Stabler et al. 2001, 2002; Simpson et al. 2004, 2005). Specifically, alginates with a high guluronic acid content hindered cellular growth for a period of up to 40 days before allowing the encapsulated cells to proliferate. Once proliferation began, cells grew in isolated pockets distributed randomly throughout the bead. Conversely, alginates with a high mannuronic acid content did not hinder proliferation of encapsulated cells, which grew in an O-ring pattern at the periphery of the bead, where oxygen was more available. The alginate property apparently responsible for this composition-dependent effect was the strength of the interaction between blocks of contiguous guluronic acid residues and Ca^{+2} cations, i.e. the “egg-box” configuration (Simpson et al. 2004).

A key property of encapsulated cell systems in regulating blood glucose levels *in vivo* is the dynamics of insulin release. The study by Chicheportiche and Reach was the first to investigate the effects of alginate encapsulation on insulin release

and concluded that small beads (300 μm in diameter) released more insulin than larger beads (800 μm diameter) (Chicheportiche and Reach 1988). A more recent study by de Haan et al. concluded that it was the porosity of the alginate/poly-L-lysine (PLL) matrix, i.e., the time that alginate was allowed to interact with PLL, that was critical to insulin release, while capsule diameter (for capsules up to 800 μm in diameter) did not affect insulin release (de Haan et al. 2003). Although these and other reports (Fritschy et al. 1991; van Schilfgaarde and de Vos 1999; Lembergt et al. 2001; Rasmussen et al. 2003) describe the effects of alginate encapsulation on the quantity of insulin released over a period of time, they do not detail the effects of encapsulation on the precise dynamics of insulin secretion in response to physiologic stimuli. Reported results indicate that, when properly designed, encapsulated cell systems exhibit the same secretory dynamics shortly post-encapsulation as do free, suspended cells (Sambanis et al. 2002). Hence, encapsulation by itself does not alter the cellular secretory response. This conclusion is compatible with recent studies on β TC3 cell metabolism utilizing ^{13}C NMR spectroscopy. Results demonstrated that differences in metabolic fluxes were minimal between freshly trypsinized and alginate-encapsulated cells, but they were significant between those and cells in monolayers (Simpson et al. 2006). However, the effects of long-term propagation of encapsulated cells, and of the associated remodeling that occurs in the capsules (Constantinidis et al. 1999a; Stabler et al. 2001), on the secretory dynamics remain unknown.

In this study, we investigated the effects of alginate encapsulation, culturing time and culture conditions, on the dynamics of insulin release from murine insulinoma β TC3 and β TC-tet cells. Specifically, insulin secretory profiles in response to glucose concentration step changes were evaluated at different time points along the life of an encapsulated culture using a single-pass perfusion system. β TC3 mouse insulinoma cells were encapsulated in APA beads generated by either high mannuronic acid or high guluronic acid content alginates to assess the effects of alginate composition, while β TC-tet cells were encapsulated in APA beads generated by high mannuronic acid

content alginate and cultured either in the presence or absence of tetracycline to assess the effect of growth regulation on the secretory profile. Results were compared against unencapsulated cell controls.

Materials and methods

Cells and cell culture

β TC3 and β TC-tet cells were obtained from the laboratory of Shimon Efrat, Albert Einstein College of Medicine, Bronx, NY. β TC3 cells were derived from transgenic mice in which a hybrid insulin-promoted simian virus 40 (SV40) tumor antigen gene was targeted against β pancreatic cells (Efrat et al. 1988). These cells maintain features of differentiated β cells for about 50 passages in culture (Efrat et al. 1988) and are glucose-responsive but hypersensitive, in the sense that they exhibit maximal insulin secretion at lower glucose concentrations relative to normal islets (Tal et al. 1992). The β TC-tet cells were derived from double-transgenic mice generated by the crossing of mice from two lineages: in the first lineage, a fusion protein containing the tet repressor (tetR) and the activating domain of the herpes simplex virus protein VP16, which converts the repressor into a transcription activator, was expressed in β cells under control of the insulin promoter. In the second lineage, the SV40 large tumor antigen (TA_g) gene was introduced under control of a tandem array of tet operator sequences and a minimal promoter, which by itself was not sufficient for gene expression (Efrat et al. 1995). As cells appear to be dependent on the continuous presence of the TA_g oncoprotein for proliferation, cell incubation in the presence of tetracycline resulted in inhibition of proliferation, as evidenced by decreased BrdUrd and [³H]thymidine incorporation (Efrat et al. 1995).

In this study, cells of passage number 42–45 (β TC3) and 33–37 (β TC-tet) were used. Cells were cultured as monolayers in T-flasks with culture medium changed every 2–3 days. Culture medium consisted of Dulbecco's Modified Eagle's Medium (DMEM, Sigma Chemical Co., St. Louis, MO) with 25 mM glucose, supplemented with 15%

horse serum (from Hyclone (Logan, UT) and Sigma for β TC3 cells and β TC-tet cells respectively), 2.5% bovine serum (Mediatech, Herndon, VA), 1% penicillin–streptomycin (Mediatech) and L-glutamine (Gibco, Grand Island, NY) to a final concentration of 6 mM. Upon reaching 100% confluence, cells were harvested by trypsin-EDTA (Sigma) for encapsulation.

Cell encapsulation

Cells were encapsulated at a density of 3×10^7 cells/ml alginate using an electrostatic droplet generator (Nisco Engineering Inc., Zurich, Switzerland) and following the procedure of Stabler et al. (2001) for preparation of alginate-poly-L-lysine-alginate beads. Low molecular weight, high guluronic acid content alginate (LVG: 73% guluronic content) or low molecular weight, high mannuronic acid content alginate (LVM: 38% guluronic content) (NovaMatrix, Oslo, Norway) were used for β TC3 cells; high mannuronic acid content alginate (40% guluronic content) (ISP Inc., San Diego, CA) was used for β TC-tet cells. The final bead size was 700–800 μ m in diameter. Bead aliquots of approximately 2 ml volume were cultured with 40 ml of culture medium in each of five T-75 flasks placed on an orbital shaker within a humidified 37°C, 5% CO₂/95% air incubator. The spent medium was completely replaced with the fresh medium every 2–3 days at the beginning of the experiment and every 1–2 days towards the end as cells grew. For the encapsulated β TC-tet cells, beads were propagated without tetracycline for 20 days, at which point the culture was split in two: one-half continued to be propagated in the absence of tetracycline, and the other half was propagated in the presence of 30 ng/ml tetracycline (Sigma) up to day 30. For all entrapments, one of the T-75 flasks was used to withdraw samples of beads for perfusion and histology examination, while the others were used for glucose concentration measurements. Three independent ($n = 3$) bead preparations were performed for each type of culture studied (β TC3 cells in LVG, β TC3 cells in LVM, and β TC-tet cells in M alginate).

Fig. 1 Single-pass perfusion system for measuring the secretion dynamics of free and encapsulated cells subjected to step changes in glucose concentration. Free cells were mixed with macroporous cellulose and then gradually loaded into the cell chamber. Both free and encapsulated cells were retained in place with cotton plugs positioned

upstream and downstream of the bed. Step changes in glucose concentration were implemented by switching from one type of medium to another using a multiport valve. Effluent samples were collected in a fraction collector for subsequent assay of secreted insulin by a rat insulin radioimmunoassay

Perfusion system and experiments

The configuration of the perfusion system is shown in Fig. 1. A spindle-shaped glass tube of approximately 0.34 ml volume was used as the cell chamber. Cotton was placed at both ends of the chamber to retain the cells, free or encapsulated. A peristaltic cassette pump (Manostat, New York, NY) was used to generate the required flow rate; a multiport valve positioned before the cell chamber was used to control the type of medium flowing through the bed. The whole apparatus except for the fraction collector was placed within a humidified 37°C, 5% CO₂/95% air incubator. Effluent samples were stored at -20°C for later assay of insulin. The flow properties were evaluated by flowing through a cell-free system a square wave of FITC-insulin (Sigma) in PBS and assaying the FITC-insulin in effluent samples.

With free β TC3 and β TC-tet cells, freshly trypsinized cells were counted by trypan blue (Sigma). A total of 6×10^6 cells were mixed with macroporous cellulose (Sigma) and gradually loaded into the cell chamber. With encapsulated

β TC3 and β TC-tet cells, approximately the same number of cells, corresponding to 0.2 ml of alginate beads, was loaded in the perfusion chamber without any cellulose. Cells were perfused at a flow rate of $0.25 \pm 10\%$ ml/min, which ensured differential operation of the bed and measurable insulin concentrations in the effluent samples. Each experiment started by flowing culture medium for 30 min to allow the cells to recover from the loading process. Basal medium consisting of glucose-free DMEM without glutamine (Sigma) was then flowed through the bed for 1 h to bring cells to their basal secretion level. A glucose square wave of 1.5 h high glucose was then implemented followed by 1.5 h of basal medium. The high glucose medium consisted of DMEM without glutamine and with 8 mM glucose for β TC3 or with 20 mM glucose for β TC-tet cells. For β TC-tet cells propagated in the presence of tetracycline, all perfusion media were also supplemented with 30 ng/ml tetracycline. To account for differences in the number of cells loaded in the chamber, secretion rates were normalized to the respective average secretion rate measured during the first 30 min following the glucose step up.

Assays

FITC-insulin concentrations were measured by a fluorescence plate reader (Spectra Max Gemini Plate Reader, Molecular Devices Corp., Sunnyvale, CA) using a standard curve constructed from known FITC-insulin concentrations. Glucose concentrations were determined by Glucose Trinder assay (Diagnostic Inc., Oxford, CT). Secreted insulin was assayed by a rat insulin radioimmunoassay (Linco Research, St. Charles, MO), in which the insulin antibody exhibited 100% cross-reactivity against mouse insulin (manufacturer's data). Numbers of β TC3 and β TC-tet cells were measured by mixing suspensions of trypsinized cells with trypan blue (Sigma, St. Louis, MO) and counting cells on a hemocytometer.

Histology

For histology, beads were washed with 1.1% CaCl_2 and fixed in 3% glutaraldehyde (Sigma). After embedding in a paraffin block, beads were sectioned, and slides were stained with hematoxylin/eosin (H/E).

Results

Control experiments

To evaluate the time lag introduced by the free volume between the multiport valve and the system outlet, and the extent of axial mixing, a cell-free cellulose bed was subjected to a square

Fig. 2 Profile of FITC-insulin at the output of perfusion system in response to a square wave at the input. Squares: glucose; diamonds: FITC-insulin

wave of FITC-insulin. Figure 2 shows the profile of FITC-insulin at the output in response to the square wave at the input. There was an approximately 4.5 min time lag for both step up and step down. This compares well with the calculated residence time $t = V/Q$ of 4 min, where $V = 1$ ml is the volume between the valve and the system outlet, and $Q = 0.25$ ml/min is the perfusion flow rate. The more gradual ascent and descent of FITC-insulin at the output relative to the input was apparently the result of axial dispersion. This performance was sufficient and appropriate for evaluating the secretory characteristics of free and encapsulated cells with the accuracy needed to address the objectives of this study.

Effects of alginate composition on secretion dynamics of encapsulated β TC3 cells

β TC3 cells encapsulated in LVG and LVM alginate exhibited different growth patterns, as illustrated by the rate of glucose consumption (GCR) and the histological observation of beads at different time points (Fig. 3). For LVM beads, cells quickly overcame any negative effects of the encapsulation procedure, and the GCR started to increase from day 4 onward. Histology slides indicated that remodeling in cell distribution inside the beads was diffusion driven: cells grew and formed clusters at the periphery of beads where oxygen was more available. By day 20, the

Fig. 3 Temporal changes in the rates of glucose consumption for β TC3 cells encapsulated in 2% LVG (open squares) and 2% LVM (solid diamonds)-based APA beads. Representative histology cross-sections of APA-encapsulated β TC3 cells are: LVM beads at day 2 (left), LVM beads at day 20 (middle), and LVG beads at day 38 (right)

integrity of the beads became compromised and the experiment was terminated. For LVG beads, there was a prolonged growth suppression of cells, with GCR being essentially constant for 20 days before starting to increase. Cell growth resulted in clusters randomly dispersed throughout the beads. The LVG beads maintained their structural integrity throughout the 40 days of culture. When normalized to GCR, the rate of insulin secretion (ISR) in the T-flask cultures was lower at the end of the long-term cultures for both types of alginate. Specifically, the ISR/GCR ratio declined from 2.79 ± 0.46 (mean $\pm$ std. dev.) μU insulin/nmole glucose on day 5 to 1.55 ± 0.33 μU /nmole on day 19 for the LVM culture ($P < 0.06$), and from 3.16 ± 0.14 μU /nmole on day 2 to 0.72 ± 0.26 μU /nmole on day 38 for the LVG culture ($P < 0.03$). These trends are all consistent with trends reported by Stabler et al. (2001).

The secretion dynamics of freshly trypsinized βTC3 cells loaded with cellulose in the perfusion chamber are shown in Fig. 4a. Free cells responded essentially immediately to glucose stimulation, as indicated by the rapid increase in insulin secretion rate from the first sample taken after the system time lag. The insulin secretion rate decreased to below 50% of its highest value after 30 min of glucose stimulation. Secretory responses of LVG-encapsulated cells are shown in the other panels of Fig. 4 and of LVM-encapsulated cells in Fig. 5. The encapsulation process did not affect the glucose-stimulated secretion dynamics, as freshly encapsulated cells in both LVG and LVM exhibited secretion profiles very similar to those of free cells (day 2 LVG: Fig. 4b; day 3 LVM: Fig. 5a). The fact that these measurements were obtained 24 h apart is not critical since at this early stage encapsulated cells did not have enough time to remodel and were thus still distributed uniformly throughout the beads. However, perfusion studies conducted on beads at the end of their culture period, LVG beads on day 38 (Fig. 4d) and LVM beads on day 20 (Fig. 5c), indicated that capsules retained their fast response to glucose stimulation, but a high rate of secretion was maintained longer under glucose induction relative to the free or freshly encapsulated cells. Results with LVG on day 20 (Fig. 4c) approximated the day 38 response

Fig. 4 Dynamic secretory response of free βTC3 cells (a) and of 2% LVG-encapsulated βTC3 cells at day 2 (b), day 20 (c), and day 38 (d) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM. Each insulin secretion rate is normalized to the respective average secretion rate during the first 30 min following the glucose step-up. Squares: glucose; diamonds: insulin secretion rate

(Fig. 4d), whereas results with LVM on day 13 (Fig. 5b) approximated the day 3 response (Fig. 5a). Finally, both types of alginate and

Fig. 5 Dynamic secretory response of APA-encapsulated β TC3 cells in 2% LVM-based capsules at day 3 (**a**), day 13 (**b**), and day 20 (**c**) subjected to a square wave of glucose concentration from 0 to 8 to 0 mM. Each insulin secretion rate is normalized to the respective average secretion rate during the first 30 min following the glucose step-up. Squares: glucose; diamonds: insulin secretion rate

all time points, insulin secretion returned to its basal rate within 15 min after the glucose step down.

The characteristics of the above secretion dynamic patterns are summarized in Table 1. The percentage of induced insulin secreted during the first 30 min was calculated by dividing the amount secreted during the first 30 min post-induction by the total amount secreted during the 1.5 h of glucose stimulation. The glucose induction-fold reported in Table 1 was calculated by dividing the total amount of insulin secreted

during the 1.5 h of induction by the total amount of insulin secreted during the following 1.5 h of exposure to glucose-free basal medium. The sharpness of the secretory profile of free and freshly encapsulated β TC3 cells is reflected by the higher values of the percentage of induced insulin secreted during the initial 30 min after the glucose step up. Following long-term culture, the percentage of induced insulin secreted during the first 30 min of a glucose challenge decreased for both LVG and LVM cultures, as the secretion profiles became broader. The induction-fold also decreased for the LVM culture, however, changes were not statistical significant for the LVG culture.

Effects of tetracycline administration on secretion dynamics of encapsulated β TC-tet cells

Whereas the effects of alginate composition and cellular remodeling on the secretion dynamics were evaluated with β TC3 cells, studies with β TC-tet cells focused on the effect of growth for a single type of alginate. Since administration of tetracycline regulates the growth of the conditionally transformed β TC-tet cells, these cells allow decoupling the effects of alginate composition and encapsulated cell growth. Figure 6 shows the glucose consumption rate (GCR) and representative histology cross-sections of encapsulated β TC-tet cells as functions of time. Encapsulated cells were propagated in tetracycline-free medium for 20 days, at which point the culture was split in two: half the amount of beads continued to be propagated in the absence of tetracycline, whereas the other half was cultured in the presence of 30 ng/ml of the antibiotic. The β TC-tet cells in ISP alginate exhibited a long lag before GCR started to increase. In the absence of tetracycline, GCR started to increase around day 20, and by day 30 histology revealed a dense peripheral band of cells, indicating that the cell remodeling was also primarily driven by oxygen availability. Beads propagated with 30 ng/ml of tetracycline maintained approximately the same glucose consumption rate, and histology at day 30 revealed random cell clusters but no dense peripheral band of cells. These results are consistent with data from monolayer β TC-tet cell cultures which, when propagated with 30 ng/ml

Table 1 Summary of secretion characteristics of free and encapsulated β TC3 and β TC-tet cells

	Free β TC3 cells	Encapsulated β TC3 cells			
		LVM day 2	LVM day 13	LVM day 20	
Percentage of insulin secreted during the first 30 min	33.50 $\pm$ 3.41 (<i>n</i> = 5)	38.67 $\pm$ 7.23 ^a (<i>n</i> = 3)	39.16 $\pm$ 12.00 ^b (<i>n</i> = 3)	24.00 $\pm$ 10.14 (<i>n</i> = 3)	
Glucose induction-fold	5.87 $\pm$ 1.58 (<i>n</i> = 5)	11.18 $\pm$ 0.95 ^a (<i>n</i> = 3)	8.36 $\pm$ 2.36 ^b (<i>n</i> = 3)	4.68 $\pm$ 1.68 (<i>n</i> = 3)	
		Encapsulated β TC3 cells			
		LVG day 2	LVG day 20	LVG day 38	
Percentage of insulin secreted during the first 30 min		39.00 $\pm$ 1.00 ^c (<i>n</i> = 3)	25.00 $\pm$ 3.46 ^d (<i>n</i> = 3)	25.67 $\pm$ 2.88 (<i>n</i> = 3)	
Glucose induction-fold		11.68 $\pm$ 8.59 (<i>n</i> = 3)	6.30 $\pm$ 3.99 (<i>n</i> = 3)	3.83 $\pm$ 0.95 (<i>n</i> = 3)	
	Free β TC-tet cells	Encapsulated β TC-tet cells			
		M-alginate Day 2	M-alginate Day 20	M-alginate Day 30 (w/o tet)	M-alginate Day 30 (with tet)
Percentage of insulin secreted during the first 30 min	24.93 $\pm$ 0.62 (<i>n</i> = 3)	30.30 $\pm$ 10.58 (<i>n</i> = 3)	40.47 $\pm$ 10.66 ^e (<i>n</i> = 3)	24.88 $\pm$ 6.84 (<i>n</i> = 3)	41.89 $\pm$ 8.96 (<i>n</i> = 3)
Glucose induction-fold	4.80 $\pm$ 2.01 (<i>n</i> = 3)	4.86 $\pm$ 0.90 (<i>n</i> = 3)	2.25 $\pm$ 0.88 ^f (<i>n</i> = 3)	1.27 $\pm$ 0.03 (<i>n</i> = 3)	2.48 $\pm$ 0.47 (<i>n</i> = 3)

^a $P > 0.3$ relative to day 13, $P < 0.06$ relative to day 20^b $P < 0.1$ relative to day 20^c $P < 0.05$ relative to days 20 and 38^d $P > 0.3$ relative to day 38^e $P < 0.06$ relative to day 30 w/o tetracycline, $P > 0.3$ relative to day 30 w/tetracycline^f $P < 0.10$ relative to day 30 w/o tetracycline, $P > 0.3$ relative to day 30 w/tetracycline **Fig. 6** Temporal changes in the rates of metabolic activity (glucose consumption rate) for β TC-tet cells encapsulated in 2% alginate-based APA beads. Representative histology cross-sections of alginate encapsulated (TC-tet) cells are (from left to right): day 2, day 20, and day 30 beads propagated with (bottom right) or without (top right) 30 ng/ml tetracycline

tetracycline after reaching 80% confluency, also stopped proliferating over a period of 10 days (results not shown). The effect of culture on the ISR/GCR ratio depended on the administration of tetracycline. In the absence of tetracycline, ISR/GCR declined from $5.8 \pm 1.76 \mu\text{U/nmole}$ on day 2 to $1.26 \pm 0.88 \mu\text{U/nmole}$ on day 30 ($P < 0.03$), whereas when tetracycline was administered from day 20 to day 30, the (ISR/GCR) ratio on day 30 was $9.75 \pm 2.80 \mu\text{U/nmole}$ ($P < 0.002$ relative to day 30 without tetracycline, and $P < 0.03$ relative to day 2). These trends are consistent with patterns of secretion observed in previous studies with β TC-tet cells (Zimmer et al. 1999; Simpson et al. 2005).

As with β TC3 cells, the encapsulation process had no immediate effect on the secretion profile of β TC-tet cells (not shown). Beads cultured

Fig. 7 Dynamic secretory response of 2% APA-encapsulated β TC-tet cells at day 20 (**a**) and at day 30, the latter of beads propagated with (**b**) and without (**c**) 30 ng/ml tetracycline between day 20 and day 30. All encapsulated cell preparations were subjected to a square wave of glucose concentration from 0 to 20 to 0 mM. Each insulin secretion rate is normalized to the respective average secretion rate during the first 30 min following the glucose step-up. Squares: glucose; diamonds: insulin secretion rate

without tetracycline had a broader secretion profile at day 30 (Fig. 7c) relative to day 20 beads (Fig. 7a). On the other hand, the secretion profile from beads propagated with tetracycline from day 20 onward (Fig. 7b) was very similar to the profile of day 20 beads, indicating that growth inhibition by tetracycline preserved the secretory profile. The spikes observed after the step down in glucose concentration were probably due to the sensitivity of β TC-tet cells to the small pressure changes during medium switches. These spikes

did not significantly affect the overall secretory profile, including the sharpness of the response to the glucose step-up.

The secretion characteristics of β TC-tet cells are also listed in Table 1. Day 30 beads cultured without tetracycline had different secretion characteristics compared to day 20 beads, in that both the percentage of induced insulin secreted during the first 30 min and the induction-fold decreased. On the other hand, there were no statistical differences in these two parameters between days 20 and 30 when the cultures were propagated with 30 ng/ml of tetracycline from day 20 onward.

Discussion

In a cell-based therapy for insulin-dependent diabetes, the cells used should, ideally, exhibit the same insulin secretion dynamics as normal islets. Thus, the secretion of insulin should be glucose-dependent and exhibit fast kinetics in response to glucose and other physiologic stimuli. Static incubation of islets in media containing different glucose concentrations can elucidate the dependence of insulin secretion on glucose concentration. However, to capture the dynamic response of insulin release in response to rapid glucose concentration changes, a single pass perfusion system is needed. Following a step up in glucose concentration, islets exhibit a biphasic insulin secretion profile. The initial acute phase of insulin release is due to the discharge of insulin stored in readily releasable granules, whereas the second more prolonged phase of insulin secretion is attributed to elevated insulin biosynthesis and/or to the preparation of another group of granules for release (Bratanova-Tochkova et al. 2002; Straub and Sharp 2002). The precise characteristics of the biphasic secretion pattern are reportedly species-dependent (Henquin et al. 2002).

Murine insulinoma β TC3 and β TC-tet cells exhibit secretion profiles similar to those obtained with islets in vitro: an acute first phase of insulin secretion followed by a second, lower and flat or slowly declining, secretion phase. Our current study shows that encapsulation in APA beads did not affect the secretory profiles, which were

essentially the same between free cells and encapsulated cells examined within 2–3 days post-encapsulation. This is consistent with results from studies comparing glucose metabolism (by ^{13}C -labeled glucose incorporation and analysis of cell extracts by nuclear magnetic resonance spectroscopy) and insulin secretion of βTC3 cells in monolayer cultures, in suspension shortly after trypsinization, and encapsulated in APA beads. Although significant differences existed between monolayer and suspended cells, differences in the metabolic profiles and insulin secretion rates of suspended and freshly encapsulated cells were minimal (Simpson et al. 2006).

A possible cause for the change in insulin secretion profile exhibited by APA-encapsulated cells with time in culture is the change in the cellular microenvironment due to cell growth. Shortly after encapsulation, cells were homogeneously distributed as single units inside the beads. During the remodeling process, cells formed clusters and deposited extracellular matrix, thus altering the cell–cell and cell–matrix interactions from those in freshly encapsulated preparations. Furthermore, the rates of transport of oxygen and other nutrients and metabolites inside the cell clusters are lower than the transport rates in the alginate gel, resulting in a broader distribution of the nutritive microenvironments experienced by cells after remodeling versus initially. With the bead diameter and cell density used in this study, all encapsulated βTC3 cells were initially exposed to oxygen concentrations above those limiting secretion (Tziampazis and Sambanis 1995). However, after remodeling, it is expected that cells at the core of the clusters secreted insulin at reduced rates or not at all due to the hypoxic conditions at these locales. Thus, a heterogeneous cell population is generated with remodeling leading to a broad insulin secretion profile. In support of this conclusion are the insulin profiles obtained from $\beta\text{TC-tet}$ cells. Growth arrest, such as that achieved with administration of tetracycline is necessary to decelerate or arrest remodeling and maintain the secretion profile of APA-encapsulated cells. It should be noted that for encapsulated $\beta\text{TC-tet}$ cells, both the concentration of tetracycline and the time of administration are critical in achieving growth arrest (Simpson et al.

2005). Thus, the *in vivo* implementation of this growth control mechanism may be challenging, although in a recent study encapsulated $\beta\text{TC-tet}$ cells successfully restored normoglycemia in spontaneously diabetic NOD mice when tetracycline was administered to the animals immediately post-implantation (Black et al. 2006).

Although encapsulated mouse insulinoma cells are useful as allografts in studies with diabetic mice, clinically relevant substitutes are expected to be based on allogeneic human cells. This is mainly because of the strong immune response elicited by xenografts even when they are encapsulated: although encapsulation prevents direct cellular recognition, antigens shed by the cells as a result of secretion and, more importantly, lysis in the capsules eventually pass through the permselective barrier and are recognized by the antigen-presenting cells of the host (Sambanis, *in press*). Recently, significant progress was made towards establishing a human pancreatic β cell line that appears functionally equivalent to normal β cells (Narushima et al. 2005). This was accomplished through a complicated procedure involving retroviral transduction of primary β cells with the SV40 large T antigen and cDNAs of human telomerase reverse transcriptase. This resulted in a reversibly immortalized human β cell clone, which secreted insulin in response to glucose, expressed β cell transcriptional factors, prohormone convertases 1/3 and 2 that process proinsulin to mature insulin, and restored normoglycemia upon implantation in immunodeficient diabetic mice (Narushima et al. 2005). It is expected that the lessons learned in the present study on the changes of secretory characteristics of encapsulated mouse insulinomas with time and conditions in culture would be directly applicable to encapsulated human cell lines, which constitute a system much closer to an actual clinical product.

In conclusion, this study demonstrated that APA encapsulation had no immediate effect on the secretory dynamics of insulinoma cells. However, the dynamic secretion pattern changed and the ISR/GCR ratio declined with unregulated growth for both APA-encapsulated βTC3 and $\beta\text{TC-tet}$ cells. When tetracycline was used to suppress $\beta\text{TC-tet}$ cell growth, the secretion

dynamics from these capsules remained the same, whereas the ISR/GCR ratio increased. It is unclear whether such changes in secretion patterns would affect the effectiveness of APA-encapsulated insulinomas in providing in vivo an optimum glycemic control. Growth arrest, either through regulation of expression of the SV40 T antigen (Tag) oncoprotein by tetracycline, as in the β TC-tet cells, or through some other mechanism, could be applied if changes in secretory profile prove to be detrimental for effective glycemic regulation.

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