Functional Analysis of a Conditionally Transformed Pancreatic β-Cell Line

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Development of β-cell lines for cell therapy of diabetes is hindered by functional deviations of the replicating cells from the normal β-cell phenotype. In a recently developed cell line, denoted βTC-tet, derived from transgenic mice expressing the SV40 T antigen (Tag) under control of the tetracycline (Tc) gene regulatory system, growth arrest can be induced by shutting off Tag expression in the presence of Tc. Here, we compared differentiated cell functions in dividing and growth-arrested βTC-tet cells, both in culture and in vivo. Proliferating cells stably maintained normal glucose responsiveness for >60 passages in culture. Growth-arrested cells survived for months in culture and in vivo and maintained normal insulin production and secretion. After growth arrest, the cells gradually increased their insulin content three- to fourfold. This occurred without significant changes in insulin biosynthetic rates. At high passage numbers, proliferating βTC-tet cells exhibited an abnormal increase in hexokinase expression. However, the upregulation of hexokinase was reversible upon growth arrest. Growth-arrested cells transplanted intraperitoneally into syngeneic recipients responded to hyperglycemia by a significant increase in insulin secretion. These findings demonstrate that transformed β-cells maintain function during long periods of growth arrest, suggesting that conditional transformation of β-cells may be a useful approach for developing cell therapy for diabetes.
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Transplantation of pancreatic β-cells represents a potentially promising therapy for diabetes. The difficulty in obtaining sufficient numbers of β-cells from isolated islets has prompted attempts to develop functional β-cell lines. A number of such cell lines have been developed from insulinomas induced in rodents (1–9). Although the majority of these lines deviate considerably from the normal β-cell phenotype with respect to insulin production and regulated secretion, a number of cell lines have manifested a high level of differentiation (4,6–9). β tumor cell (βTC) lines developed in our laboratory from transgenic mice expressing the SV40 T antigen (Tag) under control of the rat insulin II promoter produce about a third of the amounts of insulin found in normal islets and respond to multiple physiologically relevant insulin secretagogues in a manner similar to that of normal mouse β-cells (10).

An obvious obstacle to the use of transformed β-cell lines in transplantation is their uncontrolled proliferation. To address this drawback, Efrat et al. (12) used the tetracycline (Tc) conditional gene expression system (11) to regulate Tag activity in transgenic mouse β-cells. A βTC line developed from these mice, denoted βTC-tet, is strictly dependent on Tag expression for its proliferation. In the absence of Tc, the Tag transgene is expressed and maintains the cells in continuous proliferation. In the presence of Tc, Tag expression is tightly shut off, leading to a complete arrest of cell replication. The regulation is reversible, allowing resumed cell growth upon removal of Tc. This system offers an efficient way for cell expansion in culture and for regulation of cell numbers after transplantation. Indeed, βTC-tet cells are unique among rodent β-cell lines in their ability to maintain euglycemia for months in streptozotocin-diabetic syngeneic recipients in the presence of Tc (12), whereas all other known insulinoma cell lines form tumors in vivo that lead to severe hypoglycemia.

Although proliferating βTC-tet cells manifest a highly differentiated phenotype, it is of interest to determine whether growth arrest can affect their function. β-Cells in normal islets withdraw from the cell cycle when undergoing final differentiation. Induction of cell replication may shut off some of their specialized functions. Therefore, growth arrest could be expected to restore a more normal phenotype in the βTC-tet cells. Conversely, it could be expected that forced growth arrest of transformed β-cells may lead to a gradual deterioration in viability and function. To examine these possibilities, we compared insulin production and secretion in dividing and growth-arrested βTC-tet cells in culture and in vivo. Proliferating cells stably maintained normal glucose responsiveness for >60 passages in culture. Growth-arrested cells survived for months in culture and in vivo and maintained insulin production and secretion. After growth arrest, the cells manifested a gradual increase in insulin content. This occurred without significant changes in insulin biosynthetic rates. Growth-arrested cells transplanted intraperitoneally into syngeneic recipients responded to hyperglycemia by a significant increase in insulin secretion. These findings demonstrate that transformed β-cells maintain differentiated function dur-

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βTC, β tumor cell; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; IBMX, isobutylmethylxanthine; KRB, Krebs-Ringer buffer; PBS, phosphate-buffered saline; RIA, radioimmunoassay; Tag, SV40 T antigen; Tc, tetracycline; TCA, trichloroacetic acid.

TABLE 1
Effect of growth arrest on insulin content of βTC-tet cells

Incubation condition	Insulin content (ng/100 μg protein)
–Tc	2,488 ± 217
+Tc	9,056 ± 894

Data are means ± SE of seven separate experiments, each performed in triplicate. βTC-tet cells at passages 14–29 continuously growing (–Tc) or growth-arrested for 4 weeks (+Tc) were extracted for insulin assay or for protein determination. In both growing and growth-arrested cells, 100 μg protein corresponded to ~10^6 cells.

ing long periods of growth arrest, suggesting that conditional transformation of β-cells may be a useful approach for developing cell therapy for diabetes.

RESEARCH DESIGN AND METHODS

Cell culture. The generation of the βTC-tet line has been previously described (12). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) (GIBCO) containing 25 mmol/l glucose and supplemented with 15% horse serum (GIBCO), 2.5% fetal bovine serum (Hyclone), 100 U per ml penicillin, and 100 μg per ml streptomycin (growth medium). Growth arrest was induced by including 1 μg per ml Tc (Sigma) in the culture medium. After 7 or more days, this concentration of Tc was sufficient to totally inhibit [^{3}H]thymidine incorporation into βTC-tet cells (12).

Insulin content and secretion assay. Cells were plated in 24-well culture dishes (Costar) at 4×10^5 per well. Assays were performed 4–6 days after plating, when the cells were 70–90% confluent. The medium was exchanged with fresh medium 16 h before secretion was assayed. On the day of the experiment, the medium was removed and the cells were washed twice with HEPES-buffered Krebs-Ringer buffer (KRB) (119 mmol/l NaCl, 4.74 mmol/l KCl, 2.54 mmol/l $CaCl_2$, 1.19 mmol/l $MgSO_4$, 1.19 mmol/l KH_2PO_4, 25 mmol/l $NaHCO_3$, 10 mmol/l HEPES at pH 7.4, and 0.1% bovine serum albumin [BSA] [Sigma]) bubbled with 95% O_2/5% CO_2. They were then pre-incubated in KRB at 37°C for 1 h, followed by incubation in fresh KRB containing 0.5 mmol/l isobutylmethylxanthine (IBMX) (Sigma) and the indicated glucose concentration for 2 h. Each condition was assayed in triplicate. Cell clusters were formed by gentle shaking of trypsinized cells. The 5×10^5 cells were placed in 100-mm Petri dishes in 6 ml of growth medium on a rotator in a CO_2 humidified incubator for 48 h. The clusters were washed twice in KRB and pre-incubated for 1 h. They were then transferred to capped glass vials at ~5×10^4 cells per vial and incubated in 1 ml KRB bubbled with O_2 containing the indicated secretagogues in a shaking 37°C water bath for 2 h. At the end of the incubation period, the buffer was removed from the wells and the vials and centrifuged at 400*g* to remove any detached cells. The cells were extracted in acetic acid, and the amount of insulin in the buffer and cell extract was determined by radioimmunoassay (RIA), as described (10). Cellular protein was measured by the method of Lowry et al. (13).

Glucose utilization assay. Glucose utilization was measured by following the conversion of 5-[^{3}H]glucose into ^3H_2O. Cells at passage 4 were placed in culture media or culture media containing Tc (1 μg/ml) for 4 weeks. They were then plated in 24-well culture plates at 2×10^5 cells per well. After 3 days, they were incubated with KRB for 1 h, followed by incubation with KRB containing the indicated concentrations of 5-[^{3}H]glucose (Amersham) for 2 h. ^3H_2O formation was measured as described previously (10).

Glucose phosphorylation assay. Attached cells were washed with phosphate-buffered saline (PBS) and scraped into 1 ml of extraction buffer (20 mmol/l K_2HPO_4, pH 7.5, 5 mmol/l dithiothreitol, 1 mmol/l EDTA, and 110 mmol/l KCl). The cells were homogenized with a Dounce homogenizer by 35 strokes. The homogenate was centrifuged for 10 min at 12,000*g*. The supernatant was used for assays of glucose phosphorylation activity. Glucokinase and hexokinase activity was assayed by a fluorimetric method as previously described (14). Approximate V_{max} and K_m values were determined by Edie-Hofstee plots with the best-fitted lines drawn using the method of least squares.

Metabolic labeling. Cells were trypsinized and distributed into reaction tubes (2–5 × 10^5 cells/tube). The cells were washed three times in PBS and incubated in 0.5 ml methionine-free DMEM containing 10% dialyzed fetal bovine serum for 30 min at 37°C in a 5% CO_2 humidified incubator. At the end of the incubation, the medium was removed and the cells were washed three times with KRB without BSA (KRB). The labeling reaction was initiated by the addition of 100 μl prewarmed KRB containing 20 mmol/l glucose and 150 μCi [^{35}S]methionine (1,400 Ci/mmol; Amersham). Samples were incubated for the indicated times. At the end of the incubation period, the buffer was removed and replaced by cold KRB containing 2 mmol/l L-methionine. The cells were washed twice with cold KRB, resuspended in 200 μl lysis buffer (0.2 mmol/l glycine, 2.5 mg/ml BSA, 0.5% NP-40, pH 8.8, containing 1 mmol/l EDTA, 1 mmol/l phenylmethylsulfonyl fluoride, 20 μmol/l leupeptin, and 0.1 mmol/l E-64 [transepoxysuccinyl-L-leucyl-amido-(4-guanidino)butane]) and passed repeatedly through a 21-gauge needle. Lysates were centrifuged for 2 min at 14,000*g*, and the supernatants were collected and stored at –20°C pending analysis.

Measurement of insulin/proinsulin and total protein biosynthesis. Quantitative determination of total radioactive protein was performed as described by Halban et al. (15). Cell lysate supernatant (10 μl) was mixed with 0.5 ml of 0.2 mmol/l glycine-2.5 mg/ml BSA, pH 8.8 (glycine-BSA) and 0.5 ml 10% trichloroacetic acid (TCA). The tubes were centrifuged, and the precipitate was washed twice by resuspension in 0.5 ml glycine-BSA and 0.5 ml 10% TCA. Radioactivity was determined in a liquid scintillation counter. Radioactively labeled insulin/proinsulin was measured by quantitative immunoprecipitation of aliquots of cell lysate (5×10^5 cpm TCA precipitable counts) as described earlier (16). Immunoprecipitation was performed using excess anti-insulin antibody and protein A-Sepharose (Pharmacia-LKB). Nonimmune serum was used to assess nonspecific immunoprecipitable counts. Nonspecific counts averaged 2–3% of total counts immunoprecipitated in the presence of insulin antibodies. The radioactivity in the immunoprecipitates was measured in a liquid scintillation counter. Each sample was assayed in triplicate. Because the Edie-Hofstee plots are a linear transformation of the Michealis-Menten equation, the approximate $S_{0.5}$ and K_{cat} values are well represented by K_m and V_{max}, respectively.

Hyperglycemic clamp studies. For cell implantation, βTC-tet cells were trypsinized, washed in PBS, and resuspended in PBS at 5×10^6 cells per ml. One million cells were injected intraperitoneally into male syngeneic C3HeB/FeJ mice that had been made diabetic by a single dose of streptozotocin (200 mg/kg body wt i.p.). Mice were monitored daily for blood glucose levels by bleeding from the tail vein and measuring glucose using a Glucometer Elite instrument. When blood glucose was restored to the physiological range, Tc (1 mg/ml in 2.5% sucrose) was added to their drinking water to induce βTC-tet cell growth arrest. Hyperglycemic clamp studies (17–19) were performed on six of these mice after 8–10 days of Tc administration and in five animals after 5–8 weeks of Tc administration. Mice (28–35 g) were anesthetized with chloral hydrate (400 mg/kg of body wt i.p.), and an indwelling catheter was inserted into the right internal jugular vein, as previously described (17,18). The venous catheter was used for the multiple infusions, and blood samples were obtained from the tail. Mice were studied 4–6 days postsurgery in the awake, unrestrained state using the hyperglycemic clamp technique as previously described (17,18). Food was removed for 6 h before the in vivo studies. Briefly, a variable infusion of a 25% glucose solution was started at time 0 and periodically adjusted to clamp the plasma glucose concentration at ~16 mmol/l. The studies lasted 170 min and included an 80-min basal equilibration period and a 90-min hyperglycemic clamp period. Plasma samples for determination of plasma insulin concentrations were obtained at time -40, 0, 10, 20, 30, 40, 50, 60, 70, 80, and 90 min during the study. The total volume of blood withdrawn was ~0.9 ml/study; to prevent volume depletion and anemia, a solution (1:1 vol/vol) of ~1.2 ml of fresh blood (obtained by heart puncture from a littermate of the test animal) and heparinized saline (10 U/ml) was infused. Plasma glucose was measured by the glucose oxidase method (Glucose Analyzer II; Beckman Instruments, Palo Alto, CA), and plasma insulin was measured by RIA as described above using rat and porcine insulin standards.

The study protocol was reviewed and approved by the Institutional Animal Care and Use Committees of the Albert Einstein College of Medicine. Differences between groups were determined by analysis of variance.

RESULTS

Effect of growth arrest on insulin content. Studies were performed comparing proliferating βTC-tet cells with βTC-tet cells growth-arrested in the presence of Tc for either a short period (up to 2 weeks) or a prolonged period (over 3 weeks). Proliferating βTC-tet cells contained an average of 2,488 ± 217 ng of insulin per 100 μg of cell protein (Table 1). This content is comparable to that previously reported for other βTC cell lines (8–10). The insulin content of βTC-tet cells growth-arrested for 4 weeks averaged 9,056 ± 894 ng per 100 μg cell protein (Table 1). This effect of growth arrest on insulin content was progressive after the first week of incubation in the presence of Tc and plateaued after 4–6 weeks of growth arrest. To determine whether the increased insulin content

TABLE 2
Effect of growth arrest on (pro)insulin biosynthesis in βTC-tet cells

Incubation condition	(Pro)insulin (cpm · min^{-1} · μg^{-1} protein)	Total protein (cpm · min^{-1} · μg^{-1} protein)	% (Pro)insulin from total protein
–Tc	19.1 ± 1.7	71.6 ± 2.9	26.7
+Tc	17.4 ± 1.1	81.0 ± 8.9	21.4

Data are means ± SE (n = 6). βTC-tet cells continuously growing (–Tc, passage 25) or growth-arrested for 8 weeks (+Tc, passage 32) were pulse-labeled for 30 or 60 min with [^{35}S]methionine. The radioactivity associated with (pro)insulin was determined by immunoprecipitation with anti-insulin antibodies, and the incorporation of radioactivity into total protein was determined by TCA precipitation. No significant differences were noted between proliferating and growth-arrested cells, as assessed by Student's t test.

resulted from an increase in insulin protein biosynthesis, the biosynthetic rate of (pro)insulin in βTC-tet cells was tested by pulse-labeling with [^{35}S]methionine for 30 and 60 min in the presence of 20 mmol/l glucose (Table 2). These biosynthetic studies were performed in KRB in the absence of IBMX or other potentiators of glucose-induced insulin secretion. Insulin release from both Tc$^+$ and Tc$^-$ cells under these conditions is similar. No significant differences in the incorporation of [^{35}S]methionine into (pro)insulin or total protein were detected between proliferating βTC-tet cells and cells that were growth-arrested for 8 weeks.

We also examined the release of insulin into the culture medium over a 24-h period by cells that were growing and cells that were growth-arrested for 2–4 weeks. Growing cells released 22 ng insulin per 100 μg cellular protein, whereas growth-arrested cells released 16 ng insulin per 100 μg cellular protein. Thus, despite similar release rates of insulin between growing and growth-arrested cells, the content of chronically growth-arrested cells rose about fourfold (Table 1). The small amounts of insulin released in the presence of 25 mmol/l glucose and amino acids in the DMEM medium reflects the requirement of a potentiator such as IBMX for a large effect of glucose on insulin secretion.

Insulin secretion from βTC-tet cells. Because incubation of βTC-tet cells with glucose alone induced only a twofold increase in insulin secretion (data not shown), insulin secretion was analyzed in the presence of 0.5 mmol/l IBMX (Fig. 1). IBMX was included because in βTC-tet cells, as in other βTC lines (8–10), it potentiates the glucose effect, although it is ineffective as a secretagogue by itself. In the absence of glucose, insulin release averaged 1.32 ng per 100 ng of insulin content. Glucose (16.7 mmol/l) induced a 12-fold increase in insulin release. There was a concentration dependency for glucose-induced insulin release between 5 and 16.7 mmol/l glucose. A statistically significant increase in insulin release first occurred at 5 mmol/l glucose, as observed in normal islets. Correctly regulated glucose-induced insulin release from βTC-tet cells remained stable for >60 passages (more than a year in continuous culture). The glucose concentration dependency for glycolysis, as measured by ^3H_2O formation from 5-[^{3}H]glucose, paralleled the glucose concentration dependency for insulin secretion (Fig. 2). This response was associated with a predominance of glucokinase activity and a low level of hexokinase (Table 3). The presence of low levels of hexokinase and high levels of glucokinase has been a consistent characteristic of βTC lines with correctly regulated glucose-induced insulin secretion (8,9). Growth arrest for 4 weeks in these early-passage cells resulted in a small decrease in the ratio of hexokinase and glucokinase activities, from 1:4 to 1:5.75 (Table 3).

The addition of Tc to βTC-tet cells results in complete growth arrest within 1 week. No changes in insulin secretion in response to glucose were noted after 1–2 weeks in Tc (Fig. 1). However, when βTC-tet cells were growth-arrested for 3 or more weeks, glucose-induced insulin secretion became blunted (Table 4). This effect was restricted to the secretory response to glucose. Depolarizing K$^+$ in the presence of IBMX was effective in both growing cells and long-term growth-arrested cells (Table 4). Likewise, insulin release in response to a cocktail of secretagogues (22 mmol/l glucose, 1.0 mmol/l IBMX, 1.0 mmol/l tolbutamide, and 1 μmol/l TPA) increased 7.05-fold in growing cells and 6.5-fold in cells that were growth-arrested for 4 weeks.

Microscopic examination of long-term growth-arrested βTC-tet cell colonies revealed a flattened morphology, compared with the spherical shape of proliferating cells (Fig. 3). To determine whether the changes in the secretory response to glucose were associated with the altered cell morphology, insulin release was assayed in βTC-tet cells in free-floating cell clusters, which have a morphology similar to that of colonies of proliferating cells. As shown in Table 4, insulin

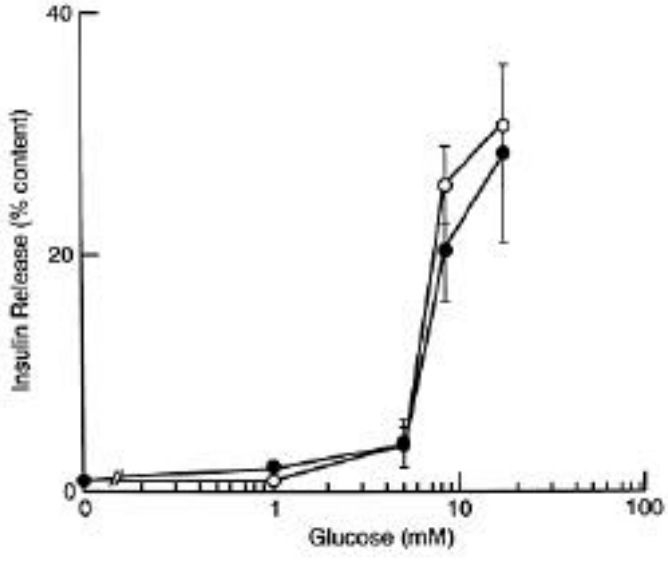

FIG. 1. Glucose-induced insulin secretion from βTC-tet cells. Cells at passages 4–8 continuously growing (○) or growth-arrested for 1 week (●) were incubated with the indicated glucose concentrations. Incubation medium and cell extracts were analyzed by insulin RIA. Data represent means ± SE (n = 6).

TABLE 3
Glucose phosphorylation activity in βTC-tet cells: effect of growth arrest

Incubation condition	Glucokinase		Hexokinase	
	V_{max} (U/g)	K_m (mmol/l)	V_{max} (U/g)	K_m (mmol/l)
–Tc	1.16	8.28	0.29	ND
+Tc	1.38	9.23	0.24	0.36

V_{max} and K_m values for glucokinase and hexokinase were calculated from Eadie-Hofstee plots of glucose phosphorylation activity at multiple glucose concentrations in extracts of βTC-tet cells at passages 3–4 under growing (–Tc) or growth-arrest (4 weeks +Tc) conditions. One unit is defined as the amount of enzyme that phosphorylates 1 µmol of substrate per minute at 25°C. ND, not determined.

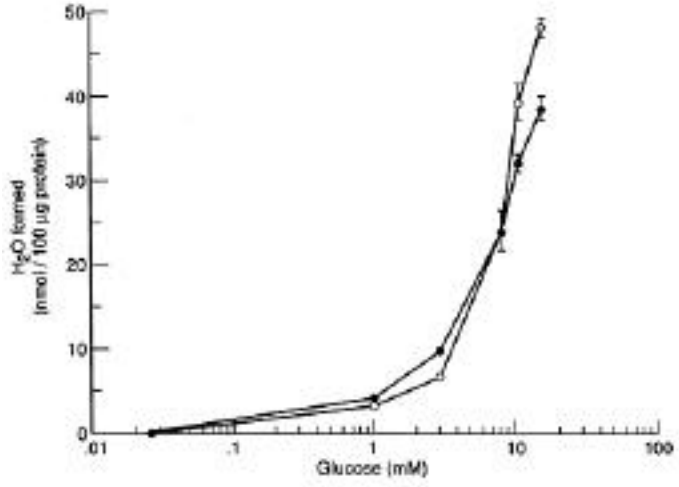

FIG. 2. Glucose utilization by βTC-tet cells. Passage 4 cells continuously growing (○) or growth-arrested for 4 weeks (●) were incubated with 0.5 mmol/l IBMX, and the indicated concentrations of 5-[^{3}H]glucose and the levels of ^3H_2O were determined. Data are means ± SE of three replicates.

release in response to both glucose and depolarizing K^+ concentrations was comparable in dividing and long-term growth-arrested cells in clusters. The clustered cells had an elevated insulin content similar to that of attached long-term growth-arrested cells (data not shown). A consistent effect of long-term growth arrest in both attached and clustered cells was a decrease in the basal insulin release. As seen in Table 4, insulin release in the presence of buffer alone was 30% lower in growth-arrested cells than in growing cells.

Effect of growth arrest on hexokinase expression. Most nonclonal βTC lines manifest a gradual increase in hexokinase activity after about 20 passages in culture (8,9). In contrast, βTC-tet cells exhibited a low hexokinase activity for >60 passages. Nonetheless, with increased passage number, these cells showed an increase in hexokinase activity (Table 5). Studies from this laboratory (8,9) indicated that such increases in hexokinase activity result in an insulin secretory response to subphysiological glucose concentrations. The upregulation of hexokinase was reversible to a large extent upon growth arrest. A decrease in hexokinase activity was first detected after 1 week of incubation in the presence of Tc (Fig. 4). Upon continued growth arrest, the hexokinase activity gradually dropped down to a third of its peak level. No significant changes were observed in the glucokinase activity during this period (Fig. 4). In addition, no significant changes were observed in either hexokinase or glucokinase activity in the proliferating βTC-tet cells assayed in parallel (Table 5, passages 76–81). The decrease in hexokinase in the growth-arrested cells was not sufficient to shift the glucose-induced insulin secretion back to the physiological glucose concentration range (data not shown), probably because the ratio of hexokinase to glucokinase (about 1:1) was still higher than in early-passage cells (Table 3) or normal islets (about 1:4). However, these findings clearly demonstrate that the increase in hexokinase activity is related to cell proliferation and is reversible upon growth arrest.

Insulin secretion from transplanted βTC-tet cells. Previous studies have shown that βTC-tet cells can correct

TABLE 4
Effect of long-term growth arrest on insulin release from βTC-tet cells

Secretagogue	–Tc		+Tc	
	Insulin release (% of content)	Fold stimulation	Insulin release (% of content)	Fold stimulation
Attached cells				
None	1.89 ± 0.24	1.0	1.11 ± 0.11	1.0
Glucose	19.07 ± 1.37	10.08	2.04 ± 0.23	1.84
K^+	33.36 ± 4.29	17.65	20.65 ± 4.93	18.60
Clustered cells				
None	1.27 ± 0.27	1.0	0.91 ± 0.25	1.0
Glucose	11.18 ± 3.66	8.80	7.56 ± 1.71	8.30
K^+	21.31 ± 6.01	16.78	18.87 ± 6.20	20.74

Data are means ± SE of 24 replicates performed on eight separate occasions (attached cells) and 11 replicates performed on three separate occasions (clustered cells). Insulin release was analyzed in βTC-tet cells under growth (–Tc, passages 8–30) or growth-arrest (3–8 weeks +Tc, passages 16–20) conditions. The cells were assayed either attached to the surface of the culture dish wells (attached cells) or in free-floating clusters in glass vials (clustered cells). Cells were pre-incubated in glucose-free KRB, followed by incubation in KRB containing 0.5 mmol/l IBMX (None), KRB containing 0.5 mmol/l IBMX plus 16.7 mmol/l glucose (Glucose), or KRB adjusted to contain 47 mmol/l potassium (K^+). The medium and cell extract were assayed for insulin. Fold stimulation is calculated relative to cells incubated in the absence of secretagogues.

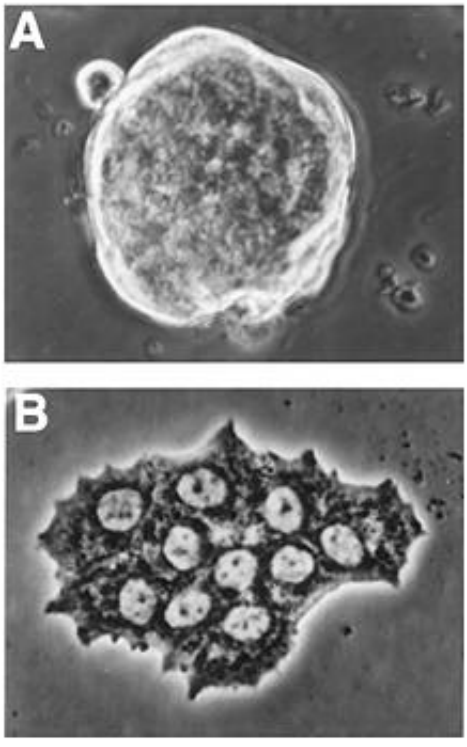

FIG. 3. Morphology of proliferating βTC-tet cells (*A*) and cells growth-arrested for 4 weeks (*B*). Cells were photographed under phase-contrast illumination. Original magnification ×720.

streptozotocin-induced hyperglycemia in syngeneic mice when implanted intraperitoneally (12). However, it remained to be established whether this effect represented regulated insulin secretion, as opposed to constitutive release. To determine whether transplanted βTC-tet cells were constitutively secreting insulin or were capable of responding to changes in plasma glucose by adjusting their insulin secretion, we used the hyperglycemic clamp technique in conscious mice. Glucose was infused to achieve steady-state plasma levels of 16–17 mmol/l. The glucose infusion rate required to achieve this glucose level was 48.1 ± 15 and 46.0 ± 11.8 mg · kg^{-1} · min^{-1} in mice transplanted with short-term (8–10 days) or long-term (5–8 weeks) growth-arrested cells, respectively. Consistent with the above metabolic data, glucose-induced insulin secretion was similar in both short-term and long-term growth-arrested cells (Fig. 5). βTC-tet cells responded to the hyperglycemia by increasing their insulin release up to 13-fold. These findings demonstrate that growth-arrested βTC-tet cells can sense the rise in plasma glucose and respond by increasing their insulin output.

DISCUSSION

Our findings demonstrate that long-term growth-arrested βTC-tet cells maintain insulin production and glucose-induced insulin secretion. A progressive increase in insulin content is consistently noted during 4–6 weeks of growth arrest. This increase is not associated with an increase in (pro)insulin synthesis or a decrease in the amount of insulin released into the culture medium. The increase in insulin content may result from an alteration in proinsulin processing or in intracellular storage and/or degradation of mature

TABLE 5
Glucose phosphorylation activity in βTC-tet cells: effect of passage number

Passage number	V_{max} (U/g)	
	Glucokinase	Hexokinase
3	1.16	0.29
22	1.37	0.68
74	1.28	5.88
76	1.06	6.91
81	1.14	7.99

V_{max} values for glucokinase and hexokinase were calculated from Eadie-Hofstee plots of glucose phosphorylation activity at multiple glucose concentrations in extracts of growing βTC-tet cells (–Tc). One unit is defined as the amount of enzyme that phosphorylates 1 µmol of substrate per minute at 25°C. K_m values averaged 7.78 mmol/l for glucokinase and 0.018 mmol/l for hexokinase.

insulin. Another reproducible effect of growth arrest is a reduction in basal insulin release compared with proliferating cells. Such low basal insulin release is typical of normal islets. These changes in insulin content and constitutive release noted in growth-arrested cells can be considered to reflect a more differentiated phenotype.

βTC-tet cells have maintained correct glucose responsiveness for insulin secretion for >1 year of continuous proliferation in culture. Glucose-induced insulin secretion from cultured cells was primarily studied in the presence of IBMX because agents that increase cAMP levels greatly augment the effect of glucose on secretion in βTC cell lines (8–10) and β-cells isolated from normal rat islets (20,21). Correct glucose responsiveness of these cells has been associated with maintenance of a low hexokinase activity and a normal activity of glucokinase, as is the case with normal islets. In contrast, all previously reported nonclonal βTC lines manifested an

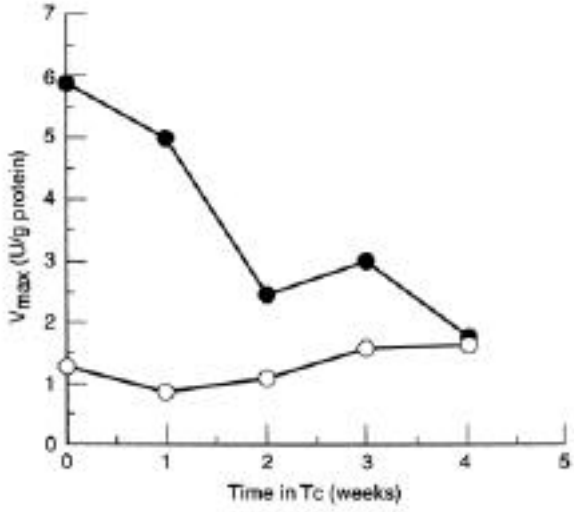

FIG. 4. Changes in glucose phosphorylation activity in growth-arrested, late-passage βTC-tet cells. Cells at passage 76 incubated in the presence of Tc for the indicated time were homogenized, and the homogenate was analyzed for glucokinase (○) and hexokinase (●) activity by a fluorimetric method. V_{max} values were calculated from Eadie-Hofstee plots of glucose phosphorylation activity at multiple glucose concentrations, with the best-fitted lines drawn using the method of least squares. One unit is defined as the amount of enzyme that phosphorylates 1 µmol of substrate per min at 25°C.

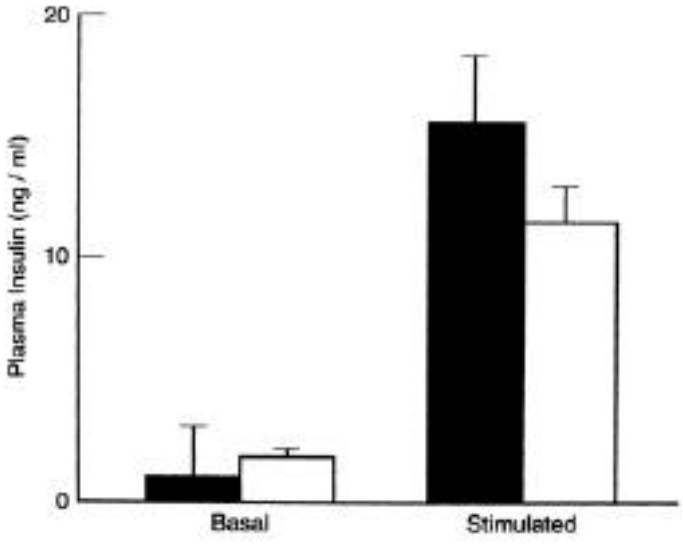

FIG. 5. Plasma insulin concentrations in diabetic mice transplanted with βTC-tet cells. Steady-state plasma insulin concentrations were measured under fasting (basal) or hyperglycemic clamp (stimulated) conditions in mice implanted with βTC-tet cells at passage 26, growth-arrested for 8–10 days (■) or 5–8 weeks (□). Data are means ± SE.

increase in hexokinase activity and a shift to the left in glucose responsiveness after approximately 20 passages in culture (8,9). Clonal lines, isolated from these parental lines before passage 20, demonstrated a greater stability of the physiological glucose responsiveness and the low hexokinase activity (9). It is not known why the nonclonal βTC-tet line maintains these differentiated properties, even when actively proliferating, for a much higher number of passages than other βTC lines. Eventually, hexokinase is upregulated in βTC-tet cell after >60 passages. The increase in hexokinase activity represents a serious obstacle in the development of transformed β-cell lines for cell therapy of diabetes, because transplantation of such cells would be expected to lead to hypoglycemia. The mechanism responsible for the increase in hexokinase mRNA and resulting activity remains unknown. It is thought that upregulation of hexokinase results from the increased metabolic requirements of the dividing cells (22). Hexokinase is probably more efficient than glucokinase in providing energy from glucose metabolism to the proliferating β-cells. Our findings demonstrate that the hexokinase upregulation is reversible. After growth arrest, which presumably decreases the metabolic requirements of the cells, hexokinase activity was downregulated about threefold. These findings indicate that a limited upregulation of hexokinase activity during propagation in culture of conditionally transformed β-cells is not a concern for subsequent usage of these cells for transplantation in vivo, because the ability to induce growth arrest in these cells may allow restoration of the normal pattern of glucose phosphorylation, which is key to correct glucose sensing and accurate insulin secretion.

Long-term growth-arrested βTC-tet cells in monolayer cultures manifested a marked decrease in glucose-induced insulin release. The mechanism responsible for this deviation from the normal response remains to be elucidated. The fact that free-floating clusters of these cells do not manifest the secretory defect noted in monolayer cultures indicates that this effect may be associated with morphological changes in the long-term growth-arrested cells attached to culture dishes. Although the mechanism behind this phenomenon is of interest, these changes have little relevance to the performance of long-term growth-arrested cells in vivo. As demonstrated by the hyperglycemic clamp studies, these cells are able to sense a rise in glucose and respond by a marked elevation of insulin secretion when implanted in the peritoneal cavity of syngeneic mice. The cells perform equally well whether they are proliferating or after long-term growth arrest. These findings emphasize the potential of this approach for developing strategies for cell therapy of diabetes.

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