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Insulin-Secreting Pituitary GH3 Cells: A Potential β-Cell Surrogate for Diabetes Cell Therapy

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In a companion article, we describe the engineering and characterization of pituitary GH3 cell clones stably transfected with a furin-cleavable human insulin cDNA (InsGH3 cells). This article describes the performance of InsGH3 (clones 1 and 7) cell grafts into streptozotocin (STZ)-induced diabetic nude mice. Subcutaneous implantation of 2×10^6 InsGH3 cells resulted in the progressive reversal of hyperglycemia and diabetic symptoms, even though the progressive growth of the transplanted cells (clone 7) eventually led to glycemic levels below the normal mouse range. Proinsulin transgene expression was maintained in harvested InsGH3 grafts that, conversely, lose the expression of the prolactin (PRL) gene. Elevated concentrations of circulating mature human insulin were detected in graft recipients, demonstrating that proinsulin processing by InsGH3 cells did occur in vivo. Histologic analysis showed that transplanted InsGH3 grew in forms of encapsulated tumors composed of cells with small cytoplasms weakly stained for the presence of insulin. Conversely, intense insulin immunoreactivity was detected in graft-draining venules. Compared to pancreatic βTC3 cells, InsGH3 cells showed in vitro a higher rate of replication, an elevate resistance to apoptosis induced by serum deprivation and proinflammatory cytokines, and significantly higher antiapoptotic Bcl-2 protein levels. Moreover, InsGH3 cells were resistant to the streptozotocin toxicity that, in contrast, reduced βTC3 cell viability to 50–60% of controls. In conclusion, proinsulin gene expression and mature insulin secretion persisted in transplanted InsGH3 cells that reversed hyperglycemia in vivo. InsGH3 cells might represent a potential β-cell surrogate because they are more resistant than pancreatic β cells to different apoptotic insults and might therefore be particularly suitable for encapsulation.

Key words: Pituitary cells; Bioengineering; Insulin gene; Cell therapy; Diabetes

INTRODUCTION

Diabetes cell therapy depends on the implantation of insulin-producing cells in sufficient number and quality to survive long enough to restore the pancreatic endocrine function and to escape from autoimmune recognition. Recent improvements in the field of immunoisolation membranes, which allow transplantation in the absence of systemic immunosuppression (6), gave impetus to search for xenogeneic islets (19) or β-cell lines (2–4,6,7,15,16,20,24,28,33) for encapsulation-based diabetes cell therapy. Encapsulated cells must be supplied with oxygen and nutrients by diffusion from the closest blood vessels, surrounding tissue, and immunoisolation membrane. The distance from the nearest blood vessels determines a gradient in oxygen and nutrients supply that is often unbearable for the β cells (6), which are normally extremely well perfused (3). Indeed, β-cell secretion and viability are extremely susceptible to hypoxia and other cytotoxic stresses (12,21,29). Immediate loss of islet tissue, as a result of oxygen supply limitation and aspecific inflammation, has also been shown in naked syngeneic transplantation (8).

β-cell vulnerability, as well as β-cell-directed cytotoxicity present in autoimmune diabetes, raises the rationale of using non-β cells for diabetes cell therapy. Previous studies suggested that GH3 cells might be a logical substrate for engineering an artificial β cell, even though they express the receptor-type protein tyrosine phosphatase IA-2a (36), which is an islet autoantigen of type I diabetes (32). Indeed, a cytotoxic antibody that binds to rat pancreatic islet cells and RIN cells, but not to GH3 cells, has been described in the NOD mouse (31). Moreover, splenic lymphoid cells cytotoxic to major histocompatibility complex (MCH)-compatible Wistar-Furth rat islets and also to MCH-incompatible

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Lewis rat islets and RIN cells, but not to GH3 cells, have been described in diabetic BB rats (26). In addition, cytotoxic cell-mediated immunoreactivity selectively against a β-cell line was also observed in the multiple low-dose streptozotocin-induced rodent model of autoimmune diabetes (27).

The rationale of developing insulin-secreting non-β cells also applies to encapsulation. Indeed, shed β-cell antigens might foster an inflammatory reaction around the capsule, leading to pericapsular fibrosis and suffocation of encapsulated cells (6). Furthermore, shed antigens from encapsulated β cells might perpetuate or reactivate autoimmunity against the residual recipient's pancreatic β cells that often survive for years after the diagnosis of type I diabetes. GH3 cells are also attractive because their original secretory products (rat prolactin and growth hormone) do not exert biological effects on human tissues due to the species specificity of the human receptors (18,37), even though their secretory products might still be immunogenic. Finally, xenogeneic InsGH3 cells, being highly phylogenetically distant, should be promptly rejected in case of leakage from encapsulation devices, thereby minimizing the safety concerns related to the use of transformed cell lines into humans.

In this study we explored the performance of insulin-secreting pituitary GH3 cell (InsGH3 cells, described in a companion article, this issue) implants performed in STZ-diabetic mice. We also compared the vulnerability of InsGH3 and βTC3 cells to a variety of toxic agents. Our data indicate that InsGH3 cells might be a potential β-cell surrogate for encapsulation-based cell therapy even though additional engineering is required.

MATERIALS AND METHODS

Cells and Cell Cultures

Rat pituitary GH3 (American Type Culture Collection), mouse pancreatic βTC3 cells, and proinsulin-transfected InsGH3 cells (clones 1 and 7) were cultured as previously described in the companion article.

Cell Growth In Vitro

Proliferation was determined by cell counting with a Coulter Counter ZM (Beckman, Coulter Inc., CA, USA). InsGH3 (clones 1 and 7), wild-type GH3, and βTC3 cells were plated onto six multiwell plates and cultured under standard conditions. Cells were then harvested and counted 2, 4, 6, and 8 days after plating.

Detection of Apoptosis

Apoptosis was determined by using a DNA fragmentation assay (13). Briefly, InsGH3 cells of both clones and βTC3 cells (<50% confluent) cultured for 5 days in 25-cm^2 tissue culture flask in their standard tissue culture medium supplemented or not with 10% FCS, or in the presence of a cocktail of cytokines (IL-1 50 IU/ml + TNF-α 1000 UI/ml + IFN-γ 1000 UI/ml) were washed and resuspended in 100 μl of lysis buffer (10 mM Tris-HCl/10 mM EDTA/0.5% Triton X-100, pH 8.0) and sonicated. After centrifugation for 20 min at 4°C (14,000 × *g*), the supernatant-containing fragmented (soluble) DNA was transferred to another tube. Lysis buffer (100 μl) was added to the pellet containing insoluble DNA. Samples were then treated with Rnase for 1 h and then with proteinase-K for another hour. After adding isopropanol, samples were incubated overnight at −20°C, and the DNA concentrations were measured by the method of Duke and Sellins (13). Fragmented DNA was calculated as 100% × soluble DNA/soluble + insoluble DNA. The soluble fraction of DNA was determined by electrophoresis on 1.8% agarose gel.

MTT Viability Assay

Viability of InsGH3 (clones 1 and 7) and βTC3 cells cultured 5 days in the presence of the same mixture of cytokines described above, or exposed for 1 h to increasing concentrations of streptozotocin (2 and 10 mmol/L; Sigma, St. Louis, MO), was measured by using the MTT (tetrazolium) colorimetric assay (22).

Western Blotting

Bcl-2 protein levels in βTC3, GH3 wild-type, and InsGH3 clones were measured by Western blotting and anti-Bcl-2 antibody (rabbit anti goat 1:100; Santa Cruz, Biotechnology, USA).

Insulin Assays

Circulating insulin levels in mice bearing InsGH3 grafts were measured by using a IRMA (SANOFI, Pasteur Institute, Paris, France), which recognizes only human mature insulin and split 65-66, and does not cross-react with the other (pro)insulin forms. Circulating human proinsulin levels were measured by ELISA (Dako Diagnostic, Ely, UK), which does not cross-react with insulin and other split proinsulin forms. Human C-peptide levels were measured by RIA (Diagnostic Product Corporation, LA, USA).

Transplantation Experiments

Male nude mice (Nu/Nu, 6–8 weeks old) were rendered diabetic by injecting a single dose of STZ (180 mg/kg body weight, IP). Diabetes was confirmed by the presence of hyperglycemia (>350 mg/dl), weight loss, and polyuria. Random (nonfasted) glycemic levels were measured (between 0900 and 1000 h) on whole blood obtained from the snipped mouse tail with a portable glucose meter (One Touch II, Johnson & Johnson, Milpitas, CA). The animals were kept under conventional

conditions in climatized rooms with free access to water and food. Three groups of diabetic mice were implanted subcutaneously between the shoulders with aliquots of 2×10^6 wild-type GH3 cells and InsGH3 cells of clones 1 and 7. After implantation, mice were followed with repeated measurements of blood glucose levels and body weight. At the time of sacrifice grafts were retrieved and RNA was extracted for the analysis of proinsulin transgene expression by Northern blotting analysis. Naive pancreases were also harvested and immunostained for insulin as previously described (9) in order to detect residual β cells. Finally, blood samples were collected for the measurement of circulating mature human insulin levels. To determine proinsulin processing by transplanted InsGH3 cells, circulating human insulin, proinsulin, and C-peptide levels were measured in the same samples drawn from normal nude mice that were transplanted 2 weeks earlier with 2×10^6 InsGH3/clone 7.

Northern Blot Analysis

At the time of sacrifice the grafts were harvested and divided into two parts. One half of each graft was used to measure the expression of the human proinsulin and rat PRL genes in either wild-type GH3 or InsGH3 cell implants. Immediately after harvesting, total graft RNA was prepared with the ULTRASPEC RNA isolation system (Biotecx Laboratories Inc., Houston, TX). Northern blot analysis was performed as described in the companion article (this issue).

Graft Morphology and Immunohistochemistry

The second half of the harvested grafts (wild-type GH3 and InsGH3 implants) underwent histologic analysis. Morphology and immunohistochemistry were performed on formalin-fixed, paraffin-embedded specimens. After deparaffinization and rehydration, 5-μm sections were stained with hematoxylin-eosin and immunostained for insulin (guinea pig anti-porcine 1:1500; Eurodiagnostica, Malmo, Sweden). Slides were blocked for 30 min in 5% normal goat serum and 0.1% Triton X-100 in phosphate-buffered saline (PBS) and exposed overnight to the primary antibodies. Sections were then washed and incubated with the secondary antibodies for 1 h at room temperature. The specific staining was detected using the ABC immunoperoxidase system (Vectastatin, Vector, Burlingame, CA) as chromogen.

In Situ Hybridization

Graft specimens were cleared of paraffin with xylene and rehydrated by sequential washings with graded ethanol solutions (70–95%). After three washes in PBS buffer, sections were deproteinated by incubation (20 min, room temperature) with proteinase K (10 μg/ml) in 10 mM Tris buffer, pH 7.5, containing 2 mM $CaCl_2$. Deproteinated tissues were rinsed in 2× SSC for 10 min and then prehybridized for 3 h at room temperature with prehybridization buffer (50% formamide, 4× SSC, 2× Denhardt's, 0.1% SDS, 100 μg/ml salmon sperm DNA). Hybridization was performed with 8 ng/μl of proinsulin digoxigenin-labeled RNA probe (sense or antisense) in hybridization solution (i.e., prehybridization buffer with 10% dextran sulfate). Slides were covered with parafilm and incubated at 42°C for 16 h in a humidified chamber. After hybridization, sections were washed twice in 4× SSC and then incubated with Rnase A (50 μg/ml) for 30 min at 3°C. Posthybridization treatment was performed by incubation of sections in 2× SSC, 0.1% SDS (twice for 15 min/each), 2× SSC (15 min), 1× SSC (15 min). To detect digoxigenin-labeled probe, tissue was rinsed briefly in TBS buffer, blocked for 7 h in 100 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% BSA, and 0.3% Triton X-100 and then incubated with an anti-digoxigenin antibody, conjugated to alkaline phosphatase (Boehringer Mannheim), diluted 1:400. The specific signals were visualized by incubation in developing buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, and 50 mM $MgCl_2$), containing 0.34 mg/ml of nitrotetrazolium blue, 0.17 mg/ml of 5-bromo-4-chloro-3-indolylphosphate, and 0.024% levamisole. Developing reaction was stopped with 10 mM Tris-HCl, pH 7.5; sections were dehydrated with graded ethanol solutions (70–95%) and mounted.

Statistical Analysis

Experiments were repeated at least three times. Data were expressed as means ± SE. Statistical analysis was performed using the unpaired Student's *t*-test for pairwise comparisons and one- or two-way analysis of variance (ANOVA) for multiple comparisons. Statistical significance was considered at $p < 0.05$.

RESULTS

Cell Growth

As shown in Figure 1, InsGH3 cells of clones 1 and 7 proliferated in vitro significantly faster than wild-type GH3 and βTC3 cells ($p < 0.01$ at day 7 and $p < 0.001$ at day 9 vs. GH3 and βTC3). βTC3 cells grew with the longest doubling time (approximately 68 vs. 24 h of InsGH3 clones) and reached a maximal density of 4.4×10^4 cell/cm^2, which was about 10-fold lower than the maximal density of InsGH3 clone 7 (1.4×10^5 cells/cm^2).

Apoptosis

Both InsGH3 clones were extremely resistant to apoptosis induced by serum deprivation and proinflammatory cytokines. In InsGH3 cells no evidence of DNA fragmentation was detected after 5 days of culture in the absence of serum, while massive apoptosis was detected

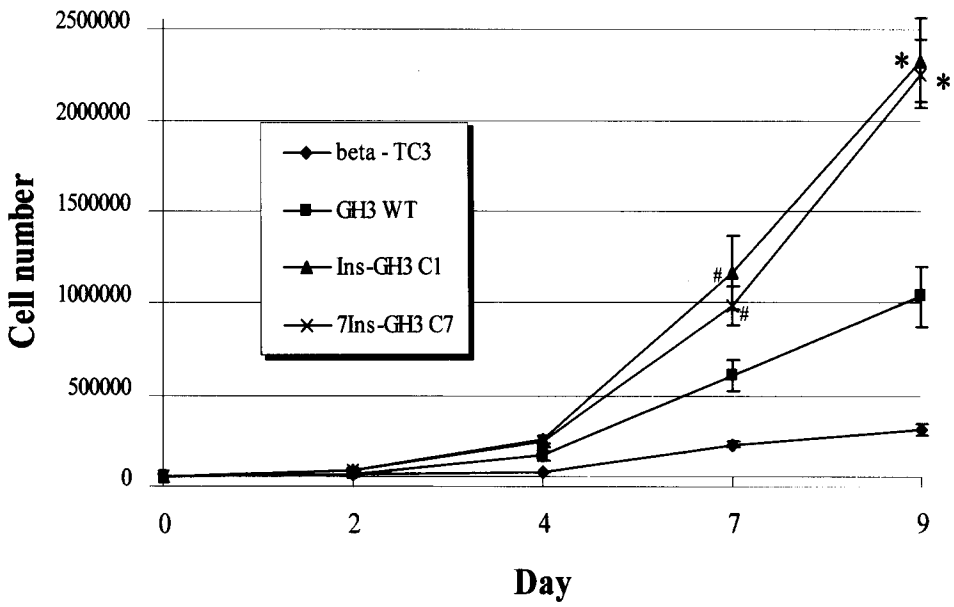

Figure 1. Cell replication in vitro. Proliferation of InsGH3 cells (clones 1 and 7), with a doubling time of about 24 h, was significantly higher than that of wild-type GH3 and βTC3 cells (*$p < 0.01$ at day 7, and #$p < 0.001$ at day 9 vs both GH3 and βTC3). βTC3 showed the lowest replication rate with an approximate doubling time of 68 h. Data are mean ± SE of six independent experiments, performed in duplicate.

in βTC3 cells (Fig. 2, top). In InsGH3 cells of clone 1, serum deprivation increased DNA laddering from a baseline of 0.02% to 0.4%, and in InsGH3 of clone 7 from 0.01% to 0.5%; in contrast, in βTC3 cells baseline laddering was 0.4% that increased to 24% in the absence of serum (Fig. 2, bottom). InsGH3 cells (clones 1 and 7) were also more resistant than βTC3 to apoptosis induced by proinflammatory cytokines. The percentage of fragmented DNA after exposure to cytokines was 9.8% in InsGH3 clone 1, 5.5% in InsGH3 clone 7, and 30% in βTC3 cells (Fig. 3).

MTT Viability Assay

This test measured the reduction of tetrazolium salt into insoluble colored formazan crystals. Exposure of InsGH3 (clones 1 and 7) to 2 and 10 mmo/L STZ did not induced any significant decrease in formazan production, thereby indicating the absence of cytotoxicity. In contrast, 2 mmol/L STZ induced a 55% decrease in formazan production by βTC3 cells, indicating a massive loss in βTC3 viability that did not decreased any further at 10 mmol/L STZ (Fig. 4).

A five-day exposure of InsGH3 (clones 1 and 7) and βTC3 cells to the same mixture of proinflammatory cytokines utilized for the induction of apoptosis resulted in a decreased viability of βTC3 and InsGH3 cells (clones 1 and 7) to 51 ± 11%, 62 ± 11%, and 57 ± 10% of controls, respectively (NS).

Bcl-2 Protein Levels

Western blotting experiments showed the absence of measurable Bcl-2 protein in βTC3 cells. In contrast, Bcl-2 protein was abundant in wild-type GH3 cells and in InsGH3 of both clones even though it was significantly higher in clone 1 than in clone 7 and in wild-type GH3 cells (Fig. 5).

Transplantation Experiments

Results of subcutaneous implantation of wild-type GH3 and InsGH3 cells (clones 1 and 7) into diabetic nude mice are shown in Figure 6. Similar with all clones, implanted cells grew tumors that remained confined to the site of implantation and that, 5 weeks after implantation, had an average diameter of about 6–7 mm. Mice transplanted with wild-type GH3 cells remained hyperglycemic and were sacrificed 5 weeks after implantation with blood glucose levels of 435 ± 23 mg/dl, undetectable circulating human insulin, and a gain in body weight of 1.9 ± 0.4 g. In contrast, mice implanted with InsGH3 cells of clone 7 showed rapid reversal of the diabetic symptoms and a progressive decrease of blood glucose levels that, 5 weeks after transplantation, were below normal range (72 ± 3 mg/dl). Because of the risk of severe hypoglycemia consequent to additional tumor growth, recipients of this group were sacrificed 5 weeks after implantation; at that time mice were healthy, with a mean level of circulating human mature insulin

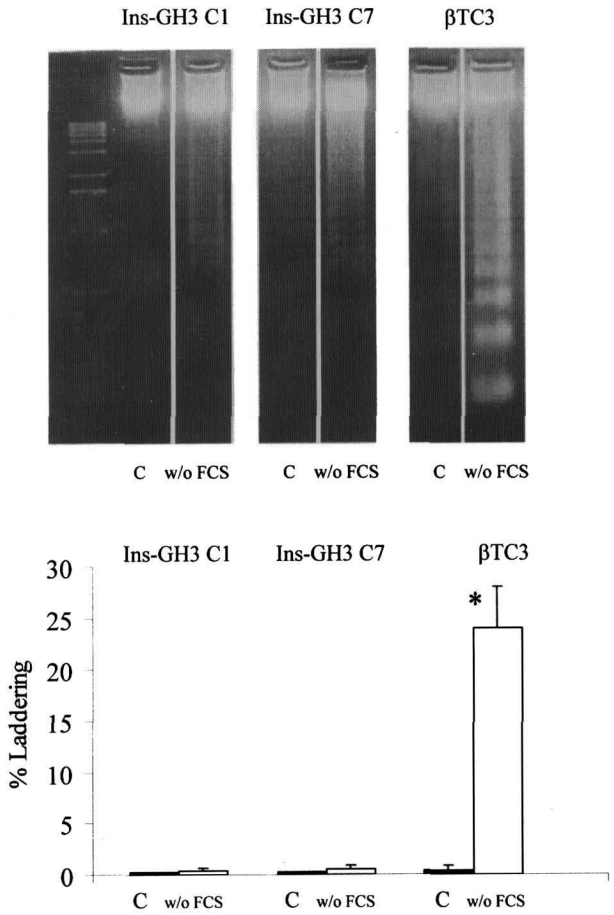

Figure 2. Apoptosis induced by serum deprivation. Upper panels show two representative gels of DNA extracted from InsGH3 cells (clones 1 and 7) (left) and βTC3 cells (right) cultured for 5 days in the absence of serum. DNA fragmentation, typical of apoptosis (laddering), was evident in serum-deprived βTC3 cells while it was absent in InsGH3 cells of both clones. Lower panel shows mean values of quantitative measurements of fragmented DNA in the different cell types under control and serum-deprived conditions. Data are mean ± SE of three individual experiments. $*p < 0.001$.

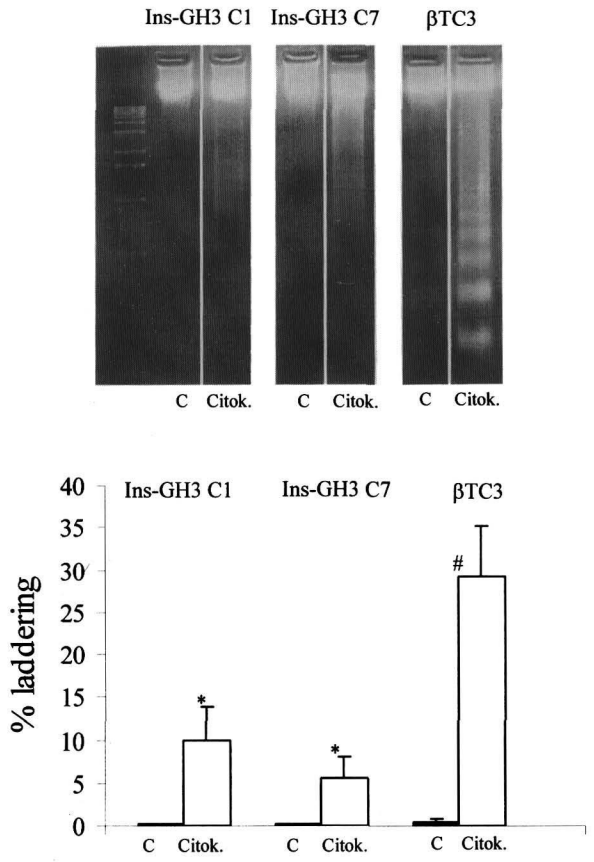

Figure 3. Apoptosis induced by proinflammatory cytokines. Upper panel shows two representative gels of DNA extracted from InsGH3 cells (clones 1 and 7) (left) and βTC3 cell (right) cultured for 5 days in the presence of cytokines (IL-1 50 IU/ml + TNF-α 1000 UI/ml + IFN-γ 1000 UI/ml). After exposure to the cytokines, DNA laddering appeared in InsGH3 cell clones but was extremely lower than in βTC3 cells. Lower panel depicts quantification of DNA fragmentation, increasing modestly albeit significantly in InsGH3 clones ($*p < 0.05$ vs. control). In contrast, cytokine-induced apoptosis was massive in βTC3 cells, as shown by 30% fragmented DNA. $\#p < 0.001$ vs. control, and $*p < 0.01$ vs. InsGH3 of clones 1 and 7. Data are mean ± SE of three individual experiments.

of 468 ± 156 pmol/L, and a gain in body weight of 6.1 ± 0.5 g. Mice implanted with InsGH3 cells of clone 1 showed a slower decline in blood glucose levels; 10 weeks after transplantation mice were healthy, the average tumor diameter was 8–9 mm, the mean blood glucose level was 148 ± 49 mg/dl, circulating human mature insulin was 414 ± 114 pmol/L, and the gain in body weight was 7.0 ± 0.6 g. Circulating human C-peptide, mature insulin, and proinsulin levels of normal nude mice bearing InsGH3 grafts were 0.28 ± 0.09 ng/ml, 32 ± 9 pmol/L, and 35 ± 11 pmol/L, respectively ($n = 3$). Northern blot analysis showed the expression of the proinsulin gene in InsGH3 grafts of either clone 1 and 7, while it was negative in wild-type GH3 grafts (Fig. 6, top). Conversely, PRL gene expression was positive in wild-type GH3 grafts, but negative in InsGH3 grafts of both clones. Note that in InsGH3 cells of clone 7 the PRL gene expression was also low before the implantation (Fig. 6, top).

Graft Morphology and Immunohistochemistry

Histology confirmed the growth of InsGH3 grafts in the form of encapsulated tumors with no evidence of capsular erosion. Hematoxylin-eosin stain of InsGH3 graft sections demonstrated the presence of healthy,

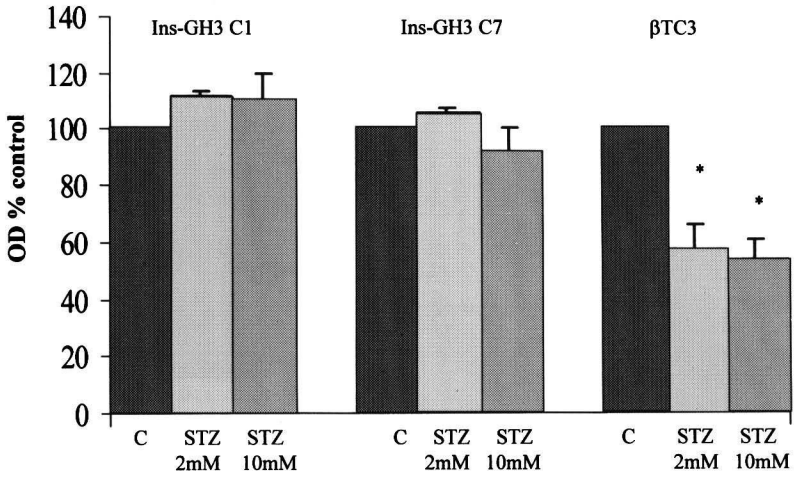

Figure 4. Streptozotocin (STZ)-induced cell damage. Viability of InsGH3 cells (clones 1 and 7) and of βTC3 cells exposed for 1 h to increasing concentrations of STZ assessed by using the MTT (tetrazolium) method. InsGH3 cell viability remained stable after the exposure to 10 mmol/L STZ; in contrast, viability of βTC3 decreased to 55% of control values already at the lower STZ concentration (2 mmol/L) and did not increased further at 10 mmol/L. Data are mean ± SE of three individual experiments. $*p < 0.001$ vs control.

well-vascularized tissue composed of cells with small cytoplasms almost completely negative for insulin that, conversely, was extremely intense in the venules draining the grafts (Fig. 7). Similar findings were observed in either clone 1 and 7 grafts (not shown). Immunohistochemistry for insulin did not show any residual β cells in the pancreases of mice implanted with InsGH3 cells (not shown).

In Situ Hybridization

Proinsulin digoxigenin-labeled RNA probes demonstrated the expression in situ of the transfected proinsulin gene in InsGH3 grafts (Fig. 8).

DISCUSSION

The aim of cell therapy is to replace or enhance the biological function of damaged tissue or organs. Encapsulation of chromaffin cells for chronic pain (23) and genetically engineered immortalized cells for neurodegenerative diseases have been demonstrated experimentally and are being tested clinically (1,17). Cell therapy of type I diabetes relies on the replacement of the function of pancreatic β cells, which are selectively targeted and destroyed by the autoimmune process responsible for the disease (38). Endocrine non-β cells are an appealing alternative for insulin gene delivery in type I diabetes because they might escape autoimmune recognition and the recurrence of graft destruction.

InsGH3 (clones 1 and 7) grew in vitro remarkably faster than βTC3 and wild-type GH3 cells (Fig. 1). Insulin might stimulate cell growth and division either by acting through its own receptor or the IGF-1 receptor (5), which is present in GH3 cells (34). Therefore, insulin might activate downstream signaling pathways in InsGH3, leading to increased cell replication. The higher replication rate of InsGH3, compared with βTC3 cells, is important because it would allow to obtain, more rapidly and at lower costs, a large mass of tissue for transplantation in large mammals.

Subcutaneous implantation of InsGH3 cells, previously characterized in vitro in the companion article (this issue), was followed by the progressive decline of blood glucose levels, reversal of diabetic symptoms, and gain in body weight in STZ-diabetic mice. Therefore, transplanted InsGH3 cells can process the furin-cleavable proinsulin gene and secrete mature human insulin in vivo. Moreover, circulating proinsulin and insulin levels were quantitatively similar in mice bearing InsGH3 cell grafts, in agreement with the results observed in vitro (see companion article). Postimplantation blood glucose reduction was faster in the recipients of InsGH3 cells of clone 7 than of clone 1, consistent with the higher insulin production of the former clone detected in vitro (see companion article). The progressive growth of InsGH3 grafts (clone 7) over time induced hypoglycemia, which is an obvious drawback toward the use of these cells for therapeutic purposes. However, this problem might be overcome by encapsulation that, by physically restraining the cells, would limit their expansion. Insulin content of transplanted InsGH3 cells was remarkably low, as shown by the insulin immunoreac-

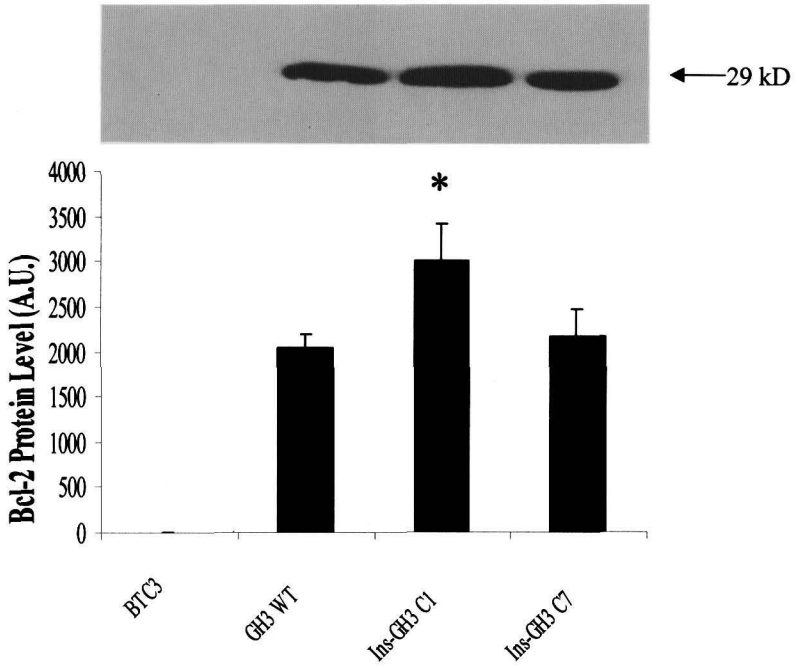

Figure 5. Upper panel shows a representative Western blot for the detection of Bcl-2 protein levels. Lanes from left to right are βTC3, wild-type GH3, InsGH3 clone 1, and InsGH3 clone 7. Lower panel depicts the densitometric analysis of different blots. Note that Bcl-2 was absent in βTC3 while it was relatively abundant in the other cell lines. Bcl-2 protein was significantly higher in InsGH3/clone 1 than in wild-type GH3 and InsGH3/clone 7. $*p < 0.05$. Data are mean ± SE of three different experiments.

tivity of their cytoplasms. Such a reduced insulin storage, together with the high plasmatic insulin levels detected in the recipients, indicates that in transplanted InsGH3 cells the constitutive release of insulin prevails on the regulated secretion, perhaps more than what is observed in vitro (see companion article). High constitutive release of insulin might put the recipient of InsGH3 cells at risk of hypoglycemia in the postabsorptive state, even if the control of graft growth is achieved by encapsulation. Therefore, strategies aimed towards reducing constitutive and possibly increasing regulated insulin secretion need to be implemented before InsGH3 cells could be considered suitable candidates for human diabetes cell therapy.

The capability of transplanted InsGH3 to produce and release insulin is remarkable, as previous studies have shown that the PRL promoter activity is selectively silenced in transplanted GH3 cells (10,11). Indeed, PRL gene expression was undetectable in transplanted InsGH3, while PRL gene expression was maintained in transplanted wild-type GH3 cells (Fig. 6). Lack of expression of the PRL gene by transplanted InsGH3 cells is an obvious advantage for our purpose. We cannot make any conclusion regarding growth hormone (GH) graft expression nor GH serum concentrations, which were not measured in InsGH3 graft-bearing mice.

Serum deprivation induced substantial apoptosis in βTC3 cells, while it was almost ineffective on InsGH3 cells (Fig. 2). Cytokines induced a similar apoptotic cell death in βTC3 cells, but had a limited impact on InsGH3 cells (Fig. 3). Alterations in Bcl-2 mRNA and protein levels have been related to apoptosis induced by cytokines either in MIN6 and RINm5F β-cell lines (21). The β-cell line (βTC1) is extremely sensitive to cytokine-induced apoptosis that, conversely, is barely detectable in the α-cell line (αTC1), which is characterized by significantly higher Bcl-2 mRNA and protein levels (29). Moreover, it has been recently demonstrated that lentivirus-mediated overexpression of Bcl-2 in βTC-tet cells significantly improves their resistance to hypoxia and cytokine-induced apoptosis (14). Therefore, our observation that Bcl-2 protein is relatively abundant in wild-type GH3 and InsGH3 cell clones could explain the resistance to apoptosis observed in InsGH3 cells, compared with βTC3 cells. It should be noted that long-term exposure to proinflammatory cytokines reduced InsGH3 and βTC3 viability, measured by the MTT assay, to a similar extent, therefore suggesting that InsGH3 cells

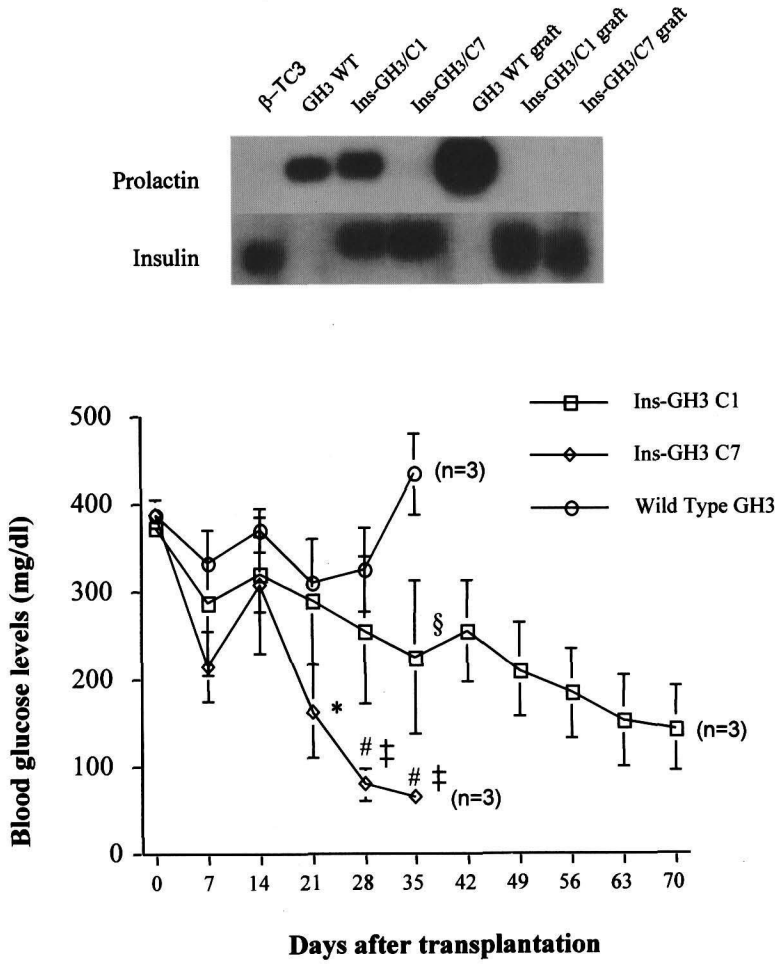

Figure 6. Upper panel shows a representative Northern blot hybridized with prolactin and proinsulin cDNA probes. Lanes from left to right represent: βTC3 cells, wild-type (WT) GH3, InsGH3/clone 1, InsGH3/clone 7 (before transplantation for all cell types), WT GH3 graft, InsGH3/clone 1 graft, and InsGH3/clone 7 graft. Note that the expression of the proinsulin gene was present in InsGH3 grafts of both clones, which, in contrast, were negative for PRL gene expression. Conversely, PRL gene was highly expressed in WT GH3 grafts. Note also that PRL gene expression was already low in InsGH3 cells of clone 7 before the implantation. Lower panel shows the glycemic levels of STZ-diabetic nude mice transplanted with either wild-type (WT) GH3 cells or InsGH3 cells of clones 1 and 7. As expected, WT GH3 grafts did not determine any reduction in blood glucose levels. In contrast, mice transplanted with InsGH3 cells of clone 7 showed a progressive decline in blood glucose that, 4 weeks after implantation, was below the normal mouse range (*$p < 0.05$ vs. WT GH3; #$p < 0.001$ vs. WT GH3; ‡$p < 0.01$ vs. InsGH3 of clone 1). Implantation of InsGH3 cells of clone 1 induced a slower decrease in glycemic levels that 5 weeks later were still in the diabetic range even though lower than before transplantation, and of those measured in mice bearing WT GH3 grafts (§$p < 0.02$). Recipients of InsGH3/clone 1 cells were allowed to survive longer after transplantation, showing a progressive decrease in glycemic levels that, 10 weeks after transplantation, reached values approaching the normal range. Data are mean ± SE.

are not protected by cytokine-mediated necrotic cell death. Noteworthy, InsGH3 cells were also resistant to streptozotocin-induced cell damage. Streptozotocin acts either via alkylation of DNA or through the release of bioactive nitric oxide (NO) (25). Whatever the cause of the insensitivity of InsGH3 cells to STZ, such a feature appears as an additional advantage for transplantation purposes. The different response of InsGH3 and βTC3 cells to STZ is not explained by a differential expression of the glucose transporter GLUT-2 that, in other β cell lines, has been pointed as the mediator of STZ toxicity (35). Indeed, we did not detect GLUT-2 protein in either

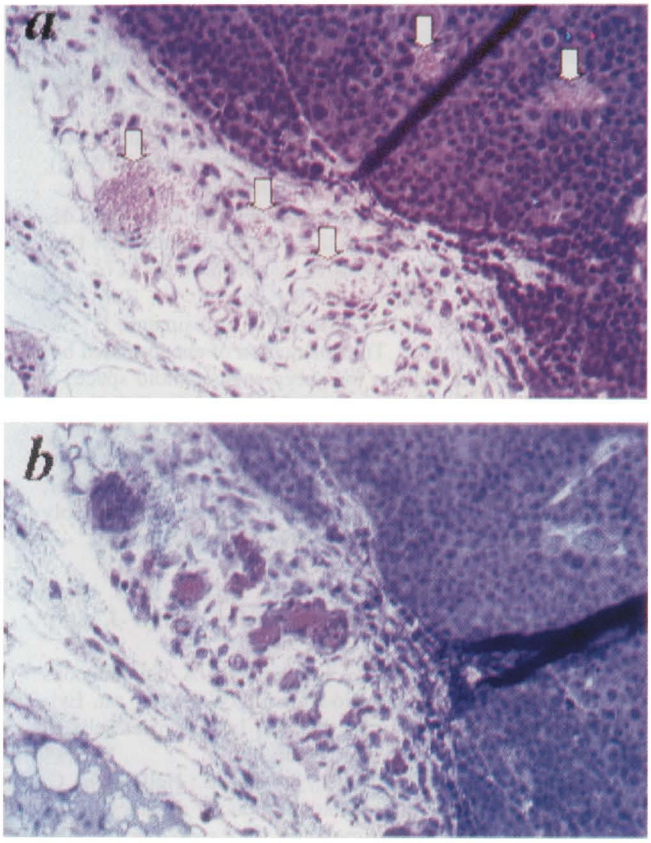

Figure 7. Histology of two adjacent sections of a InsGH3 (clone 7) graft. On the top is a hematoxylin-eosin staining showing healthy cells with small cytoplasms, well vascularized (arrows), and surrounded by a continuous capsule containing several graft-draining venules (arrows). Lower panel depicts an adjacent section stained for insulin showing the almost complete negativity of the cellular component of the graft. In contrast, insulin immunoreactivity was extremely intense in the venules draining the grafts. Original magnification: ×20.

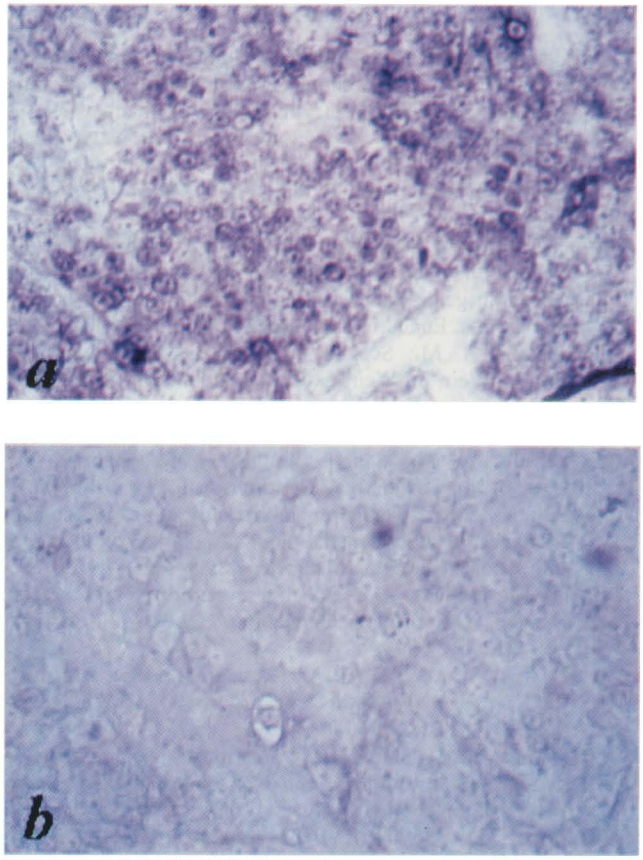

Figure 8. In situ hybridization of InsGH3 graft (clone 7) with antisense (top) and sense (bottom) proinsulin digoxigenin-labeled RNA probes showing the intracellular expression of the proinsulin gene (antisense) within transplanted InsGH3 cells. Original magnification: ×100.

wild-type GH3, InsGH3, or βTC3 cells (not shown). The absence of GLUT-2 expression by βTC3 cells, previously shown by others (30), suggests that STZ toxicity involves a GLUT-2-independent pathway in βTC3 cells.

In conclusion, because of their elevated resistance to a variety of insults, InsGH3 cells appear particularly suited for encapsulation. Moreover, InsGH3 cells will probably also allow a greater packing density within the capsules as suggested by the 10-fold higher maximal density reached by these cells in vitro compared with βTC3 cells. The achievement of physiologic regulation of insulin release by InsGH3 cells, and the reduction of their constitutive pathway of protein secretion, will greatly increase the potential of these cells for transplantation.

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