Rapid Publication

Insulin-Secreting Cells Derived From Embryonic Stem Cells Normalize Glycemia in Streptozotocin-Induced Diabetic Mice

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Embryonic stem (ES) cells display the ability to differentiate in vitro into a variety of cell lineages. Using a cell-trapping system, we have obtained an insulin-secreting cell clone from undifferentiated ES cells. The construction used allows the expression of a neomycin selection system under the control of the regulatory regions of the human insulin gene. The chimeric gene also contained a hygromycin resistance gene (pGK-hygro) to select transfected cells. A resulting clone (IB/3x-99) containing 16.5 ng/μg protein of total insulin displays regulated hormone secretion in vitro in the presence of various secretagogues. Clusters obtained from this clone were implanted (1×10^6 cells) in the spleen of streptozotocin-induced diabetic animals. Transplanted animals correct hyperglycemia within 1 week and restore body weight in 4 weeks. Whereas an intraperitoneal glucose tolerance test showed a slower recovery in transplanted versus control mice, blood glucose normalization after a challenge meal was similar. This approach opens new possibilities for tissue transplantation in the treatment of type 1 and type 2 diabetes and offers an alternative to gene therapy. *Diabetes* 49:XXX–XXX, 2000

The Diabetes Control and Complications Trial Research Group has shown that intensive insulin therapy can delay the onset and diminish the progression of microvascular complications in type 1 diabetes. However, this kind of treatment needs motivated patients and does not liberate them from insulin dependence. By restoring β-cell function with new therapeutic strategies, including transplantation of exogenous β-cells (islet allotransplantation and xenotransplantation, implanting β-cell surrogates, etc.), patients can be free from insulin therapy. On the other hand, the use of gene therapy for the treatment of diabetes still needs crucial improvements (1). By using specific promoters, the insulin gene has been successfully expressed in a broad variety of tissues, including skeletal muscle, exocrine pancreas, and salivary glands (2,3). The use of mutated proinsulin at the level of the cleavage sites allows correct processing of proinsulin to mature insulin by furins (4). However, the main obstacle of ectopic insulin expression is to mimic the modulation of the β-cell secretory pattern in non–β-cells.

The difficulty encountered in reconstructing the β-cell function ectopically makes exogenous β-cells an alternative choice for restoring β-cell mass or β-cell function in either type 1 or type 2 diabetes. Allotransplantation or xenotransplantation still imposes important problems: scarcity of material, technical difficulty, high costs, risk of infection by endogenous animal retroviruses, and immunological rejection, which lead to the inherent problems of macroencapsulation or microencapsulation strategies (5). The use of tumoral β-cell lines could be a potential solution for the scarcity problem. Clones displaying the most appropriate patterns of insulin secretion can be selected and amplified at low cost. However, the bulk of these cell lines can progressively lose this capacity with a high number of passages (6). Furthermore, tumoral cell lines display a high proliferative rate, which poses a serious obstacle for transplantation. In this respect, the recently developed β-cell line controls proliferation by using (7) the tetracycline conditioned system (8). In any case, bioengineered cell lines, once they are ready for transplantation, may be subject to immune rejection. Another attractive approach concerns β-cell regeneration from islet cell precursors. However, the identification of β-cell neogenic factors remains elusive (9). Thus, an alternative is the use of embryonic stem (ES) cells, which have the ability to differentiate into a variety of cell lineages in vitro. Using an approach similar to that described previously (10,11), we have produced an insulin-secreting cell derived from mouse ES cells that normalizes blood glucose when transplanted into streptozotocin-induced diabetic mice.

RESEARCH DESIGN AND METHODS

Generation of the construction used for transfection. A DNA molecule containing the human insulin/βgeo gene and a phosphoglycerate kinase-hygromycin resistant gene (pGK-hygro) in a pBSII-SK (Stratagene, La Jolla, CA) common vector was constructed. Human insulin gene was double digested with BssSI (New England Biolabs, IZASA SA, Barcelona, Spain) and SexA1 (Roche Diagnostics, Barcelona, Spain) at the level of exon 2, and the βgeo fragment was inserted (12). This new construction was then digested at the 3′ end of the insulin gene with *Xho*I

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Received for publication 21 October 1999 and accepted 3 November 1999. Posted on the World Wide Web at www.diabetes.org/diabetes on <<date to come>>.

BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ES, embryonic stem; FBS, fetal bovine serum; IPGTT, intraperitoneal glucose tolerance test; LIF, leukemia inhibitory factor; RIA, radioimmunoassay; STZ, streptozotocin.

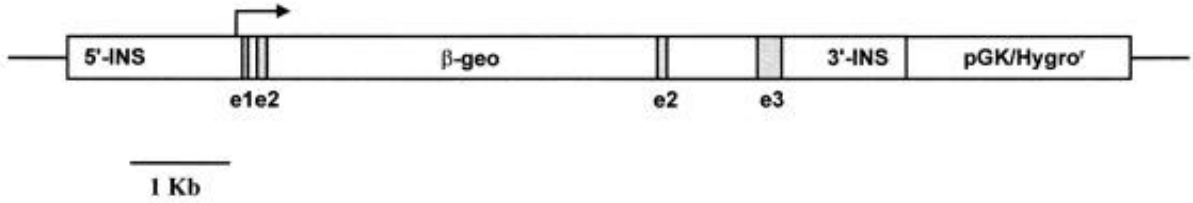

FIG. 1. Structure of the insulin–β-geo/pGK-hygro^r construction.

and blunted to insert the pGK-hygro (*Bgl*II-blunt). In both cases, right orientation was verified after ligation. Construction is depicted in Fig. 1. Plasmid was linearized with *Xba*I digestion and transfected in R1 ES cells by electroporation. Transfected clones were selected by growth in the presence of 200 µg/ml hygromycin (Calbiochem, La Jolla, CA). Transfected ES cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (GIBCO/BRL, Grand Island, NY) containing 10% fetal bovine serum (FBS) (GIBCO/BRL), 1% nonessential amino acids (GIBCO/BRL), 0.1 mmol/l 2-mercaptoethanol (GIBCO/BRL), 1 mmol/l sodium pyruvate, 100 IU/ml penicillin, and 0.1 mg/ml streptomycin. The undifferentiated state was maintained by 1,000 U/ml recombinant leukemia inhibitory factor (LIF) (GIBCO/BRL) as previously described (13).

In vitro differentiation. To induce differentiation to an insulin-secreting cell line, 2×10^6 hygromycin-resistant ES cells were plated onto a 100-mm bacterial Petri dish and cultured in DMEM lacking supplemental LIF. After 8–10 days in suspension culture, the resulting embryoid bodies were plated onto plastic 100-mm cell culture dishes and allowed to attach for 5–8 days. For ES Ins/βgeo selection, the differentiated cultures were grown in the same medium in the presence of 200 µg/ml G418 (GIBCO/BRL). For final differentiation and maturation, the resulting clones were trypsinized and plated in a 100-mm bacterial Petri dish and grown for 14 days in DMEM supplemented with 200 µg/ml G418 and 10 mmol/l nicotinamide (Sigma, Madrid). Finally, the resulting clusters were cultured for 1–5 days in RPMI 1640 supplemented with 10% FBS, 10 mmol/l nicotinamide, 200 µg/ml G418, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 5.6 mmol/l glucose.

Insulin secretion assays. For static incubations, cells were plated in 12-well dishes (Corning Glass Works, Corning, NY) at a density of ~250,000 cells per well and allowed to grow overnight in the same RPMI 1640 indicated above. Cells were washed twice for 10 min each in fresh modified Krebs buffer with 0.1% bovine serum albumin (BSA) and 3 mmol/l glucose. The Krebs buffer was always kept at 37°C and was constantly gassed with a mixture of O_2 (95%) and CO_2 (5%) for a final pH of 7.4. Cells were then transferred to glass vials in free-floating clusters at ~5×10^4 cells per vial and incubated at 37°C in 1 ml of the same fresh modified Krebs buffer with 0.5% BSA and with different secretagogues for 30 min. At the end of the incubation period, the buffer was removed from the vials. Each condition was assayed in triplicate. Perifusion studies were performed as previously described (14). Briefly, 1×10^5 ES-derived insulin-secreting cells (as free-floating clusters) were packed into a 1-cm diameter column and sandwiched between two layers of swollen Shepadex G-200 microcarrier beads (Sigma, St. Louis, MO). The column was perifused at a flow rate of 1 ml/min at 37°C with fresh modified Krebs buffer with 1% BSA plus the different glucose concentrations (3 and 22 mmol/l). The ES-derived insulin-secreting cells were first perifused in 3 mmol/l glucose for 30 min to reach a state of stable insulin release. The solutions were prewarmed at 37°C and continuously gassed. Insulin was assayed by radioimmunoassay (RIA) using the Coat-a-Count kit (DPC, Los Angeles, CA) that detects both rat and human insulin. All values were determined against a standard curve prepared with rat insulin. For measurement of total insulin-cell content, cell pellets were sonicated in 1 mmol/l acetic acid containing 0.1% BSA and cellular extract was also determined by RIA. Secretion was normalized for cell number by measuring total protein in each experiment by the method of Bradford (15).

Animals and spleen implantation of ES-derived insulin-secreting cells. Male Swiss albino (OF1) mice (B & K Universal, Barcelona, Spain), aged 8–12 weeks, were used as recipients of the implantation. Diabetes was induced with a single intraperitoneal injection of streptozotocin (STZ) (Sigma, Madrid) 200 mg/kg of body weight, freshly dissolved in citrate buffer (pH = 4.5). Before implantation, diabetes was confirmed by the presence of weight loss, polyuria, and blood glucose levels >500 mg/dl. Blood glucose was obtained from the snipped tail and measured between 9 and 11 A.M. under nonfasting conditions with a portable glucose meter (Química Farmaceútica Bayer, Barcelona, Spain). Two weeks after the injection of STZ, 15 diabetic mice were implanted with the ES-derived insulin-secreting cells and 4 mice were sham operated and kept as diabetic controls. For cell implantation, ES-derived insulin-secreting cells were washed and resuspended in RPMI 1640 supplemented with 10% FBS, 10 mmol/l nicotinamide, 100 IU/ml penicillin, 0.1 mg/ml streptomycin, and 5.6 mmol/l glucose at 5×10^6 cells per ml. With the mice under anesthesia (ketamine hydrochloride, 95 mg/kg i.p.), the spleen was exposed through a lumbar incision, and one million cells were injected into the spleen. Mice were monitored every 2 days for blood glucose levels (as indicated above) and weight. When blood glucose was restored to the physiological range, mice were monitored weekly for blood glucose and weight. Intraperitoneal glucose tolerance test (IPGTT), plasma insulin determinations, and meal challenge tests were performed in ES-implanted, sham-operated (diabetic) animals and control (nondiabetic) mice 5 weeks after euglycemia was obtained in the cell-implanted group. For all tests, mice were fasted overnight for 14 h. For IPGTT, mice were given an intraperitoneal glucose injection (2 g/kg of body weight). For meal challenge test, after 14 h of fasting, mice had free access to standard laboratory chow for 3 h and then were fasted again for another 6 h. Whole venous blood was obtained from the tail vein at 0, 30, 60, 90, 120, 180, and 210 min after injection for IPGTT, and at 0, 3, 6, and 9 h for meal challenge test. Blood glucose was measured as indicated above. Plasma insulin levels were measured by RIA for rat insulin (DPC).

RESULTS

Cell selection strategy. A cell-trapping system has been described previously to select cardiomyocytes (10) or neural precursors (11) derived from ES cells. On the basis of this approach, we have created an insulin-secreting cell clone from undifferentiated ES cells. The construction used allows the expression of a neomycin selection system under the control of the regulatory regions of human insulin gene. In addition, this chimeric gene was fused to a hygromycin resistance gene (pGK-hygro) to select transfected cells. The β-gal expression allows the in vivo detection of cells that have incorporated the construction. ES cells were cultured according to standard protocols. However, in the last stages of this incubation period, cells were cultured in bacterial Petri dishes, allowing cells to form aggregates to facilitate subsequent transplantation in the spleen of diabetic animals. The lowering of glucose concentration to 5 mmol/l during this last incubation stage has been instrumental in developing high productive insulin cell clones, as described previously for INS-1 cells (6).

ES-derived insulin-secreting cells have in vitro regulated insulin release. ES-derived insulin-secreting cells were cultured in different conditions, and their insulin content and secretory responses to secretagogues were assessed as a function of their stage of differentiation. After culture of the cells in the presence of 10 mmol/l nicotinamide and high glucose for 14 days, very low levels of insulin content were found (Table 1). In addition, no secretagogue-induced insulin release was observed in these cells (Table 1). However, when the cells were grown in the presence of low glucose (5 mmol/l) for final differentiation, a progressive increase of insulin content was observed after the first day of incubation (Table 1). However, at this stage, the secretagogue-induced insulin release was minimal. The effect of low glucose on insulin content plateaued after 5 days of culture. Under these conditions, we obtained an ES-derived insulin-secreting cell clone, designated as IB/3x-99, for which total insulin content was 16.5 ± 2.7 ng/µg protein, corresponding to ~90% of the insulin content of normal mouse islets (16). These cells showed a good secretatogue-induced insulin response to

TABLE 1
Insulin release from IB/3x-99 cells in response to various secretagogues

Culture conditions	*n*	Insulin secretion (pg · µg protein^{-1} · 30 min^{-1}) 3 mmol/l glucose	16.7 mmol/l glucose	30 mmol/l KCl	100 µmol/l tolbutamide	10 µmol/l forskolin	Insulin content (ng/µg protein)
30 mmol/l glucose (1 day)	3	2.8 ± 0.8	5.3 ± 1.1	5.4 ± 1.8	5.2 ± 1.4	7.2 ± 1.7	0.8 ± 0.1
5 mmol/l glucose							
1 day	4	15.2 ± 1.8	22.3 ± 2.4	31.5 ± 2.8	26.1 ± 1.1	33.2 ± 2.0	4.1 ± 1.2
2 days	3	42.8 ± 5.7	101.6 ± 12.6*	116.3 ± 18.0*	132.4 ± 18.3*	142.4 ± 10.9*	6.2 ± 1.4
5 days	5	46.3 ± 12.8	318.4 ± 46.4*	281.6 ± 34.8*	292.1 ± 32.0*	253.71 ± 31.9*	16.5 ± 2.7

Data are means ± SE. IB/3x-99 cells were previously cultured for 14 days in DMEM supplemented with 10 mmol/l nicotinamide. The cells were assayed in free-floating clusters in glass vials at ~5 × 10^4 cells per vial and incubated at 37°C in 1 ml of fresh modified Krebs buffer with 0.5% BSA and with different secretagogues for 30 min. Previously, the cells were preincubated in glucose-free modified Krebs buffer (30 min). The incubation medium and cell extracts were analyzed for insulin by RIA. *P < 0.001 vs. 3 mmol/l glucose. n, number of independent determinations per secretagogue.

16.7 mmol/l glucose (6.87-fold increase), 30 mmol/l KCl (6.08-fold increase), 100 µmol/l tolbutamide (6.30-fold increase), and 10 µmol/l forskolin (5.47-fold increase) (Table 1). Finally, low glucose conditions improved basal insulin secretion (Table1).

According to these results, incubations of the ES-derived insulin-secreting cells for 5 days in low glucose resulted in the best evaluated condition of cell differentiation; thus, we further characterized glucose-induced insulin release in this situation. Fig. 2*A* shows insulin release from ES-derived insulin-secreting cells cultured for 5 days in low glucose (IB/3x-99 cells), in response to glucose concentrations between 2.75 and 22.2 mmol/l. It was observed that there was a concentration dependency for glucose-induced insulin release between 5 and 16.7 mmol/l glucose that fitted to a sigmoidal curve. This pattern resembles the behavior observed in mouse pancreatic islets (14,16); meanwhile, IB/3x-99 cells reach lower insulin secretion values. The foregoing results on insulin secretion from the various conditions of culture of the IB/3x-99 cells were achieved by static incubation studies. To learn whether the robust response of the IB/3x-99 cells occurs with appropriate dynamics, we performed perifusion studies. As shown in Fig. 2*B*, perifused IB/3x-99 cells responded to a stepwise increase in glucose concentration with a rapid and transient burst of insulin secretion (5.25-fold increase). In this case, we did not observe the biphasic pattern of insulin release described in mouse pancreatic islets (14,16).

ES-derived insulin-secreting cells maintain a stable in vivo glucose response. To evaluate the capacity to maintain glucose homeostasis in vivo, IB/3x-99 cells were implanted into diabetic recipients. The body weight of sham-operated (diabetic) mice was 40% lower 8 weeks after the STZ injection. ES-implanted mice lost 23% of their body weight during the first 2 weeks after STZ injection. Thereafter, ES-implanted animals increased their body weight after the implantation at 3–4 g per week, being normalized 4 weeks after the implantation. As shown in Fig. 3, cell implantation led to correction of hyperglycemia within 1 week (Fig. 3, inset), suggesting a certain ability of IB/3x-99 cells to mimic normal β-cell function in vivo. Nevertheless, for unknown reasons, this normalization was reversible in 40% of ES-implanted mice that became hyperglycemic ~12 weeks after the implantation (Fig. 3). Note that in spite of being hyperglycemic, all of these animals maintained their body weight and had a longer survival than sham-operated diabetic mice. Immunocytochemistry shows the presence of a β-galactosidase–positive reaction in the spleen of transplanted animals (data not shown). To assess the ability of the transplanted mice to dispose of a glucose load, an IPGTT and

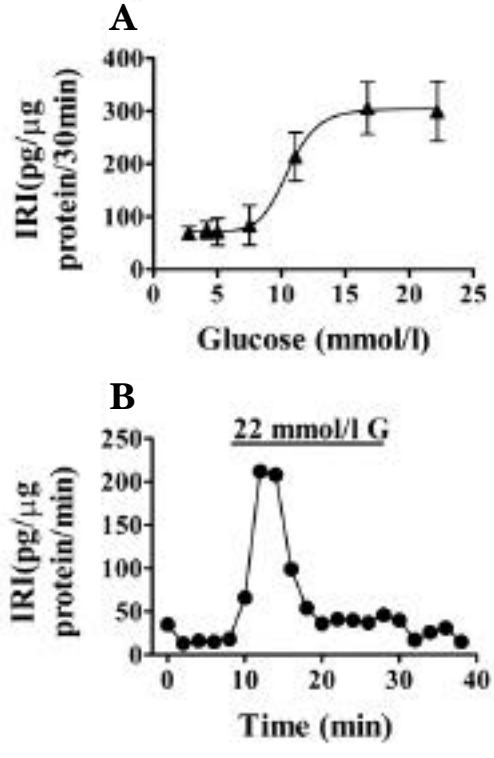

FIG. 2. Glucose-induced insulin release in IB/3x-99 cells. *A*: IB/3x-99 cells were washed twice for 10 min in fresh modified Krebs buffer with 0.1% BSA and 3 mmol/l glucose, transferred to glass vials at ~5 × 10^4 cells per vial, and incubated at 37°C in 1 ml of the same fresh modified Krebs buffer with 0.5% BSA and with different glucose concentrations (between 2.75 and 22.2 mmol/l) for 30 min. Values are expressed as means ± SE of five replicates. *B*: 1 × 10^5 IB/3x-99 cells were perifused at a flow rate of 1 ml/min at 37°C with fresh modified Krebs buffer supplemented with 1% BSA. After a 30-min stabilization period with 3 mmol/l glucose, cells were perifused for 10 min with 3 mmol/l glucose, then for 20 min with 22.2 mmol/l glucose (as indicated by the bar), and finally for 10 min with 3 mmol/l glucose. Values are expressed as means ± SE of four replicates. The incubation medium was analyzed for insulin by RIA, and determinations were run in triplicate. G, glucose.

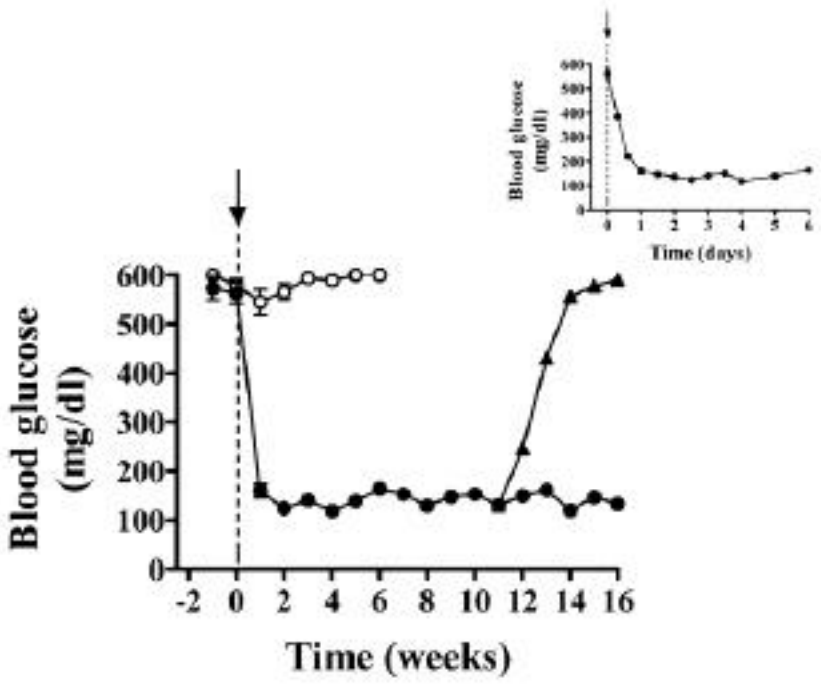

FIG. 3. Changes in blood glucose levels after implantation of IB/3x-99 cells. Blood glucose levels in sham-operated diabetic mice (○) (n = 4), ES-implanted mice (●) (n = 15), and ES-implanted mice that became hyperglycemic (▲) (n = 6). Inset: Detail showing blood glucose levels in the first week after implantation in ES-implanted mice (●) (n = 15). Blood glucose was obtained from the snipped tail and measured between 9:00 and 11:00 A.M., under nonfasting conditions, at 2- to 7-day intervals with a portable glucose meter, since the third day after STZ injection. Arrow indicates the implantation day (shown as week 0). Values are means ± SE.

a meal challenge test were performed. As noted in Fig. 4*A*, fasting plasma glucose levels were the same in both control nondiabetic and transplanted mice. Upon glucose challenge, however, ES-implanted mice showed significantly higher ($P < 0.001$) plasma glucose levels after 30 min than control nondiabetic animals. Recovery of euglycemia was delayed in transplanted mice (210 min) with respect to nondiabetic mice, whose plasma glucose values were back to normal after 120 min. Similar results were obtained during the meal challenge test (Fig. 4*B*) but with smaller differences between nondiabetic and transplanted mice, who recovered normoglycemia within 6 h. Sham-operated diabetic mice showed at all times significantly higher ($P < 0.001$) plasma glucose levels than control nondiabetic and transplanted mice. Finally, after an overnight fast, plasma insulin levels from ES-implanted mice were decreased (47%) (198.3 ± 15.7 pmol/l) compared with control nondiabetic mice (327.8 ± 35.7 pmol/l), but significantly higher ($P < 0.001$) than sham-operated diabetic animals (46.3 ± 9.1 pmol/l).

DISCUSSION

Many questions regarding gene therapy for diabetes and its precise application at the clinical level remain. Therefore, the use of engineered tissues opens new therapeutic possibilities for the treatment of this disease. This study uses a simple genetic approach, previously described (10,11), that permits the generation of insulin-producing cells from mouse ES cells. These cells are able to maintain a stable in vivo glucose response in STZ-induced diabetic mice. To achieve this, we have included some modifications in the last stages of the standard protocol used to culture ES cells (11). One of these implies the formation of cell clusters (see RESEARCHDESIGNAND METHODS) that morphologically resemble mice islets. Moreover, these clusters are easy to implant and stay in the spleen of recipients. In addition, our modeling data support the idea that aggregates or pseudoislet structures permit cell-to-cell contact, providing cell-to-cell interaction and favoring coordinated insulin secretion (17).

The use of nonstimulatory glucose concentrations in the culture medium during the last differentiation stage has been revealed as a crucial event in obtaining cell clones with a high yield of insulin production. It is well documented that glucose is a principal modulator of gene expression in β-cells and that sustained hyperglycemia is able to produce dramatic phenotypic changes that alter the pattern of insulin secretion (6,18). In addition, a recent report indicates that chronic hyperglycemia produces a loss of β-cell differentiation in a hyperglycemic rat model (19). Thus, the causal role of high glucose concentrations in β-cell gene expression and loss of differentiation strongly suggest the importance of low glucose concentrations in the differentiation process of ES cells to ES-derived insulin-secreting cells.

One problem inherent in our study is that normoglycemia reached after transplantation might be due to regeneration of β-cell mass. However, the following are reasons that argue against this possibility: *1*) None of the sham-operated diabetic mice, which remained hyperglycemic throughout the study, survived more than 8 weeks after surgery; *2*) ES-implanted mice were normoglycemic 1 week after implantation, even though it is known that pancreas regeneration in mice is a slow process, taking ~6–8 weeks in the case of 60% pancreatectomized animals (16); and *3*) 40% of normoglycemic ES-implanted mice developed hyperglycemia 12 weeks after the implantation, indicating that this process is reversible, probably depending on the half-life of the implanted cell cluster, suggesting the absence of contribution of β-cell mass regeneration to glycemia normalization. It is interesting to note that animals that became diabetic after the implantation maintained a normal body weight and were capable of surviving under hyperglycemic conditions.

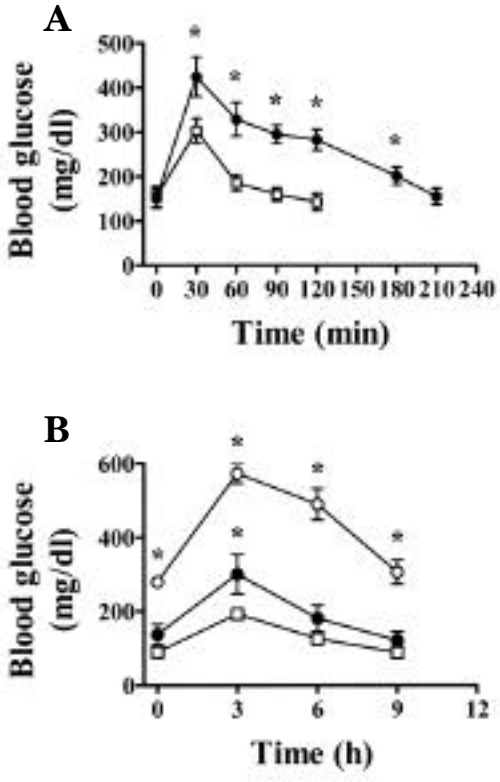

FIG. 4. Plasma glucose responses during an IPGTT and a meal challenge after implantation of IB/3x-99 cells. *A*: Glucose tolerance was tested by an intraperitoneal injection of glucose (2 g/kg of body weight) in overnight-fasted control (nondiabetic) (□) (n = 4) and ES-implanted (●) (n = 3) mice, 5 weeks after euglycemia was obtained. *B*: For meal challenge test, control (nondiabetic) mice (n = 4) (□), ES-implanted mice (n = 9), 5 weeks after euglycemia was obtained (●), and sham-operated (diabetic) mice (n = 3) (○) were fasted for 14 h, then they had free access to standard laboratory chow for 3 h, and then they were fasted again for another 6 h. Whole venous blood was obtained from the tail vein at 0, 30, 60, 90, 120, 180, and 210 min after the injection for IPGTT, and at 0, 3, 6, and 9 h for meal challenge. Blood glucose was measured with a portable glucose meter. Values are means ± SE. *P < 0.001 vs. control (nondiabetic) mice.

The use of ES-derived cells opens new possibilities for tissue transplantation in the treatment of diabetes. However, there are some problems to solve, such as tumor development and immune rejection. Previous attempts with hybridoma HM1 cells and a construct containing a gene stuffer in exon 2 (glycogen synthetase) resulted in a less efficient selection of insulin-containing cells that were tumorigenic, forming teratomas in transplanted animals (unpublished results in collaboration with A. Valera and M.J. Mayol). Both the new construct described in Fig. 1 and the maturation strategy followed (exposure to low glucose and 10 mmol/l nicotinamide) rendered clones in which no tumors were observed 16 weeks after transplantation. Furthermore, to overcome this problem, a procedure that consisted of ligating the Herpes timidine kinase gene after the pGK-hygro^r gene is currently being developed. This should allow tumor suppression by ganciclovir administration. We have not observed immune rejection within this period. Long-term blood glucose normalization suggests that ES-derived cells have a low immunogenic potential or that there exists a strand similarity between ES-derived cells and animal recipients.

In summary, we have shown that ES-derived insulin-containing cells are able to normalize blood glucose in streptozotocin-induced diabetic mice. Further studies are needed to characterize insulin-containing clones obtained with this method and to implement in vitro differentiation and clone selection methods based on these approaches. These results strongly suggest that cell therapy with ES-derived cells provides new possibilities for the treatment of type 1 and type 2 diabetes.

ACKNOWLEDGMENTS

This work was supported by grants FIS 96/1994-01 (to B.S.), FIS96/1994-02 (to J.A.R.), and PM98/0105 (to B.S.) from the Ministry of Education and Culture and by grant IFD97-1065-CO3-02 (to B.S.).

We thank Dr. P. Soriano (Fred Hutchinson Cancer Research Center, Seattle, Washington) for the βgeo gene, Dr. R.M. Mortensen (Harvard Medical School, Boston, Massachusetts) for the PGK-hygromycin gene, Dr. G.J. Bell (Howard Hughes Medical Institute, Chicago, Illinois) for the human insulin gene, and Dr. J. Rossant (Mount Sinai Hospital, Toronto, Ontario, Canada) for R1 ES-cells. We are also grateful to Drs. E. Fernandez and C. De Felipe (Universidad Miguel Hernandez, Alicante, Spain) for their help with the X-gal staining experiments and Dr. J. Salazar (University of Murcia, Murcia, Spain) for keeping transplanted animals. Useful discussions with P.J. Sharp (Roslin Institute, Edinburgh) and the members of the Concerted Action of the European Union "Bioengineered Cells for Gene Therapy of Diabetes Mellitus" (PL-96-0105) are greatly acknowledged. This text was revised by I. Ward.

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