Transplantation of Reversibly Immortalized Insulin-Secreting Human Hepatocytes Controls Diabetes in Pancreatectomized Pigs

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Type 1 diabetes results from the destruction of insulin-producing pancreatic β-cells by a β-cell–specific autoimmune process. Although converting other cell types into insulin-producing cells may compensate for the loss of the β-cell mass while evading β-cell–specific T-cell responses, proof-of-principle of this approach in large animal models is lacking. This investigation was initiated to determine whether an insulin-producing human hepatocyte line can control diabetes when transplanted into totally pancreatectomized diabetic pigs. We established a reversibly immortalized human hepatocyte line, YOCK-13, by transferring a human telomerase reverse transcriptase cDNA and a drug-inducible Cre recombinase cassette, followed by cDNA for a modified insulin under the control of the l-type pyruvate kinase (L-PK) promoter. YOCK-13 cells produced small amounts of modified insulin and no detectable endogenous L-PK at low glucose concentrations, whereas they produced large amounts of both modified insulin and L-PK in response to high glucose concentrations. Xenotransplantation of YOCK-13 cells via the portal vein into immunosuppressed, totally pancreatectomized pigs decreased hyperglycemia and prolonged survival without adverse effects such as portal thrombosis, liver necrosis, pulmonary embolism, and tumor development. We suggest that this reversibly immortalized, insulin-secreting human hepatocyte line may overcome the shortage of donor pancrea for islet transplantation into patients with type 1 diabetes. Diabetes 53:105–112, 2004

Despite intensive management, type 1 diabetes is still associated with a high incidence of complications and considerable subsequent mortality (1). Although much progress has been achieved in the field of islet transplantation in humans, islet isolation is expensive, laborious, and severely limited by a scarcity of donor pancreata (2,3). Thus, there is a growing need for another source of tissue for islet transplantation. There are several possible alternatives, including porcine islets, human-derived cell lines, and insulin-secreting cells differentiated from stem cells (4–10). The use of animal cells raises concerns regarding transmission of infectious pathogens and immunologic and physiologic incompatibilities between animals and humans (11). The long-term culture of pancreatic ductal epithelial cells, which were considered to contain tissue-specific stem cells, induced differentiation of such cells into functioning islets (12). However, the induction and differentiation of multipotent stem cells into insulin-secreting cells are not well characterized, and the use of these cells in humans will take a long time to develop. Another potential form of treatment is ex vivo gene therapy, in which cells transduced and expanded in vitro would be transplanted into the diabetic patient. Hepatocytes and muscle cells are attractive candidates for such therapy because these cells have the glucose transporters Glut2 and -4 (13–16).

We previously developed in vivo insulin gene therapy in which hepatocytes were transduced with an adenovirus-associated vector expressing a modified insulin under the control of the glucose-sensitive, hepatocyte-specific l-type pyruvate kinase (L-PK) promoter. Treatment of streptozotocin-induced diabetic rats and nonobese diabetic (NOD) mice with this gene construct resulted in the long-term remission of diabetes (13). These results suggest that hepatocytes would be good candidates for cell therapy for type 1 diabetes.

We have established human hepatocyte lines for using Cre/loxP-based reversible immortalization, which allows the rapid preparation of safe, functional, and uniform hepatocytes (17). In the present study, we focus on engineering a reversibly immortalized insulin-secreting human hepatocyte line containing the modified insulin gene. Such a cell line would provide unlimited availability with the potential to treat a large number of patients. We
present here Cre/loxP-based amplification of insulin-secreting human hepatocytes in vitro by retroviral transfer of human telomerase reverse transcriptase (hTERT) cDNA, which confers immortality (18); insertion of a drug-inducible Cre recombinase cassette (MerCreMer), which allows later excision of the hTERT gene (19); and introduction of modified insulin cDNA under the control of the hepatocyte-specific promoters. Multiple injection of the resultant YOCK-13 cells via the portal vein significantly prolonged the survival and lowered blood glucose of diabetic pigs previously undergoing total pancreatectomy.

**RESEARCH DESIGN AND METHODS**

**Generation of the YOCK-13 cell line.** The YOCK-13 cell line was derived from the reversibly immortalized human hepatocyte line TTNT-16-3. Briefly, human hepatocytes were transduced with a recombinant retroviral vector (SSR#197) containing cDNAs expressing hTERT, for immortalization, and enhanced green fluorescent protein (EGFP), for a selection marker, flanked by a pair of recombinase target loxPs. Two days after three rounds of SSR#197 transduction, cells were sorted using a FACSCalibur system (BD Biosciences Immunocytometry Systems, San Jose, CA) for recovering EGFP-positive cell populations. One of the EGFP-positive immortalized clones, TTNT-16, was selected on the basis of its hepatocyte-specific gene expression profile and negative tumorigenesis. Then, cells were transduced with a plasmid encoding a tamoxifen-inducible Cre recombinase fused with paired mutant estrogen receptor ligand–binding domains (MerCreMer) under the control of the CAG promoter (cytomegalovirus IE enhancer, chicken /H9252 estrogen receptor ligand encoding a tamoxifen-inducible Cre recombinase fused with paired mutant and negative tumorigenesis. Then, cells were transduced with a plasmid resistance genes under the control of the rat L-PK promoter. After transfection, cells were cultured with zeocin-containing ISE-RPMI medium, and resultant YOCK-13 cells via the portal vein significantly prolonged the survival and lowered blood glucose of diabetic pigs previously undergoing total pancreatectomy.
estrogen receptor ligand–binding domains (MerCreMer) (19). The resulting clone obtained after extensive screening is referred to as TTNT-16-3 (Fig. 1). We further engineered the resulting clonal cell line to express a variant of human insulin under the transcriptional control of the glucose-sensitive rat t-PK promoter (13). The insulin variant is a single-chain modified insulin, in which the connecting C-peptide of human preproinsulin was substituted by a short turn-forming heptapeptide, and does not require pancreas-specific enzymatic processing (25) for biological activity. A single cell clone, referred to as YOCK-13, was selected for further studies out of 41 tested on the basis of their in vitro properties (Fig. 1).

YOCK-13 cells proliferated indefinitely in serum-free medium without observable replicative senescence and with an approximate doubling time of 48 h. YOCK-13 cells were uniformly positive for EGFP expression by FACS analysis (Fig. 2A and B) and displayed morphological characteristics of normal human hepatocytes with a large round nucleus, a few nucleoli, and enriched cytoplasmic granules. YOCK-13 cells expressed markers of hepatocytic differentiation that include albumin, asialoglycoprotein receptor, bilirubin-uridine diphosphate glucuronosyl-transferase, cytochrome p450–associated enzyme 3A4, glutamine synthetase, glutathione S-transferase π, and human blood coagulation factor X (data not shown). Approximately 60% of the cells became EGFP negative, as determined by FACS analysis after a 7-day incubation with 500 nmol/l hydroxytamoxifen (Fig. 2A). In contrast, there was no change in the proportion of EGFP-positive cells when YOCK-13 cells were treated with 17β-estradiol (Fig. 2B), thus indicating the lack of cross-activity between hydroxytamoxifen and other related naturally occurring hormones.

EGFP-negative cells isolated by high-speed cell sorting after hydroxytamoxifen exposure were negative for both expression of hTERT and telomerase activity and remained in the reverted form with excellent viability in culture for >1 month. Tumorigenicity assays in Balb/c.scid mice were negative for both immortalized and reverted cells, whereas tumors developed ~3 weeks after inoculation of a human-transformed human liver cell line (PLC/PRF/5) (23).

To determine whether secretion of modified insulin by reverted YOCK-13 cells is appropriately regulated by glucose levels, we examined the expression of both the endogenous t-PK gene and the t-PK promoter–driven recombinant modified insulin in YOCK-13 cells cultured in the presence of low or high concentrations of glucose in
the medium (60 and 450 mg/dl, respectively). YOCK-13 cells produced very small amounts of modified insulin and no detectable endogenous L-PK at low glucose concentration, whereas they produced large amounts of both proteins in response to 450 mg/dl glucose (Fig. 3A–C). Even in the presence of high glucose concentrations, expression of

FIG. 2. GFP expression of YOCK-13 cells before and after tamoxifen treatment. Cre/loxP recombination efficiency was evaluated by assessing GFP expression ratios in YOCK-13 cells before (day 0; gray) and 2 and 7 days after (black) treatment with 500 nmol/l tamoxifen (A) or 17β-estradiol (B) as a control. Data are representative of at least three independent experiments.

FIG. 3. Expression of L-PK and modified insulin in YOCK-13 cells. A: L-PK expression was examined by Northern blot analysis of YOCK-13 cells with a radiolabeled probe before (lanes 1 and 2) and after (lanes 3 and 4) 1 week of tamoxifen treatment, in the presence of low (60 mg/dl; lanes 1 and 3) or high (450 mg/dl; lanes 2 and 4) glucose. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was also probed as an internal control. B: Expression and secretion of modified insulin protein by YOCK-13 cells into the media were examined by Western blot analysis. 1, Purified insulin protein control; 2, Purified modified insulin protein control (6.5 kDa); 3, Human fibroblast extract; 4, Reverted YOCK-13 cell extract in low glucose; 5, Reverted YOCK-13 cell extract in high glucose; 6, 1-day culture medium from reverted YOCK-13 cells in high glucose; 7, 3-day culture medium from reverted YOCK-13 cells in high glucose. β-actin was also probed as an internal control. C, Secretion of modified insulin (MI) was measured by enzyme-linked immunosorbent assay in culture supernatant after 2 days of culture in low or high glucose. D, Immunofluorescence staining with a labeled anti-insulin antibody, which also recognizes modified insulin, of reverted YOCK-13 cells cultured in the presence of low (left) and high (right) glucose concentrations.

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endogenous \(\Delta\)-PK was notably increased in reverted YOCK-13 cells compared with nonreverted cells (Fig. 3A). In addition, modified insulin was detected in reverted YOCK-13 cells by immunofluorescence staining with an anti-insulin antibody when the cells were cultured in high-glucose conditions (Fig. 3B).

We next set out to determine whether transplantation of modified insulin-producing YOCK-13 cells would be capable of decreasing hyperglycemia in animal models of insulin-dependent diabetes. We first transplanted reverted YOCK-13 cells in mice with STZ-induced diabetes. This initial experiment was only aimed at collecting immediate readouts on glycemia while avoiding the use of immunosuppressive drugs, which may interfere with glucose homeostasis. Reverted YOCK-13 cells were cultured for 24 h in the presence of high concentration of glucose (450 mg/dl) before injection into both renal capsules or the intraperitoneal cavity of STZ-treated mice. As controls, we injected medium into the renal capsule of STZ-treated mice. All the diabetic mice receiving YOCK-13 cells reverted to the euglycemic state within 24–48 h (Fig. 4). There was no significant difference in the reduction of blood glucose levels between diabetic mice having received YOCK-13 cells intrarenally or intraperitoneally, and no hypoglycemia was seen in either diabetic mice or normal mice injected with YOCK-13 cells (Fig. 4). Diabetic mice receiving medium alone did not have any reduction in blood glucose (Fig. 4).

To evaluate this approach in an animal model of size comparable to that of human patients, we chose Landrace pigs of 10 kg average weight. A normal 10-kg pig has \(\sim 3 \times 10^9\) \(\beta\)-cells, which is approximately sixfold less than the number of \(\beta\)-cells in a 60-kg man. We decided to surgically remove the entire pancreas from the pigs to eliminate any possible production of insulin from residual \(\beta\)-cells that usually remain in STZ-induced diabetic animals. An advantage of using pigs is that their bile and pancreatic ducts have completely separate openings into the duodenum; therefore, total pancreatectomy can be successfully performed without the need for bile duct reconstruction (Fig. 5A). Both control and transplanted groups received FK506 as an immunosuppressant. Because FK506 would not be sufficient to prevent immune-mediated destruction of xenotransplanted cells but would only decrease its swiftness and intensity, we decided to perform repeated injections of reverted YOCK-13 cells at regular intervals. In untransplanted control pigs, fasting blood glucose levels rapidly increased from normal levels of 88–14 to 289 ± 35 mg/dl by day 5 postpancreatectomy (Fig. 6A). Glycemia then fluctuated around 275 ± 18 mg/dl for the next few days (Fig. 6A), concurrently to the onset of terminal acidoketosis, with a mean survival time in this group of 7 ± 3 days (Fig. 6B). Hydroxytamoxifen-treated, glucose-prestimulated YOCK-13 cells were injected in the portal vein of five pancreatectomized pigs by means of a catheter (Fig. 5B), whose cell infusion port was subcutaneously embedded into the left groin region (22). One infusion of \(1 \times 10^6\) cells was performed postpancreatectomy on day 5, followed by \(2 \times 10^6\) cells twice a week until the pigs died. Blood glucose levels in YOCK-13 cell–transplanted pigs were substantially decreased compared with TTNT-16-3 cell (without modified insulin transgene)–transplanted and –nontransplanted controls (Fig. 6A), and the mean survival time of YOCK-13–transplanted pigs was 30 ± 10 days (Fig. 6B). In contrast to TTNT-16-3 cell–transplanted and –untransplanted control groups, which became lethargic and comatose, transplanted animals remained active, and the cause of death was principally attributed to protracted diarrhea and related disorders resulting from a lack of pancreas exocrine function.
To determine whether blood glucose levels are controlled in YOCK-13 cell–transplanted pigs, we performed IVGTT. During IVGTT, blood glucose levels in YOCK-13 cell–transplanted pigs peaked at 5 min after glucose injection and then slowly declined to the levels seen before glucose injection; a similar rate of decline was seen in normal, healthy pigs. In contrast, blood glucose levels in pancreatectomized, untransplanted pigs peaked at 5 min after glucose injection and remained high (Fig. 7). This result suggests that blood glucose levels are well controlled by changes in exogenous glucose.

To assess the initial seeding of the liver parenchyma with injected YOCK-13 cells and their subsequent fate, we examined liver sections of totally pancreatectomized pigs at various posttransplantation time points. Two days after the first injection of YOCK-13 cells, we observed, by hematoxylin and eosin (HE) staining, multiple clusters of transplanted YOCK-13 cells embedded throughout the liver in the vicinity of portal spaces (Fig. 8A). Immunofluorescence staining with an anti-human insulin antibody, which also recognizes modified insulin, demonstrated expression of modified insulin in the embedded cell clusters (Fig. 8B). We observed a mild accumulation of mononuclear cells around YOCK-13 cell clusters without their obvious infiltration (Fig. 8A). However, at the time of death 20–42 days postpancreatectomy, we observed severe mononuclear cell infiltration of the clusters of transplanted YOCK-13 cells (Fig. 8C) and very few cells expressing modified insulin (Fig. 8D). These observations are consistent with the anticipated progressive destruction of the embedded cells by immune rejection of the xenograft. We did not observe other pathological features at autopsy, including portal thrombosis, liver necrosis, pulmonary embolism, or tumor development in the liver or any other organs.

In recent years, allogeneic transplantation of HLA-matched pancreatic islet cells into the liver by intraportal injection has resulted in insulin independence with improved control of the glycemia of selected human patients with type 1 diabetes (3). However, a positive outcome requires adequate islet mass and the lifelong use of a powerful immunosuppressive regimen to prevent graft rejection triggered by both minor histocompatibility antigen and further T-cell–mediated attack of β-cell–specific diabetogenic autoantigens (26,27). Furthermore, the availability of human islets is severely limited by a scarcity of donor pancreata (28). Here, we provide support for the use of hepatocytes expressing modified insulin under the control of the I-PK promoter as an alternative approach.

The procedure of reversible immortalization with autorecursion of hTERT by hydroxytamoxifen-induced site-specific recombination provides a means of obtaining an unlimited supply of cells for engraftment. In addition, the major diabetogenic β-cell–specific antigens that include GAD (29) are not present in hepatocytes (30). The use of recombinant modified insulin may also enable mutagenesis of a putative diabetogenic epitope within insulin itself (31) while preserving biological activity, thereby rendering HLA-matched reversibly immortalized modified insulin-expressing hepatocytes better tolerated than pancreatic islet cells. If applied directly on a per-patient basis from hepatocytes obtained by liver biopsy, it may be possible to achieve autologous transplantation with complete and spontaneous tolerance of the engrafted cells. Previous

![FIG. 6. Effect of YOCK-13 cell transplantation into totally pancreatectomized pigs on the control of blood glucose and survival. A: Sustained decrease of hyperglycemia in totally pancreatectomized pigs that received repeated intraportal injections of reverted YOCK-13 cells (group C). Control groups were pigs receiving TTNT-16-3 cells, which do not produce MI (group B), and nontransplanted, pancreatectomized (group C). Bars represent means ± SD.](image)

![FIG. 7. IVGTT in totally pancreatectomized pigs transplanted with YOCK-13 cells. IVGTT was performed in diabetic control pancreatectomized pigs 5 days after total pancreatectomy (group A), YOCK-13 cell–transplanted pancreatectomized pigs 1 day after transplantation (group B), and normal, healthy pigs (group C). Bars represent means ± SD.](image)
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