Gene therapy for diabetes mellitus in rats by hepatic expression of insulin

(in vivo gene therapy/recombinant retroviral vectors/insulin gene expression/hepatic gene transfer)

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ABSTRACT Type 1 diabetes mellitus is caused by severe insulin deficiency secondary to the autoimmune destruction of pancreatic β cells. Patients need to be controlled by periodic insulin injections to prevent the development of ketoacidosis, which can be fatal. Sustained, low-level expression of the rat insulin 1 gene from the liver of severely diabetic rats was achieved by in vivo administration of a recombinant retroviral vector. Ketoacidosis was prevented and the treated animals exhibited normoglycemia during a 24-hr fast, with no evidence of hypoglycemia. Histopathological examination of the liver in the treated animals showed no apparent abnormalities. Thus, the liver is an excellent target organ for ectopic expression of the insulin gene as a potential treatment modality for type 1 diabetes mellitus by gene therapy.

Type 1 or insulin-dependent diabetes mellitus (IDDM) is caused by the lack of insulin and results from the autoimmune destruction of the insulin-producing β cells of the pancreas (1). In IDDM, a lack of insulin results in wasting, hyperglycemia, and death from ketoacidosis (2–4). The present treatment for IDDM includes careful monitoring of blood glucose levels, multiple injections of insulin, specialized diet, and exercise regimens. With vigorous compliance and intensive diabetes management strategies, patients can expect a 50–70% reduction in the severe microvascular complications of diabetes, but their health and life expectancy are still compromised (5). The present intensive treatment is still suboptimal for glucose control and patient noncompliance limits the universality of such programs. Thus a further treatment modality for IDDM by gene therapy was explored.

Since β cells are destroyed in IDDM, any attempt to reconstitute insulin gene expression must be directed at an ectopic organ. The liver is an obvious choice since it is the main target organ for insulin action and the principal effector organ in maintaining blood glucose homeostasis and ketogenesis. Somatic gene therapy was attempted by hepatic expression of the insulin gene in severely diabetic rats. The goal of this investigation was to test the hypothesis that ketoacidosis in severely diabetic animals can be prevented by sustained, low-level expression of the insulin gene in the liver.

MATERIALS AND METHODS

PCR Rescue of a Full-Length Rat Insulin 1 cDNA Clone. To replace the missing 33 nucleotides from the 5' end of the rat insulin 1 cDNA (6), the following oligonucleotides were designed: 5'-GCC GCC ACC ATG GCC CTG TGG ATG CGC TTC CTG CCC CTG CTG GCC CTG CTC GTC CTC TGG-3' and 5'-GCC GCC ACC ATG GCC CTG TGG ATG CGC TTC CTG CCC CTG CTG GCC CTG CTC GTC CTC TGG-3'. The PCR product was cloned into the Bluescript vector, and sequencing revealed it was identical to the rat insulin 1 cDNA sequence.

Construction of a Recombinant Retroviral Vector Encoding the Rat 1 Insulin Gene. The rat insulin 1 cDNA was cloned into pLNCX (7) and the cytomegalovirus and neo genes were deleted. The resulting plasmid, pLX/rINS, encoding the 5' long terminal repeat (LTR), the rat insulin gene, and the 3' LTR, was transected into the retroviral packaging cell line GPAM-12. Individual colonies were isolated and screened on the ability to induce insulin production from rat fibroblast 208F cells.

Retroviral Transduction of Rat Hepatocytes in Vivo and Induction of Diabetes. In vivo hepatic gene delivery using recombinant retroviral vectors was performed as described (8). Briefly, male Lewis rats, 3–4 weeks old, were subjected to a 70% partial hepatectomy. Twenty-four hours later, 4.0 ml of retroviral supernatant were infused into the portal vein. Two weeks after retrovirial transduction, diabetes was induced with streptozotocin (at a dose of 250 mg/kg), which was dissolved in 0.1 M citrate buffer (pH 4.5) immediately before intrahepital injection.

Determination of Retroviral Tissue Distribution and Expression. Three days after the induction of diabetes, three experimental and two control rats were sacrificed, and RNA and DNA was isolated from tissues. Two PCR primers—5'-AGTCGCGTGCTGGAAGTTGGA, which hybridized to the rat insulin cDNA, and 5'-CCTGACCTTGGATCTGACTTC, which hybridized to the Moloney murine leukemia virus vector—were used to generate a 345-bp product that specifically hybridized to labeled rat insulin cDNA. DNA from various organs as well as pLX/rINS, 10-fold serially diluted in Lewis rat liver genomic DNA, were used as templates for PCR analysis. The intensities of the ethidium bromide-stained bands after gel electrophoresis were compared to estimate the number of LX/rINS proviral sequences per cell in the tissues. For expression studies, mRNA was isolated from total cellular RNA, which was used to generate cDNA. The cDNA was used for PCR using the above primers.

Serum Chemistry Analyses. Blood glucose levels were determined using a One Touch blood glucose monitoring system (Lifescan, Mountain View, CA). Serum ketones were determined by spotting serum on an Ames Ketostix reagent strip. For RIA, serum samples were sent to Linco Research Immunnoassay Services (St. Charles, MO) for analysis. The insulin RIA utilizes antibodies raised against rat insulin, and the C peptide RIA utilizes antibodies raised against rat C peptide. The glucagon RIA utilizes an antibody raised against human glucagon.

Histopathological and Immunocytochemical Examination. For periodic acid/Schiff reagent and hematoxylin/eosin staining, the tissues were fixed in 10% buffered formalin, cut in

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Abbreviations: IDDM, insulin-dependent diabetes mellitus; C1, C peptide-to-insulin.

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2-μm slices, and stained (9). For fat staining (oil red O), tissues were placed in Shandon cryomatrix, immediately frozen in liquid nitrogen, and stained (9).

RESULTS

Construction of a Recombinant Insulin Retroviral Vector. LX/rINS, a Moloney murine leukemia virus (7)-based retroviral vector, encodes the complete sequence for rat preproinsulin 1 cDNA (6) under the transcriptional control of the viral LTR promoter. Transduction of 208F cells in vitro resulted in the production of 564 ng of immunoreactive rat insulin in the conditioned medium per 10⁶ cells per day (data not shown). No insulin was produced in cells transduced with a control retroviral vector, LX/hAAT (8).

Tissue Distribution of LX/rINS After Portal Vein Infusion. The procedure for retroviral-mediated transduction of rat hepatocytes in vivo involves a 70% partial hepatectomy followed 24 hr later by retroviral infusion into the portal vein (8). This results in 5–15% hepatocyte transduction and persistent gene expression for at least 6 months. The tissue distribution of the LX/rINS vector was determined by PCR analysis of genomic DNA using primers that specifically amplified the LX/rINS sequence. Of the eight tissues tested, the vector was detected in the liver at 0.01–0.1 copy per cell, corresponding to a 1–10% transduction frequency. The only alternative organ that was positive for LX/rINS sequences was the spleen, but only at 0.0001–0.001 copy per cell (Table 1). The remaining tissues contained <0.0001 copy of LX/rINS per cell, which was the level of detection of the assay. No LX/rINS vector sequences were detected in tissues from rats treated with the LX/hAAT vector or medium.

To determine if the LX/rINS was transcribed, mRNA from the liver and spleen was reverse transcribed followed by PCR analysis (rtPCR). LX/rINS specific transcripts were readily detected in the liver of LX/rINS-treated animals to a dilution of 10⁻⁴ (Table 2). Treatment of the mRNA with ribonuclease followed by rtPCR resulted in no product. No LX/rINS specific PCR product was detected in splenic mRNA, suggesting there were at least 10⁻⁴-fold more LX/rINS transcripts in the liver.

Induction of Diabetes in Rats and Insulin Gene Delivery to the Liver. The efficiency of retroviral-mediated hepatic transduction in severely diabetic rats was low and variable, due to altered liver regeneration kinetics (10, 11). Thus, the hepatocytes of nondiabetic rats were transduced with LX/rINS, LX/hAAT, or medium, followed by induction of severe diabetes with a high dose of streptozotocin at 250 mg/kg (12). Three days after streptozotocin treatment, control rats lost ~25% of their body weights while the average weight of LX/rINS-treated rats remained relatively constant (Fig. 1A). In 6 days, all 18 control rats died. In contrast, 13/16 rats transduced with LX/rINS survived for 21 days (Fig. 1B). Immunohistological staining of pancreatic sections with antibodies against rat insulin C peptide (Linco) revealed there was near-total ablation of β cells in all treatment groups 3 days after streptozotocin treatment (data not shown).

Prevention of Ketoacidosis in LX/rINS-Treated Rats. To determine what specific protective effect the LX/rINS vector was having, sera from all three groups of rats were tested for the presence of ketones. Three days after streptozotocin treatment all rats from the two control groups had serum ketone levels of 63 ± 21 mg/dl, which is considered high (12). However, all rats treated with LX/rINS had low or no serum ketones (Fig. 2A).

Insulin, Glucagon, and C Peptide Production in LX/rINS-Treated Rats. Rat sera were assayed for immunoreactive rat insulin by RIA. Fourteen days after retroviral transduction, nonfasting serum insulin levels in the control groups were 2.2 ± 1.8 ng/ml and those in rats transduced with LX/rINS were 7.1 ± 3.1 ng/ml (Fig. 2B). Three days after induction of diabetes, serum insulin levels in control rats decreased to 0.7

<table>
<thead>
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<th>Tissue</th>
<th>LX/rINS</th>
<th>LX/hAAT or medium</th>
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<tr>
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<td>0.01–0.1</td>
<td>&lt;0.0001</td>
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<tr>
<td>Spleen</td>
<td>0.0001–0.001</td>
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<td>Other organs*</td>
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*Kidney, pancreas, lung, heart, brain, and testes.

n = 3 for LX/rINS-treated rats, n = 1 for LX/hAAT- and medium-treated rats.

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Table 2. Detection of LX/rINS-specific transcripts

<table>
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<tr>
<td></td>
<td>10⁰</td>
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<tr>
<td>Liver</td>
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<td>Spleen</td>
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n = 3 for liver and spleen mRNA samples. ND, not determined.

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**Fig. 1.** Effect of streptozotocin treatment in experimental rats. (A) Percent weight change in rats following treatment with streptozotocin calculated from day 0. Each point was the average percent weight change ± SD. □, Medium treatment group (n = 8); △, LX/hAAT treatment group (n = 10); ○, LX/rINS treatment group (n = 16). (B) Percent survival of rats in the three treatment groups.
insulin vector, but there was an increase at 16 and 21 days (Fig. 2C). Thus, hepatic insulin was at least partially active in suppressing glucagon secretion.

**Histopathological Examination of the Liver.** Histological examination of the liver 14 days after retroviral transduction showed no differences between rats transduced with LX/rINS, LX/hAAT, or medium (not shown). Insulin was not detected by immunohistological staining of the liver, which was expected since the liver does not store secretory proteins. Three days after the induction of diabetes, glycogen was absent in livers from control rats but was well preserved in rats trans-
duced with LX/rINS (Fig. 3A). Also, by day 3, small intracellular fat inclusions were evident in most of the hepatocytes in liver sections from control rats, which were absent in rats transduced with the insulin vector (Fig. 3B).

**Blood Glucose Levels in LX/rINS-Treated Rats.** To determine if the high immunoreactive insulin levels posed a danger to the rats, 24-hr blood glucose profiles were established at 2-hr intervals. Fourteen days after retroviral transduction, nonfasting blood glucose levels of all rats remained constant at ~100 mg/dl throughout the 24-hr period. Three days after induction of diabetes, rats transduced with either the control vector or insulin vector had similarly elevated nonfasting levels of blood glucose to >250 mg/dl, which persisted for the entire 24-hr period (Fig. 4). Upon fasting, however, the blood glucose levels in control rats remained elevated (>250 mg/dl) for the first 10 hr and then slowly decreased over the next 14 hr (Fig. 4A). The fasting blood glucose levels in the LX/rINS-treated group, however, decreased to normoglycemic levels (90–110 mg/dl) within the first 4 hr of the fast and remained in this range for 20 hr (Fig. 4B). At no point during the 24-hr fast did the blood glucose level of any of the LX/rINS-treated rats decrease to <50 mg/dl, which is considered hypoglycemic (4).

**DISCUSSION**

Results of this investigation suggest that the liver is an excellent target organ for ectopic insulin gene expression as a potential treatment modality for prevention of ketoacidosis in severe diabetes mellitus. This conclusion is supported by previous reports that transplantation of \( \beta \) cells into the liver, or hepatic insulin expression in transgenic mice (17), alleviates the effects of severe diabetes. Recombinant retroviral transduction of the liver in vivo has achieved a low-level insulin gene expression that is sustained over time. This insulin activity prevented ketoacidosis and death associated with severe diabetes in rats induced by streptozotocin administration. Normoglycemia was also achieved in the treated animals during a 24-hr fast. There is no evidence of any adverse effects of hepatic insulin gene expression in the treated animals, which confirms previous reports that insulin production from transplanted \( \beta \) cells in the liver (18, 19), or insulin expression from the liver of transgenic mice (17), did not cause liver damage or abnormal biology.

Infusion of the retrovirus into the portal vein results in preferential transduction of the liver. The level of transduction with the LX/rINS vector estimated by the PCR analysis (1–10%) is within range of the transduction efficiency of 5–15% determined using a \( \beta \)-galactosidase reporter gene (8).

Since no significant level of LX/rINS provirus, or LX/rINS-specific transcript, was detected in tissues other than the liver, it may be concluded that the major source of ectopically produced insulin is the liver.

Treatment of the rats with a high dose of streptozotocin resulted in near-total destruction of pancreatic \( \beta \) cells in all rats (20). Consequently, within 4 days of treatment, serum insulin levels in control rats decreased to <0.2 ng/ml, the detection level of the assay (unpublished results). Therefore, insulin secretion by residual \( \beta \) cells is inconsequential. There was no appreciable change in serum insulin levels in rats treated with the insulin vector, suggesting an extrapancreatic source of insulin production. There are several indications that this procedure is efficacious in controlling severe diabetes. (i) Insulin inhibits the production of glucagon (16). Although glucagon levels in control diabetic rats increased severalfold, as expected, it remained in the normal range in rats transduced with the insulin vector. (ii) Low serum insulin and high glucagon levels promote ketoacidosis, which was evident in the control diabetic rats that all died in 4–6 days. Rats treated with LX/rINS, however, had no significant blood ketones and 13/16 survived for 21 days. (iii) Sufficient insulin activity was present in animals treated with the insulin vector to have prevented other catabolic activity in the liver such as glycogen breakdown, triglyceride accumulation, and ketogenesis (21, 22). Collectively, the results suggest that the acute clinical conditions associated with severe diabetes were prevented in rats treated with the insulin vector.

Unregulated low-level insulin gene expression from the liver does not appear to cause life-threatening hypoglycemia under the conditions of our study. Under nonfasting conditions, there was no difference in blood glucose profiles in all rats before and after induction of diabetes. Normoglycemia was achieved during a 24-hr fast in LX/rINS-treated rats with no evidence of hypoglycemia, which is consistent with fasting levels of insulin activity being present in these animals. Blood glucose levels in control rats also decreased 12 hr into the fast. This observation is consistent with reports that in human ketoacidotic diabetic patients, blood glucose levels decrease to normoglycemic levels under fasting conditions (23).

Three rats in the insulin vector treatment group died 19 days after streptozotocin administration and the remaining treated animals succumbed a few days afterward. The experimental
rats had low serum ketone levels, and the livers showed no fat deposition and no glycogen depletion, which strongly suggests that the primary cause of death was not worsening diabetes. Elevated creatinine and blood urea nitrogen (BUN) levels suggested the rats succumbed to kidney failure, possibly caused by streptozotocin, a known nephrotoxin at high doses (24). Histological examination revealed diffusely damaged proximal tubules that were strikingly different from the lesions observed in the kidneys of control rats dying from acute ketoacidosis. There is no indication that the LX/rINS vector did contribute to the kidney failure since no vector sequences were detected in the kidneys of LX/rINS-treated rats, and creatinine and BUN levels were unchanged 14 days after vector introduction.

Although low levels of insulin activity were evident in LX/rINS-treated rats, the level of immunoreactive insulin was 5-fold higher than in normal rats. An altered C:1 molar ratio suggested heptatically produced proinsulin was not fully processed to mature insulin and was thus biologically less active (15). Hepatocytes, like other cell types with a constitutive secretory pathway, proteolytically process secretory proteins by the enzyme furin, which cleaves at specific amino acid sequences (25). Furin will properly process rat 1 proinsulin to mature insulin if overexpressed in COS cells (26) or if the cleavage site in proinsulin is converted to the furin recognition sequence (27). Expression of these engineered insulin genes in the liver of diabetic animals should result in the production of mature insulin that will permit the reduction of recombinant viral doses in future experiments. Even without complete processing, we have demonstrated unambiguously that a sustained low level of insulin activity can prevent the acute lethal consequences of IDDM in rats. The implications of this research extend past the treatment of IDDM. Recent studies have shown that prophylactic insulin injected into human patients predisposed to developing IDDM delays or prevents disease onset (28). With the development of better vectors for insulin gene delivery, regulation of its expression, and processing in vivo, severe diabetes can be treated in the future by insulin production in ectopic tissues through somatic gene therapy.

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