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Directed overexpression of insulin in Leydig cells causes a progressive loss of germ cells

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ABSTRACT

The primary goal of this study was to determine the 5′region of the Insl3 gene that specifically targets the expression of human insulin to Leydig cells, and to explore whether the testicular proinsulin is efficiently processed to insulin that is able to rescue the diabetes in different mouse models of diabetes. We show here that the sequence between nucleotides −690 and +4 of mouse *Insl3* promoter is sufficient to direct the Leydig cell-specific expression of the human insulin transgene (*Insl3–hIns*). We also found that the 3′untranslated region (3′UTR) of *Insl3* was effective in enhancing transgene expression of the insulin *in vivo*. Expression analysis revealed that the temporal expression pattern of the *hIns* transgene in Leydig cells of transgenic testes is roughly the same as that of the endogenous *Insl3*. Despite the Leydig cells translate human proinsulin and secrete a significant level of free C-peptide into the serum, the Leydig cell-derived insulin is not able to overcome the diabetes in different mouse models of diabetes, suggesting a lack of glucose sensing mechanisms in the Leydig cells. A consequence of overexpression of the human proinsulin in Leydig cells was the decrease of fertility of transgenic males at older ages. Germ cells in transgenic males were able to initiate and complete spermatogenesis. However, there was a progressive and age-dependent degeneration of the germ cells that lead to male infertility with increasing age.

1. Introduction

Diabetes mellitus is a group of diseases characterised by an absolute or relative insulin deficiency and by poor glucose control (hyperglycemia) in the blood. In type I diabetes, also known as insulin-dependent diabetes mellitus (IDDM) or juvenile-onset diabetes mellitus, there is an absolute lack of insulin due to the progressive loss of pancreatic β-cells in the islets of Langerhans. Despite intensive research efforts, daily insulin injection remains the only therapy. This unsatisfactory treatment strongly affects the life style of the patients and cannot achieve the same degree of glycemic control as provided by endogenous insulin produced from the pancreatic β-cells. Therefore, transplantation of insulin-producing tissues and gene therapy are potential therapeutically strategies to overcome type I diabetes. Large-scale application of islet cell implantations to type I diabetic patients is hindered by limited availability of insulin-producing tissues and the subsequent need for life-long immunosuppressants (Remuzzi et al., 1994). An alternative approach is the ectopic expression of insulin in other tissues using gene therapy. The goal is to restore and to control insulin production in another tissue, thereby reducing the elevated blood glucose levels to a normal range without causing hypoglycemia. For successful therapy, at least three crucial issues must be adequately addressed. The ectopic tissues expressing engineered insulin must have constitutive and regulated secretory pathways and express proprotein convertases PC2 and PC1/3 that are required for proinsulin processing (Steiner, 1998). These cells must have glucose sensing mechanisms for adequate regulation of insulin secretion according to blood glucose levels.

Several approaches, which have been taken in mind these crucial issues, have addressed the feasibility of *in vivo* gene therapy for the delivery of insulin to diabetic patients. Target tissues tested include liver, muscle, pituitary, exocrine pancreas and intestinal K-cells (Valera et al., 1994; Kolodka et al., 1995; Lipes et al., 1996; Goldfine et al., 1997; Bartlett et al., 1997; Abai et al., 1999; Bochan et al., 1999; Cheung et al., 2000; Lu et al., 2005). The results are rather disappointing.

INSL3, a member of the insulin superfamily, is synthesized as preprotein in pre- and postnatal Leydig cells and play an essential role in growth of gubernaculums ligament during transabdominal

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descent of the testis (Zimmermann et al., 1997, 1999; Balvers et al., 1998; Nef and Parada, 1999; Adham et al., 2002; Koskimies et al., 2003). Although the structure of circulating INSL3 is only known in bovine (Büllesbach and Schwabe, 2002), the presence of certain conserved amino acids of the A and B chain at the N and C termini of the pro-INSL3 suggests that the mode of in vivo processing of pro-INSL3 and resulting structure of INSL3 is similar to that of insulin and relaxin (Adham et al., 1993; Hombach-Klonisch et al., 2004). It is known that the processing of the proinsulin occurs in the trans-Golgi by a regulated secretory pathway (Kelly, 1985; Rhodes and Halban, 1987). Similar to islet β cells, several endocrine cells such as intermediate lobe pituitary cells, and gastrointestinal *G* and *K* cells efficiently process proinsulin to mature, biologically active insulin via a regulated pathway of protein secretion (Lipes et al., 1996; Cheung et al., 2000; Lu et al., 2005), whereas in hepatocytes, which have only a constitutive pathway of protein secretion, proinsulin processing to insulin is extremely inefficient (Valera et al., 1994; Kolodka et al., 1995).

To assess the efficiency of the endocrine Leydig cells in processing the proinsulin to the biological active insulin and to determine the ability of the testicular insulin to overcome the diabetes in mice, we overexpressed insulin in the Leydig cells during pre- and postnatal development. The rational for this study was underpinned by our previous study showing that ectopic expression of *Insl3* in β-cells rescue the cryptorchidism in Insl3-null males. These results reveal that the β-cells efficiently processed the Insl3 gene product to the functional hormone. To achieve our goal, the insulin was targeted using the upstream regulated sequence of murine *Insl3* gene, and transgenic mice were generated. To determine whether the testicular insulin would rescue diabetes observed in the Pax-4 deficient mice, we introduced the transgenic allele into the *Pax-4*$^{-/-}$ mice.

2. Materials and methods

2.1. Generation of transgenic mice

The 0.7 kb HindIII/XbaI fragment containing the 5′flanking region of mouse *Insl3* was isolated from the p690-CAT plasmid (Zimmermann et al., 1998) and subclond into pBluescript IIKS+ (Stratagen) to produce pInsl3. To generate the Insl3–hIns/1 construct, a 1.7 kb genomic fragment containing exons 1–3 of human insulin gene (*hIns*) was amplified using the primers Hins2F: 5′-GCC GCT CTA GAC CTG AGC CCT CCA GGA CAG-3′ and Hins2R: 5′-GCC GCG CGG CCG CGG GAG GGG CTC ACA ACA GTG C-3′. The 1.7-kb amplified fragment with an artificial XbaI site at 5′-end and a NotI site at 3′-end was inserted into the XbaI/NotI-digested pInsl3 to create Insl3–hIns/1 construct. A 1.3 kb XbaI/NotI fragment containing the genomic sequence of *hIns* without the 3′untranslated region was amplified using the primers Hins2F and Hins11R: 5-GCC GCG CGG CCG CCC TGC AGG CTG CGT CTA GTT-3′ and subcloned into pInsl3 to create the pInsl3–hIns Δ3′. Finally, a 250 bp NotI/SstII genomic fragment containing the 3′untranslated region of mouse *Insl3* was amplified using the primers Minsl1F: 5′-GCC GCG CGG CCG CTG AAG GAG CAC AGG GCT CAG G-3′ and Minsl1R: 5′-GCC GCC CGC GGC GGT CTG GTC CGT GTG TGA CC-3′, and inserted into pInsl3–INS to generate the construct Insl3–INS/2. The nucleotide sequence of the amplified fragments was confirmed by DNA sequencing.

The fragments containing mouse *Insl3* promoter-human insulin hybrid gene (Insl3–hIns) were separated from the constructs Insl3–hIns/1 and Insl3–hIns/2 by HindIII/NotI, purified by agarose gel electrophoresis, and microinjected into fertilized FVB/N eggs to generate transgenic mice Insl3–INS/1 and Insl3–INS/2.

Mice were genotyped for the presence of the transgene by PCR designed to amplify a region spanning the junction between the mouse *Insl3* promoter and the human insulin gene. For genotyping the transgenic Insl3–INS/1 animals, PCR assays with primers Lyk2, 5′-GCCGCAAGCTTCCGCACCTGGGAGAGG-3′and HinsR3, 5′-GGCAGAAGGACAGTGATCTGG-3′ were performed. Thermal cycling was carried out for 35 cycles, denaturation at 94 °C for 30 s, annealing at 54 °C for 30 s, and extension at 72 °C for 45 s. Primers Lyk2 and HinsR5: 5′-AAAAAAGTGCACCTGACCCCCTGC-3′ were used for genotyping the transgenic Insl3–INS/2 mice. Copy number and number of integration sites of transgenic founders were determined by a quantitative real-time PCR approach as described previously (Boehm et al., 2004).

Transgenic lines were maintained on the genetic background of FVB/N. All animal experiments were carried out using protocols approved by the Medical Faculty of University of Göttingen and Research Advisory Committee.

2.2. Generation of Insl3–hIns: pax4$^{-/-}$ double mutant mice

Mating *Insl3–hIns* hemizygous males with *Pax4*$^{+/-}$ females (Sosa-Pineda et al., 1997) produced *Insl3–hIns: Pax4*$^{+/-}$ mice, which were then crossed to produce mice homozygous for the disrupted *Pax4* gene and hemizygous for the *Insl3–hIns* transgene (*Insl3–hIns: Pax4*$^{-/-}$). The following primers were used to discriminate between wild-type and mutant *Pax4* alleles. Primers Pax 4F: 5′-ATG GCT GTG TGA GCA AGA TCC-3′ and primer Pax 4R: 5′-TCC AGC TTC CCT TAA CTC CAG-3′ were designed to amplify a 250 bp fragment of wild-type allele. The primer LacZ-F. 5′-TTG GCG TAA GTG AAG CGA C-3′ was used with the primer LacZ-R: 5′-AGC GGC TGA TGT TGA ACT G-3′ to amplify a 500 bp fragment of mutant *Pax4* allele.

2.3. RNA analysis

Different tissues were dissected out from 3-month-old wild-type and transgenic mice or from testes of 1-, 3-, 5-, 6-, 8-, 10-, 15-, 20-, and 25-d-old males. Total RNA was prepared using total RNA Isolation Reagent (Biomol, Hamburg), electrophoresed on 1.2% agarose gels containing 2.2 M formaldehyde and transferred onto a nylon membrane. The membrane was hybridized with ^{32}P-labelled human insulin cDNA fragment. RNA integrity was checked by rehybridization of blots with a cDNA probe for human elongation factor-2.

RT-PCR assays were performed using 2 μg of total RNA and a One Step RT-PCR kit (QIAGEN). Primers to amplify the *PC1* cDNA fragment were 5′-TCA ACC AGA GCA CAT GAA GC-3′ and 5′-GCA GCA GCC TGT CAT CTC TA-3′, and those to amplify the *PC2* transcript were 5′-ATG GAG GGC GGT TGT GGA TC-3′ and 5′-CAG GTA CCA TTG CTT TGT AAA GAG A-3′.

2.4. Immunohistochemistry

Testis and pancreas from wild-type and Insl3–hIns transgenic mice were fixed in Bouin's fixative and transferred to 70% ethanol before paraffin embedding. Sections (5 μm) were cut from paraffin blocks with a rotary microtome and mounted on SuperFrost Plus glass slides (Fisher Scientific, Nepean, Canada). Sections were deparaffinized in xylene, rehydrated in a descending alcohol. Thereafter, tissue sections were preincubated for 1 h with 10% normal horse serum in 2% Tween/PBS and incubated overnight at 4 °C with 1:500 mouse monoclonal anti-insulin antibody (Sigma, St. Louis, USA), washed in PBS, and then incubated with CY3-conjugated goat anti-mouse antibody (1:500) (Sigma) for 30 min at room temperature. Slides were then washed in PBS, stained with DAPI (Vector), and examined with a BX60 microscope (Olympus, Hamburg, Germany) with fluorescence equipment and an analysis software program (Soft Imaging System, Münster, Germany).

2.5. Measurement of proinsulin and C-peptide level in protein extracts and serum

To determine pancreatic and testicular proinsulin content, whole pancreas and testis was removed from the mice, weighted, and homogenized in 20 vol. of cold acidic ethanol (75% ethanol, 1.5% concentrated HCl) followed by 48 h of agitation at 4 °C. Thereafter, insulin was quantified in the supernatants of the samples by time-resolved fluoroimmunoasssay. Insulin concentrations in testis and pancreas extracts were determined by Auto DELFIA Insulin Assay following the supplier's protocol (PerkinElmer Life Sciences, Wallac Oy, Finland). Serum C-peptide was determined by radioimmunoassay (RIA) (Linco Research, St. Charles, USA) following the manufacturer's instructions, using specific ^{125}I-labelled human C-peptide as tracer and a human C-peptide antibody. Antibody was 100% crossreactive with human C-peptide and <4% crossreactive with human proinsulin (Sensitivity of 0.1 ng/ml).

2.6. Generation of diabetic mice

To induce insulin-dependent diabetes, 2-month-old male mice were injected intraperitoneally with streptozotocin (STZ) (Sigma, Munich, Germany) (33.3 mg/kg body weight), dissolved in 50 mmol/l citrate buffer (pH 4.5) for 5 consecutive days. Blood glucose was determined four times a week in blood obtained from the tail vein using a Heamogluco-Test glucometer (Roche Molecular Biochemicals, Mannheim, Germany). Mice were considered diabetic after two consecutive blood glucose values of more than 250 mg/dl.

For glucose tolerance tests, STZ-treated transgenic and non-treated wild-type mice were fasted overnight with free access to water and thereafter injected intraperitoneally with glucose (1 mg/kg body weight). Blood samples were obtained from the tail vein before the glucose injection and at 20, 40, and 60 min after injection, and the glucose concentration was measured as indicated above.

2.7. Fertility test and sperm parameters

To investigate the fertility of the transgenic males on a FVB/N background, 3-month-old male mice were mated each with two wild-type FVB/N females. Females were checked for the presence of vaginal plug and/or pregnancy. Pregnant females were removed to holding cages to give birth. Litter size sired by each group of males was determined after 4, 8 and 12 months of breeding.