Insulin-secreting non-islet cells are resistant to autoimmune destruction

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ABSTRACT Transgenic nonobese diabetic mice were created in which insulin expression was targeted to proopiomelanocortin-expressing pituitary cells. Proopiomelanocortin-expressing intermediate lobe pituitary cells efficiently secrete fully processed, mature insulin via a regulated secretory pathway, similar to islet β cells. However, in contrast to the insulin-producing islet β cells, the insulin-producing intermediate lobe pituitaries are not targeted or destroyed by cells of the immune system. Transplantation of the transgenic intermediate lobe tissues into diabetic nonobese diabetic mice resulted in the restoration of near-normoglycemia and the reversal of diabetic symptoms. The absence of autoimmunity in intermediate lobe pituitary cells engineered to secrete bona fide insulin raises the potential of these cell types for β-cell replacement therapy for the treatment of insulin-dependent diabetes mellitus.

Insulin-dependent diabetes mellitus (IDDM) in humans and in nonobese diabetic (NOD) mice is an immune-mediated disorder in which mononuclear cells invade the pancreatic islets of Langerhans (islets), resulting in the selective destruction of the insulin-secreting pancreatic β cells, while sparing other islet cell types (1). Because the clinical applicability of pancreatic islet transplantation is severely limited by the scarce supply of suitable donor islet tissue, poor islet viability, and aggressive recurrence of autoimmunity in transplanted islet grafts (2), efforts have been made to engineer non-islet cells for insulin gene delivery in IDDM.

Hepatocytes and the anterior pituitary tumor cell line, AtT20, have been studied. Proinsulin processing to insulin is extremely inefficient in hepatocytes (3, 4), which have only a constitutive pathway of protein secretion. In contrast, AtT20 cells also have a regulated secretory pathway, with characteristic secretory granules containing the prohormone endopeptidases PC2 (5), PC1/PC3 (6), and carboxypeptidase H (7) that normally convert the prohormone proopiomelanocortin (POMC) to corticotropin (ACTH) and other peptides. When transfected into AtT20 cells, proinsulin is processed to mature insulin, identical in structure to native (i.e., β-cell derived) insulin (8, 9). A major limitation of using transfected anterior pituitary cells for insulin gene delivery is that their major endogenous secretory product is ACTH, and thus implantation of these cells into diabetic recipients resulted in a severe Cushings-like hypercortisolemic syndrome (10). In addition, like many transformed β-cell lines, AtT20 cells have an active constitutive pathway, with proinsulin comprising up to 25% of the secreted immunoreactive insulin (11). Whereas the advantages of various candidate cell lines have been compared in terms of proinsulin processing and other characteristics, their ability to survive the autoimmune attack that destroys β cells has not been evaluated. In the present study, insulin expression was targeted, using transgenic mouse tech-

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niques, to POMC-expressing pituitary cells in NOD mice. We demonstrate that the transgenic intermediate lobe pituitary cells efficiently process and secrete mature insulin via a regulated secretory pathway and yet, unlike insulin-producing β cells, they are resistant to immune-mediated destruction. In view of these immunological and biochemical features, the feasibility of using these cells as an insulin gene delivery system in IDDM was tested. The methodology used may provide a novel means of identifying other β-cell autoantigens targeted by the autoimmune cascade.

METHODS

Construction of the Transgene. The POMC–Ins transgene consisted of the POMC promoter region linked to the structural region of the mouse preproinsulin II (Ins) gene (see Fig. 1A). To excise the 5′ regulatory region of the Ins gene and yet preserve the translation initiation start site at position 1132, a novel HindIII restriction site was created at position 985 by site-directed mutagenesis using the recombination polymerase chain reaction (PCR) technique (12). A 2.4-kb genomic BamHI Ins fragment (13) was cloned into pBluescript (pBS, Stratagene). The recombinant Ins–pBS vector was linearized in two separate restriction enzyme digestion reactions with BalI (position 846) and PstI (position 1237). These templates were then amplified in two separate PCR reactions using primer 3, 5′-CAATCCAAACGCT-TCACGAACGAGGAGGTAC-3′ (corresponding to sense nucleotides 977–1008, mutagenesis sites are underlined and the region of complementarity to primer 3 is in boldface type), and primer 2, 5′-TCG TGT AGA TAA CTA CGA TAC G-3′ (corresponding to nucleotides 2050–2071 of pBS). The PstI template was amplified with primer 1, 5′-GCTGAAGCTTTTGATGTTGACGCGGATCTAG-3′ (corresponding to antisense nucleotides 994–962, mutagenesis sites are underlined and the region of complementarity to primer 1 is in boldface type), and primer 4 (the entire primer 4 was complementary to primer 2). The PCR products were mixed together and cotransfected into bacteria. The BalI/PstI fragment of a plasmid containing the HindIII mutation was then ligated into Ins–pBS that had not undergone PCR amplification. DNA sequencing of the PCR-amplified HindIII/PstI region did not reveal any cloning artifacts or polymerase errors.

By successive ligations, the HindIII Ins structural gene and the POMC-promoter [position –703 bp to position +61 bp (14)] were subcloned into pBS. This promoter region has previously been shown to confer cell-specific expression and correct hormonal regulation in anterior and intermediate lobe cells of transgenic mice (14, 15).

Abbreviations: IDDM, insulin-dependent diabetes mellitus; NOD, nonobese diabetic; POMC, proopiomelanocortin; ACTH, corticotropin; Ins, preproinsulin II; BG, blood glucose.

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Generation of the NOD Transgenic Mice. The POMC-Ins fusion gene cassette was excised from pBS by digestion with XhoI/EcoRV (see Fig. 1A). The cassette was purified for microinjection and was microinjected directly into the pronuclei of 1-cell embryos of NOD mice (16). Founders were identified by PCR and Southern blot analysis of tail DNA. One transgenic NOD line (POMC-Ins1) was studied in detail and is described here.

RNA Blot (Northern) Analysis. Total cellular RNA was isolated with RNAzol (Biotec Laboratories, Houston) and Northern blot analysis was performed as described previously (16). The blots were hybridized sequentially with 32P-labeled insulin and actin probes.

Immunohistochemistry. For paraffin sections, whole pituitaries were fixed in 10% buffered formalin at 4°C overnight. Sections (4-μm thick) were stained with hematoxylin and eosin and immunostained with rabbit anti-human ACTH-1–39 (1:2, Biomedia, Foster City, CA) and guinea pig anti-insulin (1:200, Incstar, Stillwater, MN), with goat anti-guinea pig (1:200, Linco Research Immunoassay, St. Charles, MO) as a secondary antibody, followed by rabbit PAP (1:200, Dako). For plastic sections, pituitary and islet grafts were fixed overnight in Bouin’s fixative, then washed and stored in 10% buffered formalin until they were embedded in plastic (Araldite; Ernest F. Fullam, Latham, NY). Sections (1-μm thick) were affixed to glass by heat; the plastic stain was removed by sodium methoxide. Immunoperoxidase staining was performed with 1:200 guinea pig anti-human insulin antiserum; a 1:3000 cocktail of antibodies to glucagon, somatostatin, and pancreatic polypeptide (gift of Michael Appel, University of Massachusetts) or rabbit anti-human ACTH-1–39 antiserum (Biomedia). Primary antibodies were incubated at 4°C for 12 hr (ACTH) or for 48 hr (insulin and cocktail).

For frozen sections, pituitaries were fixed in 4% paraformaldehyde in PBS at 4°C overnight. Tissues were cryoprotected in increasing concentrations of sucrose (10%, 15%, and 20%) in PBS at 4°C and embedded in TISSUE-TEK O.C.T. (Miles, Elkhart, IN). Pituitaries were sectioned at −20°C onto S-P Brand Superfrost Plus glass slides (Baxter Diagnostics, Deerfield, IL). Sections were co-incubated with guinea pig anti-human insulin (Incstar, 1:800) and rabbit anti-porcine ACTH (1:150) antisera overnight at 4°C. Primary antibodies were detected with goat anti-guinea pig IgG-Cy3 (Sigma, 1:200) and goat anti-rabbit IgG-FITC (Sigma, 1:50).

Pituitary and Islet Isolation and Transplantation. Islets were obtained from young (<6 weeks) female homozygous POMC–Ins1 transgenic NOD mice by collagenase infusion of the pancreas through the common bile duct as described (17). Aliquots of 100 islets (>75 and <250 μm in diameter) were hand-picked under a stereomicroscope. Intermediate lobe pituitaries from transgenic female NOD mice were dissected into fragments <250 μm. Grafts consisted either of 100 islets placed under the capsule of one kidney and the diced transgenic intermediate lobes placed under the contralateral kidney capsule or of 100 islets mixed with the transgenic intermediate lobe fragments placed into a single site under the kidney capsule. Recipients consisted of diabetic [blood glucose (BG) levels >350 mg/dl for at least 1 week] female NOD mice. Two weeks after transplantation, the grafts were visualized under a stereomicroscope, excised, and fixed in Bouins’ solution for histological processing.

For the therapeutic transplantations, diabetic female NOD mouse recipients (n = 6) were transplanted under the kidney capsule with four diced intermediate lobe transgenic pituitaries. The control group consisted of diabetic female NOD mice (n = 3) transplanted with four diced intermediate lobes from non-transgenic NOD mice. After transplantation, mice were followed with serial BG analyses using a One Touch II meter (Lifescan, Mountain View, CA) and body weights. Five weeks after transplantation, mice were killed and their grafts were fixed in Bouins’ solution for histological examination.

Metabolic Labeling of Freshly Isolated Pancreatic Islets and Primary Cultures of Transgenic Pituitary Cells. Transgenic intermediate lobe pituitary tissue was digested in M-199 containing 1 mg/ml collagenase and 0.5 mg/ml IV hyaluronidase at 37°C. Pituitary cell clusters >10–20 μM were hand-picked, washed, and placed in M-199 with 10% fetal bovine serum and Pen/Strep at 37°C. After 72 hr, the primary pituitary cell cultures were washed with methionine-free RPMI 1640 medium and radiolabeled with 0.4 μCi (1 Ci = 37 GBq) of L-[35S]methionine for 15 hr in the same medium containing 10 mM L-leucine and 10% fetal bovine serum. After removal of the radiolabeled media, the cells were washed with 2.0 ml of modified Krebs–Ringer bicarbonate buffer containing 20 mM Hepes, 0.1% bovine serum albumin, and 11 mM glucose, and incubated for 30 min in 500 μl of the same medium at 37°C. The medium was removed and the cells were incubated for 90 min first in the same medium (“basal”) and then in the same medium 1 mM forskolin and 1 mM 2-isobutyl-1-methylxanthine (“stimulated”). At the end of each incubation period, the medium was removed and placed at −20°C pending analysis.

Pancreatic islets (n = 100), isolated from Sprague–Dawley rats, were radiolabeled for 15 hr in methionine-free RPMI 1640 medium containing 11 mM glucose, 2 mM leucine, 10% fetal bovine sera, and 0.25 μCi [35S]methionine. After this incubation period, the medium was removed and the islets were placed in 1 ml modified Krebs–Ringer bicarbonate buffer containing 2 mM methionine, 2.8 mM glucose, 20 mM Hepes, and 0.1% BSA. After a 90-min preincubation period at 37°C, the medium was removed, replaced with 1 ml fresh medium, and further incubated for 120 min. At the end of this incubation period, this medium (“basal”) was collected and placed at −20°C. The same islets were then incubated for a further 60 min with the same medium containing 16.7 mM glucose. This medium (“stimulated”) was then removed and placed at −20°C.

Pituitary and islet cell lysates and the media samples were then immunoprecipitated for (pro)insulin and analyzed by alkaline-urea PAGE as previously described (18).

RESULTS

Expression of Insulin in the Pituitaries of Transgenic NOD Mice. Northern blot analysis revealed an abundant 550 bp insulin transcript in the pituitary, identical in size to the endogenous pancreatic insulin transcript. In contrast, RNAs from hypothalamus, brain, thymus, spleen, lymph nodes, testes, kidney, liver, and salivary gland did not show any detectable signals (Fig. 1B).

Immunocytochemistry of the pituitaries from the transgenic animals (Fig. 2A) showed that a small percentage of cells in the anterior lobe (A) and the great majority of cells in the intermediate lobe (I) stained positive for insulin (Top), similar in distribution to ACTH immunostaining (Middle). The posterior pituitary (P) was devoid of specific insulin immunostaining and showed background signals similar to nontransgenic control pituitaries (Bottom). Colocalization of insulin and ACTH (or POMC) immunoactivity to the same pituitary cells was demonstrated by double immunolabeling the same frozen section (Fig. 2B). Of note, the ACTH antibody used in these studies was raised against the entire ACTH molecule (i.e., 1–39) and would thus be expected to recognize its cleavage products, α-MSH [ACTH(1–13)], and corticotropin-like intermediate lobe peptide [CLIP, ACTH(18–39)] that are present in the intermediate lobe.

To further characterize transgene expression, insulin content of the anterior and intermediate lobes and the pancreas of 6-week-old transgenic NOD mice were measured by acid ethanol extraction followed by radioimmunoassay. These studies revealed that the great majority of the pituitary-derived insulin was made in the intermediate lobe with immunoreactive insulin content averaging 0.56 ± 0.05 μg per intermediate lobe (n = 5), compared with only 0.02 μg of immunoreactive insulin per anterior lobe (n = 5). In contrast, pancreatic insulin content in young NOD mice averaged 23.7 ± 0.9 μg per gland (n = 5). To ascertain whether the transgenic insulin was secreted into the circulation, a 48-hr fast was performed. Because pituitary cells lack key elements of the

glucose sensing apparatus including the glucose transporter GLUT-2, insulin secreted from the pituitary would not be expected to respond to ambient glucose levels (19). Indeed, with a 60% drop in BG in control NOD mice during the fast (from 112 ± 6.0 to 46 ± 1.6 mg/dl (n = 12)), serum insulin levels fell below the detection limits of the radioimmunoassay. In contrast, despite similar drops in BG levels in heterozygous and homozygous transgenic mice (from 102 ± 7.6 to 41 ± 2.2 mg/dl and 107 ± 7.4 to 43 ± 1.0 mg/dl, respectively), serum insulin levels after the 48-hr fast were markedly elevated, averaging 43.3 ± 4.2 (n = 8) and 94.6 ± 8.6 microunits per ml (n = 8), respectively. These in vivo data demonstrate that the ectopically produced insulin was secreted into the circulation.

**Proinsulin Is Efficiently Processed to Insulin via a Regulated Pathway of Secretion in Transgenic Intermediate Lobe Pituitary Cells.** To examine the molecular forms of insulin synthesized and secreted by the transgenic intermediate lobe pituitaries and to determine whether, characteristic of regulated secretory cells, secretion in the intermediate lobe pituitaries would be coupled to extracellular stimuli, primary intermediate lobe pituitary cultures were established from the transgenic animals. Intermediate lobe pituitary cells were labeled for 15 hr with [35S]methionine, washed, and the insulin forms secreted in response to the secretagogues forskolin and 3-isobutyl-1-methylxanthine (IBMX) during subsequent chase periods were identified by immunoprecipitation followed by alkaline urea-PAGE. These studies showed that, similar to proinsulin processing in normal islets, little insulin was detectable in the media in the basal state, but secretion was greatly enhanced in response to forskolin and IBMX (Fig. 3). The predominant secretory product in the media of transgenic intermediate lobe pituitary cells was mature insulin. Thus, similar to islet β cells, intermediate lobe pituitary cells efficiently process proinsulin to mature insulin via the regulated pathway of protein secretion.

**Insulin-Producing Intermediate Lobe Pituitary Cells Are Not Susceptible to Autoimmune Attack.** Because insulin has been implicated as an autoantigen in IDDM (20, 21) it was of interest to examine whether the expression of insulin in the pituitary, which is not normally involved by autoimmunity in NOD mice, would engender the development of ectopic lymphocytic infiltrates (i.e., hypophysitis). However, serial examination of the pituitaries from these transgenic NOD mice (and from other POMC-Ins transgenic NOD mouse lines that we generated) did not reveal the presence of ectopic lymphocytic infiltration, even after the onset of diabetes. To exclude the possibility that either the anatomical location of the pituitary gland or the sustained production of insulin in the transgenic NOD mice might be impairing immune responsiveness to the ectopically expressed insulin, we transplanted transgenic pituitaries and control islets under the kidney capsules of “naive” (nontransgenic) overtly diabetic NOD recipients. As expected, when control islet grafts were removed from the kidney capsule of the recipients 2 weeks after transplantation, the grafts showed severe infiltration with complete loss of the insulin-staining β cells, with only non-β cells remaining (Fig. 4A). In striking contrast, the transgenic pituitary grafts, placed under the capsule of the contralateral kidney, were devoid of lymphocytic infiltration (Fig. 4B) and showed abundant staining for insulin and ACTH. Thus, the ectopic expression of insulin in the pituitary did not elicit the development of pathologic lesions.

One possible explanation for these findings was that in the absence of antecedent cell injury or of a “triggering event” similar to that which leads to insulinitis, the transgenic pituitaries might not contain sufficient numbers of antigen-presenting cells to activate the relevant lymphocytes to effect destruction of the insulin-expressing pituitary cells. To address this concern and to examine the relative ability of the intermediate lobe pituitary cells to withstand inflammatory damage, islet and transgenic pituitary cells were mixed together and engrafted into a single site under the kidney capsules of diabetic NOD mice. This resulted in the development of severe lymphocytic infiltration over the entire graft, including areas containing pituitary tissue (Fig. 4C, arrowhead). Although there was complete destruction of insulin-producing β-cells in the islets, the insulin-positive cells of the pituitary remained intact. Indeed, the only insulin-positive cells that remained in the grafts colocalized with ACTH staining and were thus pituitary derived. These studies indicate that, even when placed in direct contact with islet-specific pathogenic lymphocytes (presumably, many of which are insulin-specific (22)) the insulin-expressing transgenic pituitaries were not susceptible to immune-mediated destruction.

**Transplantation of the Transgenic Pituitaries into Diabetic NOD Mice Restores Normoglycemia.** The ability of the transgenic intermediate lobe pituitary cells to efficiently process and secrete mature insulin, along with their resistance to autoimmune attack and injury, suggested that these cells could be used as a vehicle for insulin replacement in IDDM. Indeed, we found that transplantation of four intermediate lobe pituitaries under the kidney capsule of spontaneously diabetic NOD mice resulted in a significant gain in body weight (Fig. 5A) and in the complete remission from diabetic symptoms. This was associated with the progressive return to near normal levels of BG (Fig. 5B) and with BG levels decreasing from 484 ± 21 mg/dl pretransplantation to 150 ± 43 mg/dl after transplantation (n = 6). In parallel with this drop in BG, random insulin levels increased from 4 ± 0.2 microunits per ml pretransplantation to 42 ± 9 microunits per ml.
posttransplantation, in a similar range to random insulin levels of nondiabetic control mice [39 ± 9 microunits per ml (n = 6)]. At the end of the transplantation period, immunohistochemistry of the grafts of the recipients showed abundant insulin staining with no evidence of lymphocytic infiltration. As expected, the pancreatic islets of these animals were severely atrophied and devoid of insulin-positive cells, confirming that the enhanced insulin levels posttransplantation were due to the transgenic tissue implants (data not shown). These results demonstrate that the intermediate pituitary-derived insulin is biologically active and are consistent with our biochemical studies which showed that the great majority of insulin secreted by the transgenic pituitaries is fully processed, mature insulin. As expected, diabetic NOD mice receiving nontransgenic (control) intermediate lobe pituitaries had no reduction in serum BG levels, and had increasingly severe diabetic symptoms which resulted in their demise within 3 weeks after transplantation.

**DISCUSSION**

This study describes the generation of transgenic NOD mice in which insulin expression was targeted to POMC-expressing cells of the pituitary. We demonstrate that, similar to islet β cells, the POMC-expressing intermediate lobe pituitary cells from transgenic mice efficiently process proinsulin to mature, biologically active insulin via a regulated pathway of protein secretion. Unlike pancreatic β cells, however, the insulin-expressing pituitary cells are not immunologically attacked in diabetic NOD mice. Indeed, transplantation of the transgenic intermediate lobe pituitary tissues into spontaneously diabetic NOD mice restored normoglycemia and reversed diabetic
symptoms. The absence of autoimmune infiltration in intermediate lobe pituitary cells engineered to secrete insulin provides encouraging in vivo evidence of the potential of these cell types for gene replacement therapy in IDDM.

Intermediate lobe pituitary cells have several advantages compared with the previously described ACTH-producing anterior pituitary cells for targeted insulin gene delivery (10, 19). First, due to tissue specific differences in prohormone processing, ACTH is
Further processed in intermediate lobe cells to α-MSH [ACTH-(1–13)] and CLIP [ACTH-(18–39); refs. 22 and 23], neither of which are known to have adverse metabolic effects. Second, despite its relatively small size (<25% of the mass of the anterior pituitary), the insulin content of the intermediate lobe was high, ≈31-fold greater than the anterior lobe, paralleling expression of endogenous POMC (Fig. 2A and B). Third, the intermediate pituitary grafts had markedly better viability than anterior pituitary lobe grafts: when transplanted long-term (>100 days) under the kidney capsule the anterior pituitary cells, with the exception of the lactotrophs, became atrophied, whereas the intermediate pituitary grafts remained viable and continued to produce abundant amounts of hormones, similar to previous studies (24). These findings, reminiscent of changes after pituitary stalk transection, are most likely due to the differential regulation of these two cell types: whereas secretion from most anterior lobe cells is dependent upon stimulation by trophic hypothalamic hormones, secretion from intermediate lobe pituitary cells is predominantly under tonic inhibitory control by dopamine (25) and γ-aminobutyric acid (26).

Insulin has been implicated as an autoantigen in IDDM (20, 21); however, pathologic lesions did not spontaneously develop in the insulin-secreting pituitaries of the transgenic NOD mice. Likewise, in contrast to transplanted islets, transplantation of the transgenic pituitaries under the kidney capsules of diabetic (nontransgenic) NOD recipients did not provoke autoimmune infiltration or destruction of the grafts. Although these findings suggest that insulin per se is not a pathogenic target of autoimmune in NOD mice, we currently do not know the overall requirements for any protein, including insulin, to be recognized as an autoantigen in IDDM. It is notable that other than insulin, the tissue distribution of other putative key target islet autoantigens (including glutamic acid decarboxylase) is not β-cell restricted (27). It is possible that some of these β-cell autoantigen-expressing non-islet tissues (including the intermediate lobe of the pituitary) have intrinsic properties (e.g., reduced expression of stimulatory and adhesion molecules, or diminished susceptibility to cytokine-mediated damage), that curtail the initiation of immune responses (28) and/or susceptibility to destruction. In addition, if the primary autoantigen(s) is to be recognized in the context of MHC antigens, there may be tissue-specific differences in the nature and amount of antigenic peptides processed and presented by these non-islet tissues to cells of the immune system.

Regardless of the exact mechanism(s) underlying the ability of the insulin-producing intermediate lobe pituitaries to elude immune system recognition and attack, this feature is clearly advantageous for transplantation purposes. A limitation of this current gene delivery system is that insulin secretion in the intermediate lobe pituitary cells was not glucose regulated. It has recently been shown, however, that transfection of AtT20 pituitary cells with the β-cell glucose transporter GLUT-2 could confer glucose-stimulated insulin synthesis and secretion (19). Thus, while further molecular manipulations will be probably be necessary for these non-islet cell types to be useful as gene replacement therapy in IDDM, these studies demonstrate in vivo the potential of this approach.

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**Fig. 5.** Effect of transplanting transgenic insulin-producing intermediate lobe tissues into spontaneously diabetic NOD mice. (A) Percent weight change and (B) BG levels in diabetic NOD mice after transplantation with transgenic intermediate lobe pituitaries (●) or with nontransgenic control intermediate lobe pituitaries (▲). Each point was the mean percent weight change, or the mean BG level, ± SE.