Elevated mouse hepatic betatrophin expression does not increase human beta-cell replication in the transplant setting

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Abstract

The recent discovery of betatrophin, a protein secreted by the liver and white adipose tissue in conditions of insulin resistance and shown to dramatically stimulate replication of mouse insulin-producing beta-cells, has raised high hopes for the rapid development of a novel therapeutic approach for the treatment of diabetes. However, at present the effects of betatrophin on human beta-cells are not known. Here we employ administration of the insulin receptor antagonist S961, shown to increase betatrophin gene expression and stimulate beta-cell replication in mice, to test its effect on human beta-cells. While mouse beta-cells, both in their normal location in the pancreas or when transplanted under the kidney capsule, respond with a dramatic increase in beta-cell DNA replication, human beta-cells are completely unresponsive. These results put into question whether betatrophin can be developed as a therapeutic for human diabetes.
Introduction

A common feature of both type 1 and type 2 diabetes is an insufficient number of insulin-producing beta-cells in the endocrine pancreas. While promising in principle, targeted expansion of human beta-cells as a therapeutic strategy for diabetes has not been possible (1). After beta-cells have been specified from pan-endocrine precursors in fetal life, postnatal beta-cell mass expansion is the result of replication of preexisting beta-cells, and is not dependent on tissue-resident progenitor cells, at least in rodents (2). The basic proliferation rate of beta-cells in adult mammals is very low under normal physiological conditions, typically less than 1% (3). However, when metabolically challenged, such as during pregnancy, diet-induced insulin resistance, and experimental beta-cell ablation, beta-cells have the capacity to expand by proliferation, at least in rodents (4). In particular, hepatic insulin resistance has been shown decades ago to be a powerful promoter of beta-cell replication in mice (5-7). Recently, increased glycolytic flux in beta-cells, as occurs when blood glucose levels are elevated, was proposed as one mediator of increased beta-cell replication (8). Whether additional signals contribute to beta-cell replication during the insulin resistant state remains unknown.

Using a pharmacological inhibitor of insulin action, Yi and colleagues screened for a potential secreted molecule that might represent an additional mitogenic signal to beta-cells in conditions of insulin resistance (9). They identified betatrophin, also known as lipasin (10) or angiopoetin-like 8 (11), as a secreted protein synthesized in liver and to a lesser extent in white adipose tissue, as a potential beta-cell mitogen (9). Betatrophin mRNA expression was induced by four- or six-fold in response to treatment of mice with the insulin receptor antagonist S961 in white adipose tissue or liver, respectively (12). Most strikingly, overexpression of a myc-tagged betatrophin protein in the liver via hydrodynamic injection resulted in a 17-fold increase in beta-
cell replication in mice, a stimulation of replication similar to that seen by inducing insulin resistance through treatment with S961 (9). While the mechanism of action of betatrophin is currently unknown, its description has lead to wide-spread hopes that this hormone might represent a new therapeutic avenue for diabetes. An important question remaining is whether betatrophin can also stimulate replication of human beta-cells, which we addressed here through transplantation of human islets into immuno-deficient mice with elevated betatrophin expression in the liver.
Research Design and Methods

**Mice and glucose measurements.** Immunodeficient Nod-Scid (NOD.CB17-Prkdcsid/J) female mice were purchased from Jackson Laboratory (Cat No. 001303) and used for experiments at eight weeks of age. Random blood glucose levels were measured from tail vein blood using glucometer Breeze2 (Bayer AG, Leverkusen, Germany).

**Islet transplantation.** Adult C57BL6/J mice were used for islet isolation as described (3), with three preparations obtained from two months old mice and two from 12 months old mice. 500 human islets were transplanted under the left kidney capsule and 100 mouse islets were transplanted under the right kidney capsule of each mouse.

**S961 treatment.** S961 was received from Dr. Lauge Schafffer (Novo Nordisk) (12). Vehicle (H2O) or 10nmol S961 was loaded into Alzet 2001 osmotic pumps and implanted subcutaneously on the back of mice one week after islet transplantation. Mice were sacrificed for tissue harvesting seven days after S961 or vehicle treatment.

**Real-time PCR analysis and Immunohistochemistry.** Total RNA isolation and real time PCR were performed as described (3). Liver and gonadal white adipose tissues were dissected and snap-frozen for subsequent RNA isolation. 72 hours before euthanizing the mice, 1g/L BrdU (Sigma-Aldrich Corp., St. Louis, MO, USA) was administrated in the drinking water. Kidneys were dissected and fixed with 10% formalin (Fisher PROTOCOL, Pittsburgh, PA, USA). The pancreas was dissected, flattened by forceps, fixed in 4% para-formaldehyde, and paraffin-
embedded. Tissues were sectioned to 5µm thickness. Deparaffinized and rehydrated slides were subjected to antigen retrieval by pressure cooker in 10mmol/l pH 6.0 citric acid buffer. Simultaneous immunofluorescent staining was performed for BrdU and insulin. The primary antibodies used were guinea pig anti-insulin (1:1000 dilution, Dako North America, Inc., Carpinteria, CA, USA) and rat anti-BrdU (1:500 dilution, AbD Serotec; Raleigh, NC, USA). Secondary antibodies were Cy2-anti-guinea pig (1:200) and Cy3-anti-rat (1:200). The beta cell proliferation rate was quantified as the number of BrdU+/insulin+ double positive cells divided by the number of insulin positive cells. Similar double immunofluorescent staining was performed for the proliferation marker Ki67 and insulin. The primary antibodies used were mouse anti-Ki67 (1:500, BD Biosciences, San Jose, CA, USA) and guinea pig anti-insulin. Secondary antibodies were Cy2-anti-guinea pig (1:200) and Cy3-anti-mouse (1:200). The beta cell proliferation rate was quantified as the number of Ki67+/insulin+ double positive cells divided by the number of insulin positive cells. As least 1,000 beta cells were counted, respectively, for human islets under the kidney capsule, mouse islets under the kidney capsule, and endogenous mouse islets in the pancreas.

Terminal deoxynucleotidyl transferase dUTP-mediated nick-end labeling (TUNEL). Apoptosis was assessed by TUNEL staining (Roche Applied Science, Minneapolis, MN, USA) according to manufacturer’s instruction. More than 1,000 cells each were counted for endogenous islets, transplanted mouse islets, and transplanted human islets.
Results and Discussion

We asked whether human islets transplanted under the kidney capsule of immunodeficient mice increase their proliferation rate in response to increased betatrophin levels. First, we established that immunodeficient NOD-Scid (NOD.CB17-Prkdcsid/J) mice respond to the insulin receptor inhibitor S961 in a fashion similar to that seen by Yi and colleagues in C57BL/6J mice (9). As shown in Figure 1A, delivery of S981 using osmotic minipumps indeed caused hyperglycemic in NOD-Scid mice within 24 hours, consistent with prior findings (9), and this effect persisted for the entire treatment period. Next, we evaluated whether expression of betatrophin is induced in the liver of NOD-Scid mice. Messenger RNA levels of betatrophin were induced about 5-fold as determined by quantitative reverse transcription PCR (Fig. 1B), similarly to what was published previously for C57BL/6 mice (9). However, betatrophin mRNA levels were not increased in gonadal white adipose tissue after S961 treatment as had been reported by Yi and colleagues (9), possibly due to the different mouse strains employed or fat depots analyzed (Fig. 1C). It is worth noting that the increase in adipose tissue betatrophin mRNA reported previously was not statistically significant (9).

Next, we assayed replication of beta-cells in the endocrine pancreas of S961-treated NOD-Scid mice by determination of incorporation of the thymidine analogue BrdU (bromodeoxyuridine). A shown in Fig. 1G, the number of replicating beta-cells was dramatically increased in S961-treated mice compared to vehicle controls (Fig. 1D). Quantification of the data showed a mitogenic effect exceeding 20-fold, in line with what had been observed in C57BL/6J mice (Fig. 1I)(9). Co-immunostaining for insulin and Ki67, another proliferation marker, also showed a 20-fold increase in replication rate after S961 treatment (Fig. 1K), similar to what had been observed in C57BL/6J mice (9). The difference in labeling index between the two methods
is explained by the fact that BrdU captures all cells proliferating during the 72 hour period of BrdU administration, while Ki67 staining only detects those cells that are replicating at the time of tissue harvest.

Having validated the experimental model, we were now in the position to test the effects of S961 treatment and elevated betatrophin levels on human beta-cell replication. To this end, we transplanted islets obtained from five deceased, non-diabetic organ donors aged 4 to 53 years (see Table 1 for donor information) under the kidney capsule of S961-treated NOD-Scid mice. In contrast to the dramatic increase in proliferation seen in the mouse endocrine pancreas documented above, human beta-cells did not respond with increased BrdU incorporation to S961 treatment and elevated betatrophin expression (Fig. 1H versus Fig. 1E). Quantification of BrdU+/insulin+ double positive cells confirmed that the percentage of replicating human beta-cells was less than 0.5% in both vehicle and S961-treated mice (Fig. 1J). Co-immunostaining for insulin and Ki67 also confirmed that the engrafted human beta-cells did not respond to insulin resistance in the same manner as the beta-cells of the mouse pancreas (Fig. 1K).

An important caveat of this study is the fact that we could not analyze human beta-cells in their normal location, but only after transplantation under the kidney capsule. One could envision, for instance, that the betatrophin produced in the liver does not reach the islets in the kidney capsule in the same concentration as is seen by beta-cells in the endocrine pancreas. In addition, it is possible that betatrophin exerts its effects not directly by binding to a receptor on beta-cells, but indirectly, for instance by altering beta-cell innervation, or by activating another cell or signal not present under the kidney capsule. To address this issue, we simultaneously transplanted mouse islets under the right and human islets under the left kidney capsule of the same NOD-Scid mouse. Strikingly, mouse islets were responsive to S961 treatment regardless
of location, and showed a robust increase in beta-cell replication even when placed under the kidney capsule (Fig. 1F, I, J, K). In fact, the S961-stimulated replication rate of transplanted mouse beta-cells was similar to that seen in beta-cells in the endocrine pancreas of the same mice. Co-immunostaining for Ki67 and insulin confirmed that mouse islets transplanted under the kidney capsule respond to S961 treatment with a significantly increased replication rate. Since human beta-cells might be more susceptible to hyperglycemia, we performed TUNEL staining to test if these cells had become apoptotic, but found no evidence of increased beta-cell death (Fig 2).

In summary, we have confirmed that the insulin-resistant state produced by treating mice with the insulin receptor antagonist S961 causes a dramatic increase in murine beta-cell replication, and that this effect occurs regardless of whether beta-cells are in their native environment in the endocrine pancreas, or in the ectopic location under the kidney capsule. This dramatic increase in replication rate is accompanied by the previously documented increase in betatrophin expression in the liver (9), although we did not determine the increase in plasma betatrophin levels. Human islets transplanted under the kidney capsule were not responsive, and maintained negligible levels of beta-cell replication even when betatrophin production was elevated. One caveat of the presented data is whether mouse betatrophin can act on its human receptor. As this receptor is presently unknown, this issue cannot be addressed directly.

A second important issue is the age of the beta-cell responding to a mitogenic signal, as there is a well-documented decline in beta-cell replication rate with age in both mouse and human (13). While the age of the recipient mice was 2 months, we used mouse islets from both 2 months (young adult, post-puberty) or 12 months old (aged mice, postfertile) donors, which both responded to the S961 treatment with massively increased proliferation. While the basal
proliferation rate was four-fold lower in the 12 months old mouse islets compared to the 2 months old islets, the fold induction by S961 treatment was the same (Fig. 1J,K). The human organ donors ranged in age from 4 to 53 years, thus including a donor of an even younger relative age than the mice employed (Table 1), suggesting that even young human beta-cells are not responsive to betatrophin. In conclusion, these data put into question whether betatrophin is a mitogen for human beta-cells, and whether betatrophin will be a useful therapeutic approach for human diabetes.

Acknowledgments:

Supported by U01-DK089529 and R01-DK088383 to KHK. Human pancreatic islets procurement was supported by NIH/NIDDK grant U01 DK070430 and by Beckman Research Center/NIDDK/Integrated Islet Distribution Program, grant 10028044. The authors have no financial conflicts of interest to disclose. Human islets were also provided by the National Disease Research Interchange, with support from NIH grant U42 RR006042. We thank L. Schäffer of Novo Nordisk for providing the S961 compound, and Dr. Lazar of the University of Pennsylvania for critical reading of the manuscript.

Author Contributions: Y.J., J. L., M.Y., researched data. A.N. contributed to discussion, reviewed/edited manuscript. K.H.K. wrote manuscript. KHK is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.
Fig. 1. Elevated betatrophin expression does not stimulate replication of human beta-cells. (A). Random blood glucose levels of NOD/Scid mice treated with vehicle (n=6) or the insulin receptor antagonist S961 (n=10). Glucose levels in S961-treated mice were significantly elevated from day 1 to 7 (p<0.001). (B) Hepatic betatrophin mRNA levels in mice treated with vehicle (n=3) or S961 (n=4) as determined by qRT-PCR. Messenger RNA levels were normalized to those of TBP (TATA-box binding protein) as internal control, and are expressed as fold over vehicle. *, p < 0.05 (C) Gonadal white adipose tissue betatrophin mRNA levels in mice treated with vehicle (n=8) or S961 (n=6) as determined by qRT-PCR. (D-I) Beta-cell replication in NOD/Scid mice treated with vehicle or S961 was determined by BrdU incorporation (red signal). Beta-cells were identified by insulin immunofluorescence staining (green). (D) Beta-cells in the endocrine pancreas of vehicle-treated mice. (E) Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. (F) Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. (G) Beta-cells in the endocrine pancreas of S961-treated mice. Note the frequent BrdU-positive beta-cells (yellow arrow). (H) Transplanted human islets recovered from the kidney capsule of S961-treated mice. Insulin/BrdU double-positive cells are very rare. (I) Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. Note the frequent BrdU-positive beta-cells (yellow arrow). (J) Beta-cell replication was quantified as the percentage of BrdU+/insulin+ double-positive cells of the total number of insulin positive cells from the six conditions shown in C-H. n=5 for vehicle and for S961. **, p < 0.01. Beta-cell replication in transplanted mouse islets from either 2 months-old or 12 months-old donor mice was also quantified separately. (K) Beta-cell replication was determined as the percentage of Ki67+/insulin+ double-positive cells of the total number of insulin-positive cells in the six conditions shown in D-I. n=5 for vehicle and for S961.
**, p < 0.01. Beta-cell replication in transplanted mouse islets from either 2 months-old or 12 months-old donor mice was also quantified separately.

**Fig. 2.** No evidence for increased beta-cell apoptosis in S961-treated mice. (A-F) Apoptosis in NOD/Scid mice treated with vehicle or S961 was determined by TUNEL staining (red signal). Beta-cells were identified by insulin immunofluorescence staining (green). (A) Beta-cells in the endocrine pancreas of vehicle-treated mice. (B) Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. (C) Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. (D) Beta-cells in the endocrine pancreas of S961-treated mice. (E) Transplanted human islets recovered from the kidney capsule of S961-treated mice. (F) Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. (G) Positive control for the TUNEL staining assay using DNase-treated tissue.
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Table 1

Summary information for the deceased organ donors used for the human islet transplantation studies.

| Donor ID | Age | Sex | BMI | Race  | Cause of death |
|----------|-----|-----|-----|-------|----------------|
| AAER055  | 53  | M   | 29  | Hispanic | Head Trauma    |
| AAFC089  | 46  | M   | 28.8| White  | NA             |
| ICRH051  | 4   | F   | 16.1| Hispanic| Head Trauma    |
| AAGW388  | 43  | M   | 30.6| White  | Head Trauma    |
| ICRH-52  | 18  | M   | 23.5| White  | Head Trauma    |
Fig. 2. No evidence for increased beta-cell apoptosis in S961-treated mice. (A-F) Apoptosis in NOD/Scid mice treated with vehicle or S961 was determined by TUNEL staining (red signal). Beta-cells were identified by insulin immunofluorescence staining (green). (A) Beta-cells in the endocrine pancreas of vehicle-treated mice. (B) Transplanted human islets recovered from the kidney capsule of vehicle-treated mice. (C) Transplanted mouse islets recovered from the kidney capsule of vehicle-treated mice. (D) Beta-cells in the endocrine pancreas of S961-treated mice. (E) Transplanted human islets recovered from the kidney capsule of S961-treated mice. (F) Transplanted mouse islets recovered from the kidney capsule of S961-treated mice. (G) Positive control for the TUNEL staining assay using DNAse-treated tissue.

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