Agonist of growth hormone-releasing hormone as a potential effector for survival and proliferation of pancreatic islets

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Therapeutic strategies for transplantation of pancreatic islet cells are urgently needed to expand β-cell mass by stimulating islet cell proliferation and/or prolonging islet cell survival. Control of the islets by different growth factors provides a potential venue for augmenting β-cell mass. In the present study, we show the expression of the biologically active splice variant-1 (SV-1) of growth hormone-releasing hormone (GHRH) receptor in rat insulinoma (INS-1) cells as well as in rat and human pancreatic islets. In studies in vitro of INS-1 cells, the GHRH agonist JI-36 caused a significant increase in cell proliferation and a reduction of cell apoptosis. JI-36 increased islet size and glucose-stimulated insulin secretion in isolated rat islets after 48–72 h. At the ultrastructural level, INS-1 cells treated with agonist JI-36 revealed a metabolic active stimulation state with increased cytoplasm. Coincubation with the GHRH antagonist MIA-602 reversed the actions of the agonist JI-36, indicating the specificity of this agonist. In vivo, the function of pancreatic islets was assessed by transplantation of rat islets under the kidney capsule of streptozotocin-induced diabetic non-obese diabetic-severe combined immunodeficiency (NOD-SCID) mice. Islets treated with GHRH agonist JI-36 were able to achieve normoglycemia earlier and more consistently than untreated islets. Furthermore, in contrast to diabetic animals transplanted with untreated islets, insulin response to an i.p. glucose tolerance test (IPGTT) in animals receiving islets treated with agonist JI-36 was comparable to that of normal healthy mice. In conclusion, our study provides evidence that agonists of GHRH represent a promising pharmacological therapy aimed at promoting islet graft growth and proliferation in diabetic patients.
was detected in INS-1 and rat islets by Western blotting (39.5 kDa). Rat pituitary was used as a positive control (Fig. 1B).

**Immunohistochemical Confirmation of the Expression of GHRH Receptor Protein in Insulinoma Cells and Rat and Human Islets.** Immunohistochemical analysis showed pronounced GHRH-R immunostaining of INS-1 cells (Fig. 2A and D), rat (Fig. 2B and E), and human (Fig. 2C and F) islets. To confirm the localization of GHRH-R on β-cells, costaining for insulin was performed (Fig. 2F).

**Ultrastructural Analysis of Insulinoma Cells Before and After Incubation with the GHRH Agonist.** INS-1 cells under normal culture conditions were characterized by secretory granules close to the cell membrane (Fig. 3A). The cell surface itself extended long filopodia and other membrane protrusions (Fig. 3B). Treatment of islet cells with 10^{-6} M JI-36 produced an enlargement of the cell membrane and the volume of the cytoplasm. This was accompanied by the disappearance of membrane protrusions (Fig. 3C). Furthermore, mitochondria and lysosomes were also enlarged; the latter contained numerous vesicles, indicating intracytoplasmatic digestion of the contents (peptides, proteins) of secretory vacuoles, after the vacuoles fuse with lysosomes. Additional changes became obvious in the cell nucleus, demonstrating an increased amount of heterochromatin as well as nucleoli (Fig. 3D). These morphological changes suggest an increased active metabolic state of the islet cells.

**Cell Proliferation Studies on Insulinoma Cells.** Incubation of INS-1 cells with JI-36 (10^{-6} to 10^{-9} M) for 24–96 h caused a significant and dose-dependent increase in cell proliferation rates. The most effective concentration of the agonist was 10^{-6} M, with a 50% increase after 72 h (Fig. 4A and C). Coincubation of INS-1 cells with the GHRH agonist JI-36 (10^{-6} M) and the GHRH antagonist MIA-602 (10^{-6} M) for 72 h reversed the proliferation-stimulating effect of the agonist.

**Cell Apoptosis Studies on Insulinoma Cells.** Incubation of INS-1 cells with JI-36 (10^{-6}-10^{-9} M) for 24–96 h resulted in a significant decrease in degree of cell apoptosis as measured by the reduction of activity of caspases 3 and 7. The maximal antiapoptotic effect was seen after 72 h; the most effective concentration of the agonist causing this effect was 10^{-6} M (Fig. 4B and C).

**Determination of Islet Number and Islet Volume.** Cultures of isolated rat islets in the presence of JI-36 showed no relevant change in number of islets over time compared with control islets (Fig. 5A). Calculation of islet equivalents (IEQ) by relative conversion into islets of 150 μm diameter showed a significant increase in IEQ/islet ratio, indicating a relative “islet growth” after 48 h, and up to 72 h, following exposure to JI-36 (Fig. 5B).

Immunohistochemical staining of the islets, after 72 h in culture with JI-36, for insulin and the proliferation marker Ki-67, showed colocalization of the two markers, indicating an induced proliferation, specifically although not exclusively in β-cells (Fig. 5C and D).

**Measurement of Islet Membrane Integrity.** Rat islets were evaluated by fluorescent microscopy using FDA/PI staining. We observed no difference in islet viability between the groups after 24, 48, and 72 h in culture (72-h time point: 93 ± 2.2% for control islets, 96 ± 3.3% for islets exposed to JI-36; n = 4). Morphological appearance following dithizone staining also did not differ between treatment groups.

**Effects of JI-36 on Glucose-Stimulated Insulin Secretion.** In a static model of glucose-stimulated insulin secretion, exposure to JI-36...
glycemic, two had impaired graft function, and one showed graft failure (Fig. 7A). In the i.p. glucose tolerance test (IPGTT), the JI-36 group showed an insulin response comparable to that of normal healthy mice, whereas control islets in animals, classified as cured on the basis of attaining normoglycemia before challenge, exhibited delayed and inadequate responses to glucose challenge (Fig. 7B).

Islet grafts retrieved on day 27 after transplantation were immunostained for insulin and showed stable graft integration (Fig. 7C).

Discussion

The main finding of the present study is that GHRH agonist JI-36 improves β-cell survival and growth as well as metabolic function. We have shown the expression of mRNA and protein for GHRH in both rodent and human islets. The GHRH agonist reduced programmed cell death of β-cells. This was reversed by an antagonist of GHRH. Finally, pretreatment with GHRH agonist improved β-cell engraftment and metabolic function of islets following transplantation under the kidney capsule in the streptozotocin-induced diabetic mice. Furthermore, islets treated with the GHRH agonist before transplantation into diabetic NOD-SCID mice were able to produce normoglycemia in these mice earlier and more consistently than islets sham-treated without JI-36. In addition, JI-36 exposed islets showed a stronger response upon glucose challenge compared with untreated islets in vitro and in vivo.

GH itself and IGF1, as well as GH-releasing peptides such as ghrelin and other GH secretagogues, have been shown to increase β-cell proliferation in transplanted human and fetal rat islets (25, 26). This study, however, shows the potential role of a GHRH agonist in islet cell proliferation and survival. The detection of the GHRH receptor on β-cells in rat and human islets supports the view that GHRH may exert a direct signal transduction within the pancreas independent and/or in addition to the effects mediated by the GH/IGF1 pathways.

Though ghrelin and other GH secretagogues may have pleiotropic actions with potentially unexpected side effects, the administration of GHRH may offer a more physiological approach due to its direct actions. Our ultrastructural analysis shows an increase of β-cell cytoplasm with a reduction of cell extensions and filopodia. Interestingly, a recent study has shown a beneficial effect of another hypothalamic-releasing hormone, corticotrophin-releasing hormone, on β-cell proliferation (27), further emphasizing an important connection between the hypothalamic-pituitary axis and the integrity of insulin-producing cells in the pancreas. Synthetic agonists of GHRH such as JI-36 are more potent and longer acting than native GHRH or other growth factors. This may open new therapeutic options. Because there are millions of patients with type 1 diabetes, and the availability of pancreatic islet donors is extremely limited, reaching less than a few hundred per year, there is a desperate need for the development of methods for increasing the efficiency of β-cell function and islet cell mass. In vitro expansion of islet cell function and mass by the use of growth factors is therefore of great interest. If future studies can show that this strategy can be safely applied in vivo, treatment with GHRH analog may have a tremendous impact also on the prevention and treatment of type 2 diabetes patients. A major feature of diabetes mellitus type 2 is the progressive loss of β-cell mass over time, very similar to the situation with transplanted human islets.

We and others have previously shown that by improving the quality of islets and by a careful quality control of the islets before transplantation, the results can be substantially improved (2). Furthermore, multiple studies performed recently have clearly shown that β-cells are able to replicate under basal conditions and that β-cell mass can be augmented in response to a variety of physiological and/or pathophysiological stimuli (28). Indeed, it has become obvious that the major source of new β-cells during adult life is more likely due to the proliferation of preexisting β-cells than the differentiation of progenitor or stem cells in the pancreas (29).
Therefore, improving β-cell function and replication in vivo may be an important therapeutic strategy for both the prevention and the cure of diabetes mellitus. Although our study was mainly performed in rodents, we have also shown expression of the receptor in human islet cells. On the basis of previous studies with other growth factors, it is appropriate to extrapolate that human islets will have the same potential to expand and improve islet cell mass in a fashion similar to the results observed in our animal models. In addition to refining quality of islet cells and islet cell function before transplantation, it may be possible to improve islet engraftment and reduce the number of islets needed for a successful outcome by using a short-term in vivo exposure to the agonist. Previous work has shown that temporary systemic administration of growth factors such as hepatocyte growth factor (HGF) may improve graft survival and blood glucose control in vivo (30). This, however, requires further study in vivo to adequately address safety issues and the risk of uncontrolled proliferation and tumorigenesis.

In summary, the current long-term efficacy of clinical islet transplantation is rather low. One of the major underlying factors for this outcome is the loss of islet mass over time. Therefore, the exploration of mechanisms promoting islet proliferation and growth is critically important for further progress in the field. The application of synthetic GHRH agonist for islet proliferation in vitro as well as graft function and survival in vivo in therapies of diabetes, and our study showing the importance of local autocrine and paracrine GHRH in β-cell regulation and growth, suggest a promising regenerative therapeutic potential for patients with diabetes.

Materials and Methods

Peptide Analogs Preparation. GHRH agonist JI-36 and GHRH antagonist MIA-602 were synthesized in the laboratory of author A.V.S. (17, 19, 20).

Rat Insulinoma Cell Line. Rat insulinoma cells (INS-1) were cultured in RPMI medium 1640 (PAA) supplemented with 2 mM L-glutamine, 10% FBS, 1 mM Na-pyruvate, 50 μM 2-mercaptoethanol, and 100 U/mL penicillin-streptomycin (Gibco) in a humidified 5% CO₂,95% O₂ atmosphere at 37 °C. The culture medium was changed every other day. Cells were grown for 72 h before experimentation. GHRH agonist JI-36 (10⁻⁶ to 10⁻⁸ M) and GHRH antagonist MIA-602 (10⁻⁶ to 10⁻⁸ M) were used for 24-96 h, respectively.

Isolation of Rat Pancreatic Islets. Pancreatic islets were isolated from male Wistar rats according to guidelines established by the University of Dresden.
for Organ Transplantation. Islets were isolated using a modified dithizone (Sigma-Aldrich) and sized using an eyepiece reticle and inverted microscope. Islets were cultured in CMRL 1066 (Mediatech) containing 2.5% human serum albumin at 37 °C in a 5% CO2 incubator before experimentation.

Ilet Equivalent Determination. Triplicate samples of 100–300 islets were stained with dithizone (Sigma-Aldrich), which binds zinc ions present specifically in islet β-cells, and sized using an eyepiece reticle and inverted microscope (32). All islets with a diameter ≤50 μm were divided into classes of 50-μm increments (i.e., 50–100, 100–150, 150–200, etc.) for calculation of islet equivalents (IEQ). Each diameter class was converted into the mean volume of 150-μm diameter islets by a relative conversion factor. These factors allow converting the total islet number from any preparation into IEQ.

Exposure of INS-1 Cells and Rat and Human Islets to GHRH Analogs. INS-1 cells were grown for 72 h before experimentation; islets were collected immediately after the isolation procedure and divided into three treatment groups: (i) culture media with vehicle (DMSO) as a control group, (ii) culture media containing GHRH agonist JI-36 (10−8 M), and (iii) culture media with JI-36 plus GHRH antagonist MIA-602 (10−6 M). Media change and addition of the analogs was performed after 24 h and 48 h in islet cultures and every other day in INS-1 cell cultures.

Fluorescein Diacetate-Propidium Iodide Viability Staining. Small aliquots of islets were transferred in PBS-containing Petri dishes. Fluorescein diacetate (FDA) and propidium iodide (PI) were added to the samples at a final concentration of 0.5 and 75 μM, respectively. Using a fluorescence microscope, 100 islets were assessed for cell viability by estimating the percentage of viable cells (green) vs. nonviable cells (red) within each islet. The percentage of viable cells was then calculated (33).

Measurement of Insulin Secretion by Static Challenge with Glucose. For static insulin secretion in response to glucose challenge, islets were transferred into Petri dishes containing oxygenated Krebs-Ringer bicarbonate buffer (137 mM NaCl, 4.7 mM KCl, 1.2 mM KH2PO4, 1.2 mM MgSO4·7 H2O, 2.5 mM CaCl2·2 H2O, 25 mM NaHCO3, 0.25% BSA) and preincubated in 3.3 mM glucose at 37 °C (5% CO2) for 30 min. Groups of 8–10 islets from the equilibration cultures were transferred to fresh oxygen-saturated media containing either 3.3 or 16.7 mM glucose and then incubated at 37 °C. Glucose levels were measured at 0, 10, 30, 60, and 120 min. Groups of samples were tested concurrently. Glucose levels were measured by ELISA (Millipore) and values normalized to extracted islet DNA (Quant-it Picogreen; Invitrogen).

In Vivo Ilet Functional Assessment. NOD-SCID mice (MTZ breed) with induced diabetes were used as islet recipients following guidelines established by the University of Dresden Institutional Animal Care and Use Committee. Diabetes was induced by a single i.p. injection of 180 mg/kg streptozotocin (Sigma-Aldrich). Serum glucose was then measured daily using an Ascensia Elite glucometer (Bayer). Mice were considered diabetic if nonfasting blood glucose was >350 mg/dL for 2 or more consecutive days. Rat islet preparations were used for transplantation. Islets from each preparation were divided into two groups, and JI-36 (10−8 M) or vehicle (DMSO) was added to the culture media. Islets were cultured for 48 h before transplantation. After culture, samples of 300 IEQ were washed in transplant media (Ringer acetate with 5% glucose and 10% FBS) and transplanted to the left kidney capsule. The animal experiments and housing were in accordance with institutional guidelines and German animal regulations.

Posttransplant Follow-Up. The mice were observed for 30 d after transplantation. The nonfasting blood glucose levels were measured daily during the first week and twice a week thereafter. On day 25, mice were subjected to an IPGTT. Two days later, grafts were removed. This led to a recurrence of the diabetic state. This suggests that restoration and maintenance of normoglycemia was to the result of islet graft function.

Definition of Metabolic Control. On follow-up, sustained nonfasting blood glucose levels ≤10 mM ≤180 mg/dL) were defined as “cure,” 10–18 mM (180–320 mg/dL) as “partial function” of transplanted islets, and levels above 18 mM (≥320 mg/dL) as “graft failure.”

Glucose Tolerance Test. Mice were fasted overnight (at least 6 h) before examination. A glucose solution was given at 3 g/kg body weight i.p., and blood glucose was recorded before injection and 15, 30, 45, 60, 90, and 120 min following glucose injection. Nontransplanted mice were used as controls and tested concurrently.

Fig. 7. Ilet transplantation (300 islet equivalents) beneath the kidney capsule of streptozotocin-induced diabetic NOD-SCID mice. (A) After transplantation, the control group showed a delayed decrease in blood glucose levels, and only three of six animals showed stable normoglycemia during the follow-up period. Animals receiving a graft of islets pretreated with JI-36 showed rapid and persistent recovery from diabetes (five of six animals). (B) On day 25 following islet transplantation, animals with normal glucose control were subjected to an IPGTT. Though control animals responded in a delayed and insufficient manner to the glucose challenge, the group treated with JI-36 was able to revert initial hyperglycemia to normal ranges within 2 h. Shaded area highlights normal range of blood glucose. (C) Pancreatic islets were treated with JI-36 before transplantation beneath the kidney capsule. Representative immunostained islet for insulin (green) shows stable graft integration after 27 d.
For details of RT-PCR, Western blot analysis, immunohistochemical analysis, fluorescent immunohistochemistry of transplanted pancreatic islets, electron microscopy, measurement of cell proliferation, caspase activity, and statistical analysis, see SI Text.

ACKNOWLEDGMENTS. We thank Linda Gebauer for technical help, Silke Langer for her assistance with immunohistochemistry, and Doreen Streichert for help with electron microscopy. We thank Kathleen Eisenhofer and Martina Haberland for help in the preparation of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft Grant SFB 655 “From Cells to Tissues” (to M.E.B. and S.R.B.), the Dresden Tumor Centre of Excellence, the Centre for Regenerative Therapies Dresden, and the Paul Langerhans Institute Dresden. Studies in Miami were supported by the Medical Research Service of the Veterans Affairs Department and University of Miami Miller School of Medicine Departments of Pathology and Medicine, Division of Hematology/Oncology (A.V.S.) and the Austin Weeks Family Endowment for Urologic Research (N.L.B.).

20. Martina Haberland for help in the preparation of the manuscript. This work was supported by the Deutsche Forschungsgemeinschaft Grant SFB 655 “From Cells to Tissues” (to M.E.B. and S.R.B.), the Dresden Tumor Centre of Excellence, the Centre for Regenerative Therapies Dresden, and the Paul Langerhans Institute Dresden. Studies in Miami were supported by the Medical Research Service of the Veterans Affairs Department and University of Miami Miller School of Medicine Departments of Pathology and Medicine, Division of Hematology/Oncology (A.V.S.) and the Austin Weeks Family Endowment for Urologic Research (N.L.B.).

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Total RNA was isolated from frozen INS-1 cells or islet test. Differences were considered significant at P < 0.05.

Western Blot Analysis. Western blot analysis was performed as previously described (1). Briefly, INS-1 cells and rat and human islets were washed twice in ice-cold PBS and lysed in ice-cold CellLytic™ M Cell Lysis Reagent (Sigma-Aldrich) containing 1% protease inhibitor mixture (Sigma-Aldrich). Cell lysates were matched for protein content; equal amounts of protein were loaded on each lane, separated by SDS/PAGE and transferred to nitrocellulose membrane. After blocking, membranes were immunostained with rabbit polyclonal GHRH antibodies (ab-28692 against human and ab-76263 against mouse and rat; Abcam), mouse monoclonal antibody to insulin (Clone AE9D6, un conjugated from BioGenex Laboratories), and monoclonal mouse anti-human K_57 antigen (Clone MIB-1, un conjugated from DakoCytomation). The signal was amplified using the Ventana amplification kit and visualized using avidin-biotin labeling and 3,3′-diaminobenzidine. Slides were counterstained with hematoxylin. Staining with isotype control antibodies was performed to confirm staining specificity.

Electron Microscopy. INS-1 cells were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer and refixed in 1% osmium tetroxide solution as described previously (2). After dehydration in an ascending ethanol series, specimens were embedded in Epon. Ultrathin sections (60 nm) were prepared. To obtain a suitable contrast, slices were stained with lead acetate and uranyl acetate and analyzed using an electron microscope (Zeiss 906).

Measurement of Cell Proliferation. Cell proliferation was assessed using CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega) kit according to the manufacturer’s description.

Caspace Activity. Activity of caspases 3 and 7 was assessed using Caspace-Glo 3/7 Assay (Promega) following the manufacturer’s description.

Statistical Analysis. In all experiments, statistical differences between experimental groups vs. appropriate controls were determined using ANOVA. Data are presented as mean ± SEM. Statistical significance was tested by ANOVA with Bonferroni’s or Student’s t test. Differences were considered significant at values of P < 0.05.
