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ORIGINAL ARTICLE

Growth Hormone Increases Beta-Cell Proliferation in Transplanted Human and Fetal Rat Islets

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ABSTRACT

Objective The aim of the study was to increase the number of human islet beta-cells after transplantation with injections of human growth hormone (hGH). **Interventions** Human islets and fetal rat islets were transplanted under the left kidney capsule and under the right kidney capsule, respectively in nude normoglycemic mice which were then given a daily injection of 200 µg hGH for 1-4 weeks. **Main outcome measure** Beta-cell proliferation was determined using thymidine incorporation and the beta-cell area was assessed using light microscopy. **Results** Mice given hGH increased their body weight one week after transplantation and had a more efficient removal of glucose after 3 and 4 weeks. Treatment with hGH resulted in increased beta-cell proliferation in human and fetal rat beta-cells, and the beta-cell area tended to increase. However, serum insulin concentrations and pancreas insulin content remained unchanged. **Conclusions** hGH increased the proliferation of transplanted human beta-cells as well as improving the glucose tolerance of the transplanted mice.

INTRODUCTION

During the last decade, islet transplantation evolved as a promising cure for type 1 diabetes with 80% of the patients being off insulin after one year. However, the most recent data from these studies indicate that only 11% of the recipients remain insulin independent after 5 years [1]. There are obviously a number of problems to overcome; the revascularization of the islet transplant has to be improved and the instant blood-mediated inflammatory reaction (IBMIR), which leads to activation of the complement system and subsequent blood clot formation, should be avoided, and as recently described, amyloid deposits are formed in the grafted islets [2, 3]. Another problem is the shortage of tissue and the need to increase knowledge as to how to expand the islet tissue and number of beta-cells both before and after transplantation.

For many years, growth hormone (GH) has been known to be a potent stimulator of proliferation, insulin release and insulin biosynthesis as well as insulin gene expression in beta-cells from rodent islets and human fetal islet-like cell clusters [4, 5, 6]. Insulin-secreting cells express receptors for GH and, in concert with prolactin and placental lactogen, GH is believed to play an important role in the growth and development of pancreatic beta-cells [7, 8, 9].

In this study, nude C57BL/6 mice were transplanted with human pancreatic islets under the left kidney capsule and fetal rat islets under the right kidney capsule. The mice were given daily injections of human growth hormone for 1-4 weeks. Glucose tolerance and serum insulin were followed, and beta-cell area and proliferation determined.

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Key words Cell Proliferation; Human Growth Hormone; Insulin-Secreting Cells; Islets of Langerhans; Transplantation

Abbreviations GH: growth hormone; hGH: human growth hormone; IBMIR: instant blood-mediated inflammatory reaction

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MATERIAL AND METHODS

Animals

Male nude (nu/nu) C57BL/6 mice were purchased from Bomholtgaard (Ry, Denmark). The animals were housed in groups of five in Macrolone size IV cages (59x38x20 cm) and fed a standard rodent diet (R3, B&K Universal, Sollentuna, Sweden) and tap water ad libitum. All animals were kept in a 12/12 hour light/dark environment at a room temperature of 25±1°C and 30-70% relative humidity.

Human Islet Isolation

A total of 6 human islet preparations were isolated from heart-beating organ donors at the Central Unit of Beta-Cell Transplant (Brussels, Belgium) and transported by air to Uppsala. The age of the donors ranged from 23-67 years (55±3) and the mean culture time of the islets before shipping was 13±2 days. On average, the islets consisted of 56±3% insulin-positive, 22±3% glucagon-positive and 8±1% dead cells. The

insulin content was 0.88±0.11 µg insulin/µg DNA before shipping. At Uppsala University, the islets were kept in culture for another 4-5 days, in RPMI 1640 (without glucose) supplemented with 5.6 mM glucose, 10% FCS (Sigma, St Louis, MO, USA) 100 U/mL benzylpenicillin and 0.1 mg/mL streptomycin with the medium changed every second day. Before transplantation, islet glucose oxidation was determined as an indication of islet viability.

Fetal Rat Islet Isolation

Fetal islets were isolated from 20-21 day old fetuses of pregnant Sprague Dawley rats. After decapitation of the fetuses, the pancreata were removed and digested using mild collagenase isolation as described previously [10]. After washing, the tissue was seeded in attachment culture dishes containing RPMI 1640 supplemented with 10% FCS, 100 U/mL benzylpenicillin and 0.1 mg/mL streptomycin. After 2-3 days, the islets were detached by gently blowing culture medium through a pipette and were then picked up using a braking pipette.

Glucose Oxidation

A viability check of the human islet preparations after culture was carried out by measuring glucose oxidation rates. Duplicate groups of 30 human islets were incubated in vials with 100 µL KRBH (Krebs-Ringer bicarbonate buffer supplemented with 10 mM Hepes) with the addition of uniformally labeled ^{14}C D-glucose (specific radioactivity 0.3 mCi/mmol, Amersham-Pharmacia Biotech, Amersham, UK) and non-radioactive glucose at a final concentration of 1.7 or 17 mM. The vials were fixed in a 20 mL outer glass flask, gassed with air/5% CO_2 and capped air tight. After 90 min of incubation at 37°C in a shaking water bath, 100 µL of 0.05 mM antimycin A dissolved in 70% ethanol was added to stop metabolism. With the addition of 100 µL of 0.4 M Na_2HPO_4 (pH 6.0), the CO_2 formed was released. Hyamine hydroxide® was added to the outer vial and the flasks were incubated for another 120 min to allow the liberated CO_2 to be trapped by the hyamine hydroxide®. Scintillation fluid was added and radioactivity was determined using a liquid scintillation counter.

Transplantation

For transplantation, fetal rat islets as well as human islet preparations were used. For each experiment, a total of eight mice with a mean body weight of 26.4 g, were given islets from the same human islet preparation. Each animal was given two grafts, fetal rat islets under the left kidney capsule and human islets under the right capsule. Four mice were given injections of hGH and four served as controls (see below). The fetal rat islet grafts consisted of 100-150 islets and the human grafts of 0.3 µL islet tissue (approximately 1,000 islets). The animals were anesthetized with avertin, the kidney was dissected free and an incision was made in the kidney capsule. Subsequently, a subcapsular tunnel was made with a thin glass stick and the islets inserted, 2-4 mm from the incision, using a braking pipette.

Growth hormone treatment started the day after transplantation. A daily injection of 200 µg hGH/mouse in 250 µL of buffer solution (Genotropin, Miniquick, Pharmacia, Uppsala, Sweden) was administrated subcutaneously; the control mice were given only buffer solution. The animals were euthanized by cervical dislocation after 1, 2, 3 or 4 weeks of treatment. One hour before killing, the animals were injected intravenously in the tail with 1 µCi/g body weight ^3H-thymidine. Before killing the mice, blood was obtained by retro-orbital bleeding and a glucose tolerance test was performed in the morning (see below).

Growth Hormone in Serum

The concentration of growth hormone in the serum, samples collected at about 10 am, was determined by means of an AutoDELFIA hGH assay (Wallac Oy, Turku, Finland). To get some idea of the change during the day, serum GH concentrations were determined three times a day in the non-transplanted mice, given hGH injections for 15 days at 7, 11 and 15 days.

Insulin Determinations

Insulin was extracted from the recipient pancreas by means of acid ethanol and measured using a RIA as described previously [11]. Serum concentrations of insulin were determined using a rat insulin ELISA (cross-reactivity human insulin 120%, Mercordia, Uppsala, Sweden).

Glucose Tolerance Tests

Blood was taken from the tails of the mice before carrying out the intravenous glucose tolerance tests. Blood was then withdrawn after 10, 30, 60 and 120 min an intravenous injection of 30% glucose solution (10 µL/g body weight). These tests were carried out once a week and in the morning before killing the animals. Serum glucose concentrations were determined using a glucometer (ExacTech, MediSence Sverige AB, Sollentuna, Sweden).

Immunohistochemistry and Autoradiography

The mice were euthanized by cervical dislocation; the graft-bearing kidneys were removed and fixed in formalin, dehydrated in ethanol and embedded in paraffin. The graft was sectioned in 5 µm samples and mounted on glass slides. The slides were stained for insulin with an antibovine insulin antibody (ICN Pharmaceuticals, Costa Mesa, CA, U.S.A.), dipped in 50% film-emulsion in 0.75 mM ammonium-acetate, and kept overnight in a lightproof chamber. The film was exposed for 4 weeks before being developed, fixed and counterstained with Mayer's haematoxylin.

Microscopic Evaluation

Insulin-positive cells in the autoradiogram were counted using a light-microscope and cells with more than 10 black silver grains over the nucleus were regarded as residing in the S-phase of the cell cycle. The labeled cells were calculated as a percentage of the number of insulin-positive cells. In each preparation, every third section and 5,000-10,000 cells were counted. The beta-cell area of the transplant was measured using a MOP-screen (Videoplan, Munich, Germany) connected to a microscope (Leitz, Wetzlar, Germany). The insulin-positive area was expressed as a percentage of the total area of the graft.

ETHICS

The experimental protocol involving animals was approved by the Regional Laboratory Animal Ethics Committee in Tierp, Sweden.

STATISTICS

Values are expressed as mean±SEM. The students t-test or two-way ANOVA and the Bonferroni post-hoc test were used to calculate P values when a statistically significant interaction was demonstrated between the independent variables. Statistical analysis was carried out by using the Sigma Stat package (http://www.aspiresoftwareintl.com/html/sigmastat.html; Aspire Software International, Ashburn, VA, U.S.A.). Two-tailed P values less than 0.05 were considered statistically significant.

RESULTS

Animals

A total of 6 transplantation experiments, each with eight transplanted animals, were performed. In one of them, the human islet preparation was found to be

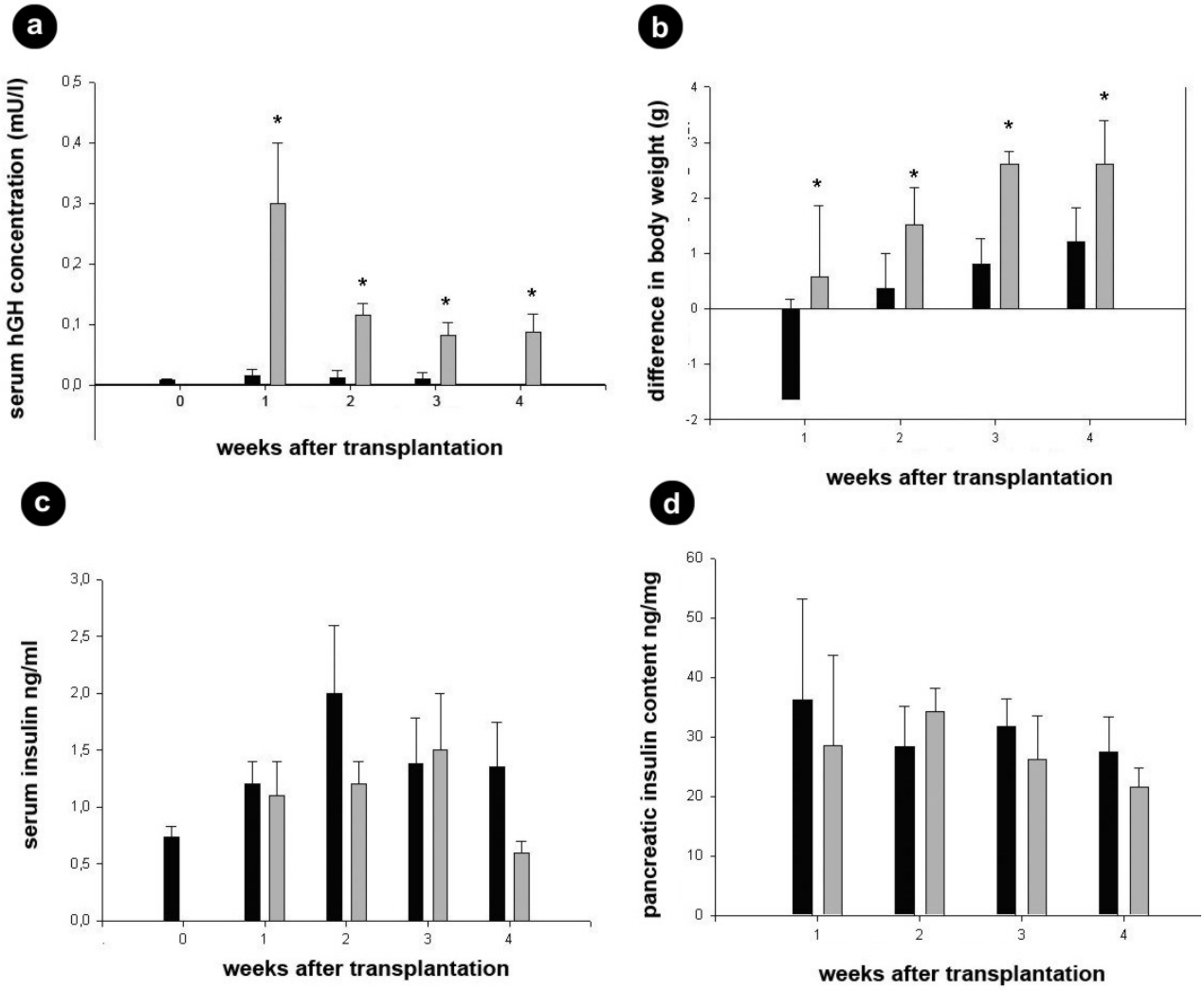

Figure 1. a. Serum concentrations of hGH. Blood samples were collected in the morning. In control mice concentrations were below the detection limit of the assay after 4 weeks of treatment. Week zero corresponds to the mean hGH concentration of all animals before transplantation. **b.** Body weight data are expressed as body weight difference (body weight after treatment (1-4 weeks) minus the body weight before the experiment). Weight before transplantation in the control group was 26.3±0.4 g and, in the GH-treated group, it was 26.6±0.4. **c.** Serum insulin**. d.** Pancreatic insulin content.
Control mice (black bars) and hGH-treated animals (grey bars); n=5 in each group.
* P<0.05. Data were analyzed using two-way ANOVA and Bonferroni as a post-hoc test.

contaminated at the onset of the experiment and therefore only fetal rat islets were given to these mice. In one group of recipients most of the animals died from unknown causes during the experiment and, therefore, had to be excluded.

Human Islet Viability

The viability of the human islets was analyzed by measuring their rate of glucose oxidation. A 10-fold stimulation of the glucose oxidation rate was determined during short-term incubation at 1.7 mM glucose (29±5 pmol/30 islets/90 min, n=6) *vs*. 17 mM glucose (279±74 pmol/30 islets/90 min) (P<0.001).

Serum Concentrations of Growth Hormone

In non-transplanted mice, given hGH according to the same schedule as in the transplanted animals, there was a dramatic increase in serum GH concentrations shortly after the injection in the afternoon (data not shown). The serum concentrations of human growth hormone in the grafted animals increased after one week of daily injections and remained so until the end of the treatment (Figure 1a). There was, however, a tendency towards decreasing concentrations after the first week.

Body Weights

The body weight of the mice before the islet transplantation was the same; (26.3±0.4 g for the controls and 26.6±0.4 g for the GH-treated animals). The controls decreased their body weight immediately after the transplantation procedure but then steadily gained weight. The GH-treated animals, however, increased their body weight after only one week and then continuously throughout the whole observation period. The weight difference between the two treatment groups attained statistical significance at all time points investigated (Figure 1b).

Serum Insulin and Pancreas Insulin Content

Serum insulin concentration and pancreatic insulin content did not change significantly during the time period the experiment lasted (Figure 1cd).

Glucose Tolerance of the Transplanted Mice

During the experiment, animals treated for 3 and 4 weeks with hGH increased their ability to clear glucose (Figure 2).

Labeling Index and Percentage of Beta-Cells in the Graft

Histological examination of the islet grafts revealed that the fetal islet rat transplant was homogenous, with islet-like areas of beta-cells, often separated by strands of connective tissue (Figure 3a). In the human islet transplants, however, the beta-cells were more spread out and areas of other endocrine cells, as adipose and connective tissue were more abundant (Figure 3b).
The labeling index of the beta-cells in both the fetal rat

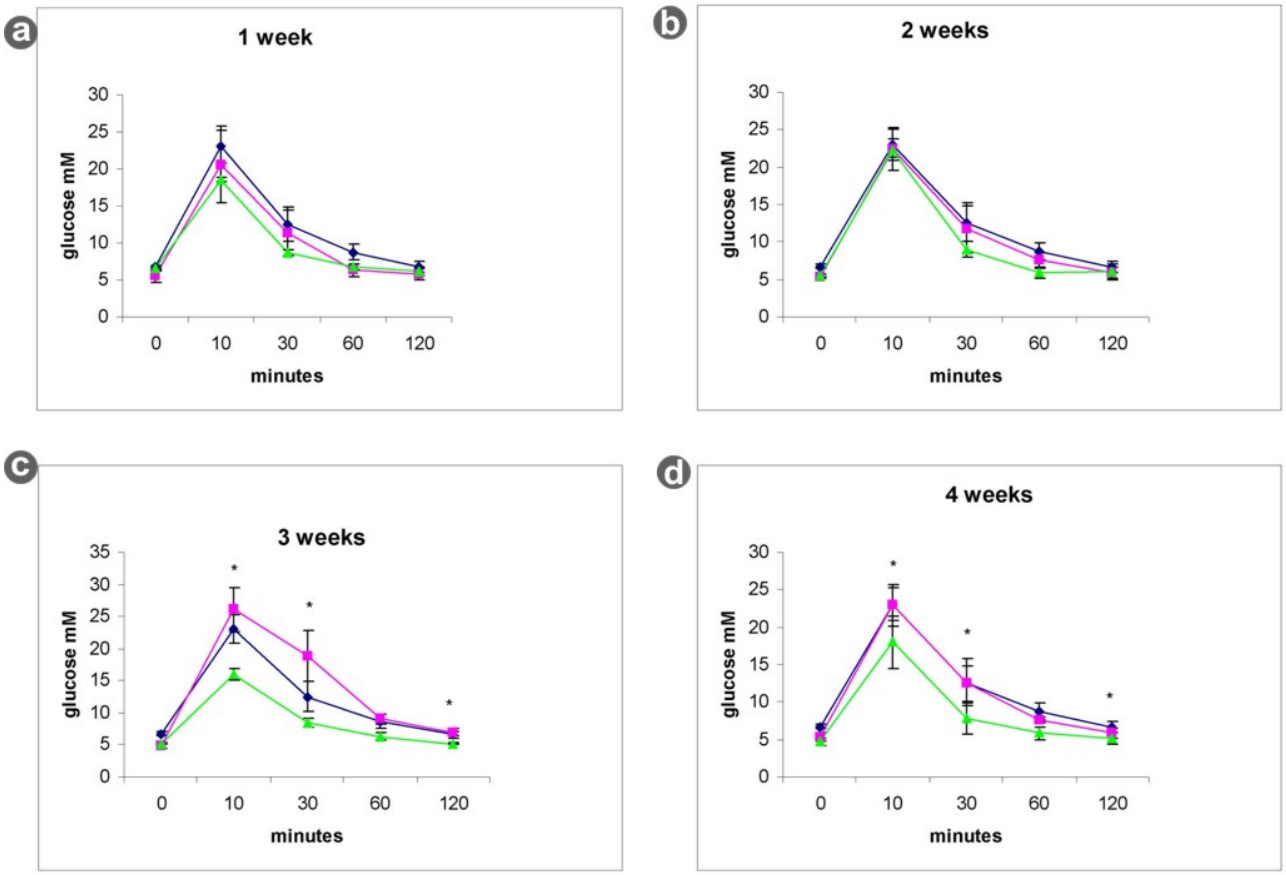

Figure 2. Glucose tolerance. Blood was withdrawn from mice before and at 10, 30, 60 and 120 min after an intravenous injection of glucose. In each figure, the glucose tolerance curve before transplantation is indicated (black) as well as the results for the transplanted control mice (magenta) and the GH-treated mice (green) after 1-4 weeks. **a.** After 1 week. **b.** After 2 weeks. **c.** After 3 weeks. **d.** After 4 weeks.
* P<0.05. Control mice were compared with GH-treated mice by using the Student's t-test (n=5 in each group).

and the human islet grafts increased in animals given hGH but the effect was statistically significant only after 1, 2 and 3 weeks for the fetal rat islets and 1, 2 and 4 weeks for the human islets (Figure 4ab). hGH treatment also increased the area of beta-cells in both types of grafts but only attained statistical significance after 1 and 3 weeks in the fetal rat islets and 3 weeks in the human islets (Figure 4cd).

DISCUSSION

In this study, human islet preparations and fetal rat islets were transplanted into the renal subcapsular space of nude mice. The recipients were then treated with daily injections of hGH for one to four weeks. Before transplantation, the human islet preparation was evaluated using glucose oxidation to assess the presence of functional beta-cells. There was a ten-fold increase in the glucose oxidation of these human islet preparations when raising the glucose concentration from 1.7 to 17 mM glucose, indicating high beta-cell viability. Indirectly, these data may also be taken to indicate a high percentage of beta-cells in the prepared islets since only such cells respond to an acute glucose challenge in this way. Fetal rat islets were included as a positive control since it is well documented that such islets proliferate after hGH treatment in vitro [12, 13].

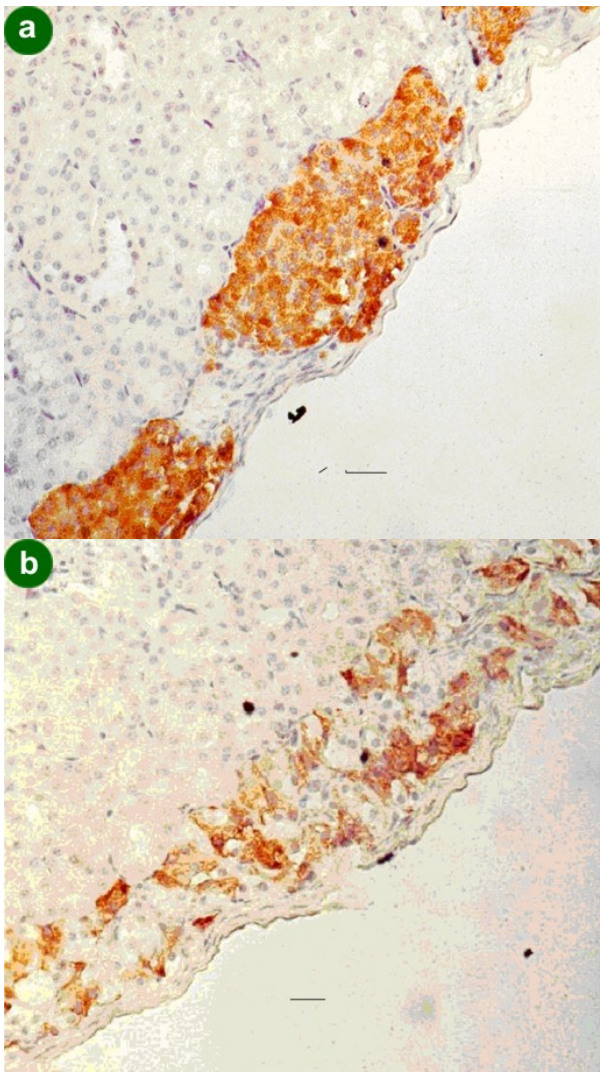

Figure 3. Autoradiographs of transplanted fetal rat islets (**a.**) and human islets (**b.**) under the kidney capsule two weeks after transplantation. Insulin-positive cells (brownish) were counted using a light-microscope and in cells with more than 10 black silver grains over the nucleus regarded as residing in the S-phase of the cell cycle. Labeled cells were calculated as a percentage of the total number of insulin-positive cells. Scale bar 20 µm.

As expected, the administration of hGH increased the serum concentration of GH dramatically after only one week. There was a marked variation in serum GH concentration throughout the day and at the time points checked; concentrations peaked at 3 pm right after administration. It was found that mice given hGH injections increased their body weight in contrast to the control mice, which even lost weight during the first week, suggesting a faster and smoother recovery after the surgical procedure in hGH-treated animals. Furthermore, the increase in body weight was expected with ubiquitous GH receptors present and also a possible rise in leptin concentrations, increasing the appetite of these mice.

In both human islet and fetal rat islet transplants, increased beta-cell proliferation was estimated as early as one week after transplantation, followed later on by an increased beta-cell area. Overall, these data support the idea that hGH increases not only fetal rat beta-cell proliferation but also human beta-cell proliferation. This is in line with studies in young healthy adults where low doses of GH administration reduced fasting glucose levels and increased beta-cell function [14].

The effects on beta-cell proliferation in the transplants were most prominent after one week and then declined. This probably reflects the decrease observed in serum concentrations of GH, which may be due to antibodies which develop with time against the administered hormone. It could also depend on a feedback inhibition of GH receptors since there is a well-known inverse relationship between GH and its receptors [15].

There were no differences in serum insulin concentrations during the four weeks of the study, suggesting that the endogenous beta-cells had decreased their release of insulin or that the observation period was too short. Similar, we found no decrease in the pancreatic insulin content. The presence of a number of beta-cell differentiation markers, Pdx-1, Nkx 6.1 and NeuroD in the endogenous pancreas was evaluated during the first two weeks of the study. There were no significant changes observed but a tendency towards increased levels of Nkx 6.1 was seen after two weeks of treatment which might reflect changes in insulin secretion (data not shown).

In this study, non-physiologically high concentrations of GH were used, trying to mimic the in vitro concentrations previously shown to effect rat islet proliferation. High doses of GH have clinically resulted in contrasting effects, diabetogenic effects [16, 17, 18] but also hypoglycemia, mimicking insulin action [19, 20, 21]. Differences have also been demonstrated using high or low doses of GH in humans. The use of low

doses of GH in young adults improved beta-cell function as well as fasting serum glucose levels [14]. Previous studies raised the concern that GH treatment could increase the frequency of acute renal graft rejection; however, many controlled trials have demonstrated that this is not the case and GH treatment does not induce a higher number of rejection episodes [22, 23, 24, 25, 26, 27, 28, 29]. Furthermore, hGH has also been demonstrated to be involved in cell survival as a protective substance in the context of various cell death inducers [30, 31, 32]. In beta-cells, the activation of STAT5 has been proposed to be the mechanism involved [5].

In conclusion, hGH treatment seems to induce beta-cell proliferation in transplanted human and fetal rat beta-cells, and improve the glucose tolerance of the transplanted mice, suggesting that this might be one possible way of improving islet transplantation in the future.

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Interest disclosures The authors declare that there is no conflict of interest which would prejudice the impartiality of this scientific study

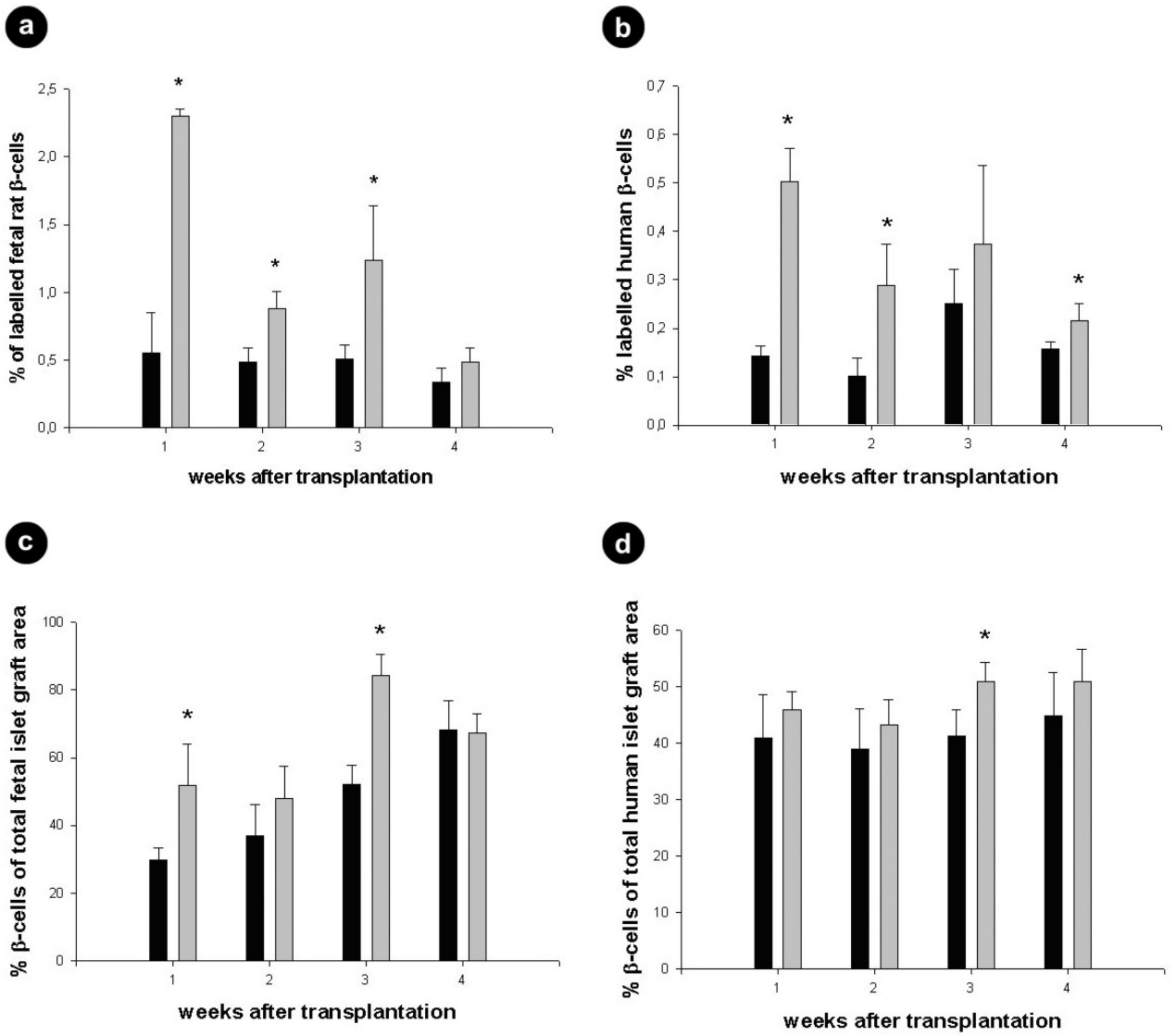

Figure 4. The labeling index as calculated by the percentage of labeled beta-cells in fetal rat islet transplants (**a.** n=5) and human islet transplants (**b.** n=4). The percentage of beta-cells of the total graft area as determined using insulin immunostaining and a MOP-screen connected to a Leitz microscope in fetal rat islet transplants (**c.** n=5) and human islet transplants (**d.** n=4).
Control mice (black bars) and hGH-treated animals (grey bars).
* P<0.05. Data were analyzed using two-way ANOVA and Bonferroni as a post-hoc test.

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