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ORIGINAL ARTICLE

Glucocorticoids reduce pro-inflammatory cytokines and tissue factor *in vitro* and improve function of transplanted human islets *in vivo*

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Summary

Factors that upregulate the inflammatory status of islets probably contribute to detrimental processes leading to islet loss and impaired post-transplant function. Glucocorticoids have the potential to counteract inflammation and thus improve islet quality and function. However, glucocorticoids have diabetogenic properties and are known to hamper islet function *in vivo*. We examined the effect of glucocorticoids on human islets *in vitro* and *in vivo* after 48 h of exposure to different concentrations of methylprednisolone. Protein and/or mRNA levels of insulin, interleukin (IL)-8, macrophage chemoattractant protein (MCP)-1, tissue factor (TF), and IL-10 were assessed by enzyme immunosorbent assay and real time quantitative reverse transcription-polymerase chain reaction. Viability was assessed with fluorescein diacetate–propidium iodide staining, adenosine triphosphate (ATP) content and caspase activity. Six-hundred islet equivalents (IEQ) were transplanted to severe combined immunodeficiency disease mice and graft function assessed by glucose measurements and intraperitoneal glucose tolerance tests. Glucocorticoids reduce mRNA and protein levels of TF, MCP-1 and IL-8, and enhance ATP content. Insulin secretion was initially inhibited; however, after 7 days in culture, it was superior to controls. Islets exposed to methylprednisolone cured diabetic mice more effectively than control islets. In conclusion, glucocorticoids have potent anti-inflammatory properties on human islets without permanent effects on insulin metabolism. Brief glucocorticoid exposure improves function of transplanted human islets *in vivo*.

Introduction

For patients with unstable type 1 diabetes, episodes of unawareness of hypoglycemia and poor metabolic control, islet transplantation has shown to induce a metabolic stability superior to that of exogenous injected insulin [1]. With the introduction of a glucocorticoid-free immunosuppressive regimen by the Edmonton group, repeated islet transplantations regularly lead to insulin-independence [2]. Disappointingly, sustained metabolic control after islet transplantation has been difficult to achieve for the majority of patients [3].

It is well established that shortly after intraportal islet transplantation a large part of the islets are destroyed because of islet-blood interactions [4–6]. Functional and experimental studies suggest that the pretransplant inflammatory status of islets are involved and reinforces such reactions [7–9].

Factors such as brain death, ischemia and islet isolation have been shown to upregulate inflammatory mediators on islets and thus may contribute to a detrimental process leading to islet loss and impairment [10–12]. Currently, deceased donors remain the main source for islet replacement. Donor characteristics such as medical history, age,

length of hospitalization, and cause of death are known factors for transplant outcome. Brain death induces multiple interrelated events including bursts of pro-inflammatory mediators from numerous cell types [7–12]. Several studies have demonstrated that such inflammatory mediators are associated with a significant reduction in islet yield after isolation and impaired islet function *in vivo* and *in vitro* [7,10,13,14]. It is therefore conceivable to postulate that prevention or reduction of deleterious effects of brain death would mitigate islet loss and improve islet engraftment and survival after transplantation.

Most centers culture islets for 48 h prior to transplantation. This allows pretransplant quality evaluation of the islets and could represent a golden time period for *ex vivo* manipulation of islets prior to transplantation for optimal engraftment. Different strategies for reducing pro-inflammatory cytokines and immunogenicity have been discussed [11,15–19].

Glucocorticoids possess strong anti-inflammatory effects by inhibition of pro-inflammatory cytokines and mediators by gene repression and induction [20]. As such, glucocorticoids could have the potential to reduce the expression of pro-inflammatory cytokines following brain death, during islet culture and in islet transplantation, thus improve islet quality and function. However, glucocorticoids also have diabetogenic properties and are known to hamper islet function *in vivo* and to induce insulin resistance [21–25].

This study aimed to investigate the hypothesis that the glucocorticoid methylprednisolone is efficient in reducing the inflammatory status of human islets and thus has the potential to improve graft function following islet transplantation. As glucocorticoids have known diabetogenic properties which could counteract positive effects on inflammation, a secondary aim was to explore the direct effect of glucocorticoids on insulin metabolism and viability in human islets.

We exposed human islets for 48 h *in vitro* with increasing concentrations of methylprednisolone and studied the effect on the expression and secretion of the pro-inflammatory cytokines macrophage chemoattractant protein (MCP)-1 and interleukin (IL)-8 from human islets, as well as the expression and content of tissue factor (TF). We also studied the effect on insulin secretion, both dynamically and statically. To study *in vivo* function, human islets were transplanted to severe combined immunodeficiency disease (SCID) mice.

Materials and methods

Islet isolation

Following consent obtained from the organ donor registry or relatives, pancreata from deceased donors were transported from the donor hospital to the laboratory for islet isolation in Oslo or Uppsala, using the two-layer method of Kuroda *et al.* [26]. Islets were isolated according to the automated method, refined by the Nordic Network for Islet Transplantation [27,28]. Briefly, pancreata were digested using a Liberase^{TM} (Roche, Roche Diagnostics, Indianapolis, IN, USA) perfusion followed by continuous-density Ficoll gradient purification in a refrigerated COBE 2991 centrifuge (COBE Blood Component Technology, Lakewood, CO, USA). Islet preparations were maintained in culture medium (CMRL 1066; ICN Biomedicals, Costa Mesa, CA, USA) supplemented with 10% ABO-identical serum or fetal calf serum (Invitrogen, Carlsbad, CA, USA), HEPES, L-glutamine, gentamicin, fungizone (Gibco, Invitrogen, Paisley, Scotland, UK), and ciprofloxacin (Bayer Healthcare, Leverkusen, Germany) at 37 °C (5% CO_2) for 2–7 days. The volume and purity were determined by microscopic sizing after staining with dithizone (Sigma-Aldrich, St Louis, MO, USA), and viability was assessed as insulin secretion in response to glucose challenge in either a static or dynamic perfusion system (see below).

Glucocorticoid exposure to human islets

Human islets were collected immediately after the isolation procedure and cultured with or without different concentrations of the glucocorticoid methylprednisolone (Solu-Medrol®; Pfizer, Oslo, Norway) for 48 h. In some experiments, the islets were subsequently washed twice, and culture was continued for 5 days. Control islets were given vehicle (0.9% NaCl in sterile water). In all experiments, cell pellets and cell-free supernatants were harvested and stored at indicated time points at −70 °C until further analysis.

Measurements of insulin secretion by static and dynamic challenge with glucose

For static insulin secretion in response to a glucose challenger, approximately 20 islets were handpicked and transferred into 24-transwell trays (Costar, Cambridge, MA, USA) containing Krebs–Ringer bicarbonate buffer (11.5 mM NaCl, 0.5 mM KCl, 2.4 mM $NaHCO_3$, 2.2 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES and 2 mg/l Albumin) and preincubated in 1.67 mM glucose at 37 °C (5% CO_2) for 30 min before the islets were incubated for 1 h in 1.67 mM glucose (basal), and then incubated for 1 h in 20.0 mM glucose (stimulated). Cell pellets and supernatants were collected at indicated time points and stored at −20 °C until analyzed. For dynamic insulin secretion, 15 islets were handpicked and added to each of six perfusion chambers, layered between inert polystyrene beads. A Krebs–Ringer bicarbonate buffer was used at a flow rate

of 0.2 ml/min. The perfusion system was initiated after a 30-min equilibration period in 1.67 mM glucose, followed by a 60-min stimulation period with 20 mM glucose and finally back to 1.67 mM glucose. Samples were collected at 6-min intervals, immediately ice-chilled, and stored at −20 °C until analyzed.

Enzyme immunoassays

Cell-bound TF protein in islet cells were measured using enzyme immunosorbent assays (EIA) (American Diagnostic, Greenwich, CT, USA) after lysing the cells completely with 1% Triton X-100. The insulin content after static and dynamic challenge with glucose was measured by EIA (Mercodia AB, Uppsala, Sweden). The insulin and TF content was normalized by the total cellular DNA content in lysed islets measured using PicoGreen® dsDNA Quantitation Kit (Molecular Probes, Eugene, OR, USA). The amount of IL-8 and MCP-1 was assayed in cell-free supernatants by EIA (R&D systems, Minneapolis, MN, USA).

Real time quantitative reverse transcription-polymerase chain reaction

Total RNA was isolated from frozen islet pellets with RNA Isolation Kit High performance using the MagNA Pure LC instrument (Roche Applied Science, Indianapolis, IN, USA) according to the manufacturer's description, and stored at −80 °C until further analysis. cDNA from total RNA was synthesized using the cDNA High Capacity Kit (Applied Biosystems, Foster City, CA, USA). Primers were designed using the Primer Express software, version 2.0 (Applied Biosystems). Primers sequences can be provided upon request. Real time quantitative reverse transcription-polymerase chain reaction (RT-qPCR) using sequence-specific probes were performed on an ABI PRISM 7000 sequence detector using SyBr Green assay I (Eurogentec, Seraing, Belgium) according to manufacturer's instructions. The specificity of the SyBr Green assays was confirmed by melting point analysis and gel electrophoresis. Gene expression of the housekeeping gene GAPDH was used for normalization.

Fluorescein diacetate–propidium iodide viability staining

Small aliquot of islets was transferred in phosphate-buffered saline (PBS) to 10 × 35 mm counting Petri dishes. Fluorescein diacetate (FDA) and propidium iodide (PI) were added to the samples at a final concentration of 0.67 and 75 μM, respectively. Using a fluorescent microscope, 50 islets were then assessed for cell viability by estimating the percentage of viable cells (green) versus the percentage of nonviable cells (red) within each islet [29]. The percentage of viable cells was then calculated.

Mitochondrial function

Intracellular adenosine triphosphate (ATP) content was measured using a CellTiter-Glo® (Promega, Madison, WI, USA) kit according to the manufacturer's description. For functional analysis, an XTT assay (Roche, Mannheim, Germany) was performed. Briefly, cells were cultured for 48 h, and during the last 6 h co-cultured with 75 μl of XTT-labeling reagent (Boehringer Mannheim, Mannheim, Germany; final concentration 0.3 mg/ml). The colored complex of formazan was monitored spectrophotometrically, significantly correlating with the overall mitochondrial dehydrogenase activity [30].

Caspase activity

Caspase 3 and 7 activity was assessed using Caspase-Glo® 3/7 assay (Promega) following the manufacturer's descriptions.

Mouse preparation and islet transplantation

Male inbred SCID (ICR-PRKDC SCID) mice (weighing 25 g; Taconic M&B, Ry, Denmark) were used as recipients. Diabetes was induced by two consecutive intraperitoneal injections of streptozotocin (Sigma-Aldrich), each at 250 mg/kg body weight. An animal was considered diabetic if its blood glucose level exceeded 20 mmol/l (>360 mg/dl) for two or more consecutive days. Three independent human islet preparations were used for transplantation. Islets from each preparation were divided into two groups, and methylprednisolone (1000 ng/mg) or vehicle (0.9% NaCl in sterile water) was added to the culture medium. Islets were cultured for 48 h prior to transplantation. Two animals were transplanted in each group per islet preparation. After culture, batches of 600 IEQ human islets were washed twice in transplant medium (Ringer acetate with 5% glucose and 20% HSA), and transplanted under the left kidney capsule. The animal experiments and housing were in accordance with institutional guidelines and national legislation conforming to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996).

Definition of metabolic control

On follow-up, sustained nonfasting blood glucose levels of ≤11 mmol/l were defined as cure, 11–20 mmol/l as

partial function of transplanted islets and levels above 20 mmol/l as graft failure.

Post-transplant follow-up

The mice were observed for 4 weeks. Animals were divided into two groups: (i) 600 IEQ of human islets exposed to culture medium containing glucocorticoids (methylprednisolone, 1000 ng/ml) and (ii) 600 IEQ of human islets exposed to culture medium containing vehicle (controls). The nonfasting blood glucose levels of the animals were measured twice a week during the first week. Thereafter, blood glucose levels were assessed once a week. At 4 weeks, cured mice were subjected to an intraperitoneal glucose tolerance test (IPGTT). Two to three days later, grafts were removed to confirm the recurrence of diabetes.

Intraperitoneal glucose tolerance test

Mice were fasted for at least 6 h before examination. A 20% glucose solution was given at 10 μl/g i.p. and blood glucose was measured before injection and 15, 30, 45, 60, 90, and 120 min after injection of the glucose solution. Nontransplanted mice were used as controls and tested at the same time.

Statistical analysis

Data are presented as mean ± SEM. Statistical significance was determined using one-way analysis of variance (ANOVA) with *post hoc* analysis using Tukey's comparisons. For comparison of two groups a two-tailed *t*-test was used. Kaplan–Meier analysis with log-rank test was used to compare cumulative proportion of animals reaching normoglycemia. Probability values were considered significant at a level of $P < 0.05$.

Results

Effects of glucocorticoids on glucose-stimulated insulin secretion

In a static model of glucose-stimulated insulin secretion, 48 h of methylprednisolone exposure resulted in a dose-dependent inhibition of insulin secretion after 1 h in high glucose medium (20.0 mM) reaching 59 ± 8% compared to control ($n = 6$, $P < 0.01$) (Fig. 1a). There was no significant difference between the groups in insulin secretion after 1 h in low glucose medium (1.67 mM, data not shown). Samples taken from culture media at day 0 and 2 from high dosage methylprednisolone and controls

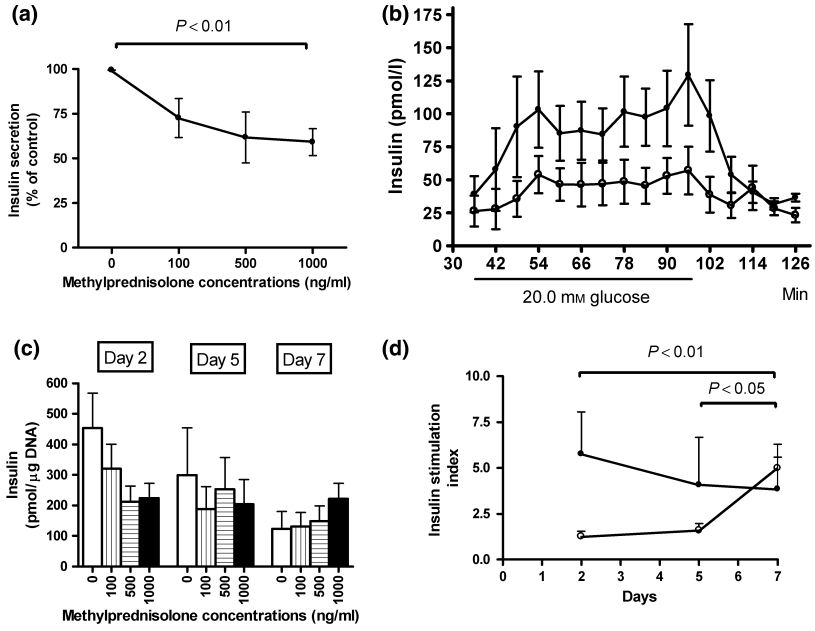

Figure 1 Effects of 48-h methylprednisolone exposure on glucose-stimulated insulin secretion *in vitro*. (a) Insulin secretion, measured by EIA after 1 h in high glucose (20.0 mM glucose) medium. (b) Dynamic insulin release. Islets ($n = 15$) were perfused with 1.67 mM glucose and 20.0 mM glucose (duration indicated by the line beneath the *X*-axis). (•) Control islets and (○) methylprednisolone (1000 ng/ml) exposed islets. (c) Islets were cultured for 2 days in medium supplemented with methylprednisolone, and 5 days in methylprednisolone-free media and insulin secretion was measured in a static model for insulin secretion on respectively day 2, 5 and 7. The figure depicts insulin secretion measured by EIA after 1 h in high glucose medium. (d) Insulin stimulation index (calculated as stimulatory/basal insulin secretion in a static model for insulin secretion) during the 5-day period after methylprednisolone removal. (•) Control islets and (○) methylprednisolone (1000 ng/ml) exposed islets. Data are presented as mean values ± SEM. The experiment was conducted on six pancreata from independent donors.

showed similar increases of insulin released into the culture medium (73 ± 25% vs. 62 ± 35%, $n = 3$, $P = 0.82$).

The methylprednisolone-induced reduction of insulin secretion was accompanied by a significant increase in intracellular insulin content (reaching 377 ± 82% compared to control at 1000 ng/ml of methylprednisolone, measured after 1 h in high glucose medium, $n = 6$, $P < 0.05$).

To assess the dynamics of the reduced insulin secretion in human islets exposed to methylprednisolone, we analyzed the insulin secretion using a dynamic perfusion model (see Methods). As shown in Fig. 1b, methylprednisolone (1000 ng/ml) inhibited the insulin secretory capacity, but the pattern of insulin secretion observed in control islets was retained, at lower amplitude. The total amount of insulin secreted, calculated as area under curve (AUC), for methylprednisolone exposed islets was reduced to 59 ± 10% of control ($n = 6$, $P < 0.01$).

Subsequently, islets were cultured without methylprednisolone for an additional 5 days. A marked reduction in glucose-stimulated insulin secretion for the control islets was observed, deteriorating from 453 ± 115 pmol/μg DNA 48 h after isolation, to 123 ± 57 pmol/μg DNA 7 days after islet isolation (Fig. 1c). Islets exposed to methylprednisolone (1000 ng/ml) secreted 224 ± 47 pmol/μg DNA 48 h after isolation, a level which remained virtually unchanged throughout this time-period (222 ± 51 pmol/μg DNA at day 7). Calculated as percentage of control, islets exposed to methylprednisolone (1000 ng/ml) performed significantly better at day 7 (216 ± 51% compared to control, $n = 6$, $P < 0.05$).

Using stimulation index (glucose-stimulated/basal insulin secretion), we noticed that while the control islets showed a steady deterioration, the glucocorticoid-exposed islets had a significant improvement, from 1.2 ± 0.3 on day 2 to 5 ± 1.3 on day 7 ($n = 6$, $P < 0.01$) (Fig. 1d). The improved stimulation index was because of a decrease in the basal insulin secretion; however, there were no difference in the basal insulin secretion between the groups at the different time points [166 ± 53 vs. 173 ± 37 pmol insulin/μg DNA at day 2, and 55 ± 17 vs. 53 ± 13 pmol insulin/μg DNA at day 7, for methylprednisolone (1000 ng/ml) exposed islets and controls, respectively]. Most of the improvement occurred between days 5 and 7 (thus between 3 and 5 days after cessation of exposure to methylprednisolone) ($n = 6$, $P < 0.05$) (Fig. 1d).

The intracellular insulin content after 1 h in high glucose medium for islets exposed to methylprednisolone (1000 ng/ml), which we found to be significantly higher 48 h after isolation, normalized by day 7 (107 ± 8% of controls).

Glucocorticoids added to culture medium reduce TF and pro-inflammatory cytokines in human islets

As shown in Fig. 2, the transcription of TF (a), MCP-1 (c) and IL-8 (e) were significantly reduced in a dose-dependent manner, after exposure to increasing concentrations of methylprednisolone for 48 h ($n = 6$). The methylprednisolone-induced reduction in transcription was confirmed at the protein level assessed by EIA, which showed significantly reduced protein levels of TF (b), MCP-1 (d) and IL-8 (f) compared to controls after 48 h in culture. To assess the inflammatory profile of islets in culture, and concomitantly study the effect of glucocorticoid withdrawal, we subsequently removed methylprednisolone from the culture medium, and analyzed TF content immediately, 3, 5 and 7 days after drug removal. The intracellular concentrations of TF rose until day 3, and then decreased throughout the rest of the period. The concentrations of TF for methylprednisolone (1000 ng/ml) exposed islets remained lower than controls during this whole period, being 424 ± 72 vs. 814 ± 42 pg/ng DNA immediately after drug removal ($P < 0.001$), 913 ± 56 vs. 1222 ± 73 pg/ng DNA at day 3 ($P < 0.01$), 703 ± 74 vs. 872 ± 103 pg/ng DNA at day 5 (NS) and finally 577 ± 86 vs. 688 ± 102 pg/ng DNA at day 7 (NS) ($n = 6$). High glucose (20.0 mM) in the culture medium abrogated the anti-inflammatory effect of methylprednisolone and resulted in increase in TF expression to 155 ± 27% of control after 48 h in culture ($n = 3$).

Effects of glucocorticoids on human islet viability

Forty human islets ($n = 5$) were counted prior to and after culture with or without methylprednisolone (1000 ng/ml) for 48 h. We observed a similar decrease of islets in both groups (methylprednisolone exposed islets 6.1 ± 1.3% reduction and control islets 9.5 ± 0.9% reduction) during the culture period. After counting, islets were lyzed, and DNA content was determined. The DNA content was the same in both groups (1.31 ± 0.04 and 1.29 ± 0.04 μg DNA, for methylprednisolone exposed islets and controls, respectively, $n = 5$) (Fig. 3a). Furthermore, we evaluated islets by fluorescent microscopy using FDA/PI staining, and observed no differences in islet viability between the groups after 48 h in culture [85 ± 5% for methylprednisolone (1000 ng/ml) exposed islets, 87 ± 6% for control islets] (Fig. 3b). At the same time-point, islets were lyzed and intracellular ATP content was measured. Exposure to 1000 ng/ml of methylprednisolone for 48 h increased the intracellular ATP content slightly to 138 ± 11% of control ($n = 4$, $P < 0.05$) (Fig. 3c). Moreover, methylprednisolone tended to increase mito-

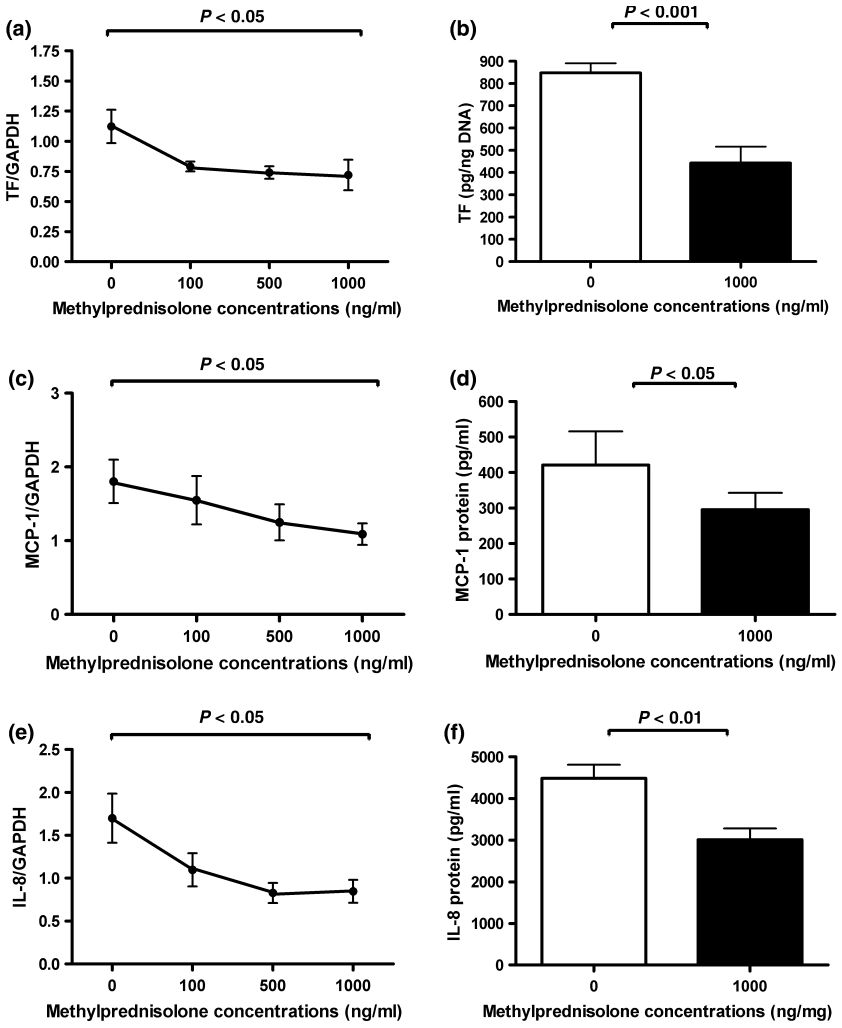

Figure 2 Effects of 48-h methylprednisolone exposure on mRNA and protein levels of TF, MCP-1 and IL-8 in human islets. (a) TF expression, measured by RT-qPCR. (b) TF in cell lysate, measured by EIA. (c) MCP-1 expression. (d) Level of MCP-1 protein in supernatant, measured by EIA. (e) IL-8 expression. (f) IL-8 protein in supernatant, measured by EIA. Data are presented as mean values ± SEM. The experiment was conducted on six pancreata from independent donors.

chondrial dehydrogenase activity (112 ± 7% of control, $n = 4$, $P = 0.11$). In contrast, no differences in the mRNA expression of either caspase-3 (79 ± 18% of control, $n = 6$, $P = 0.66$) or caspase-8 (95 ± 8% of control, $n = 6$, $P = 0.94$) were seen between the islets cultured with methylprednisolone (1000 ng/ml) for 48 h compared to control. These results were accompanied by no differences in caspase 3 and 7 activity (93 ± 14% activity compared to controls, $n = 3$). However, the mRNA expression of the anti-inflammatory cytokine IL-10 was significantly enhanced ($n = 6$, $P < 0.05$) (Fig. 3d).

Glucocorticoid exposed islets perform better *in vivo*

Two animals, one in each group, died because of technical failure. Consistently between islet preparations, animals receiving methylprednisolone exposed islets performed better. When evaluated at 4 weeks, 4/5 animals that received islets exposed to methylprednisolone (1000 ng/ml) were cured compared with 1/5 in the control group (Log-rank test, $P = 0.04$) (Fig. 4a). Blood sugar levels during the follow-up period indicated partial function in one and two animals in the methylprednisolone and control group, respectively (data not shown). Cured animals in the methylprednisolone group showed slightly elevated responses in the IPGTT, comparable to that of normal healthy mice (Fig. 4b). However, glucose levels recovered to normal values within 60 min after injection of glucose.

Discussion

The long-term efficacy of clinical islet transplantation is rather low. Therefore, new insight and re-evaluation of different aspects of islet transplantation could be useful for a further progress in the field. Factors that increase expression of pro-inflammatory genes and cytokines and thus upregulate the inflammatory status of islets such

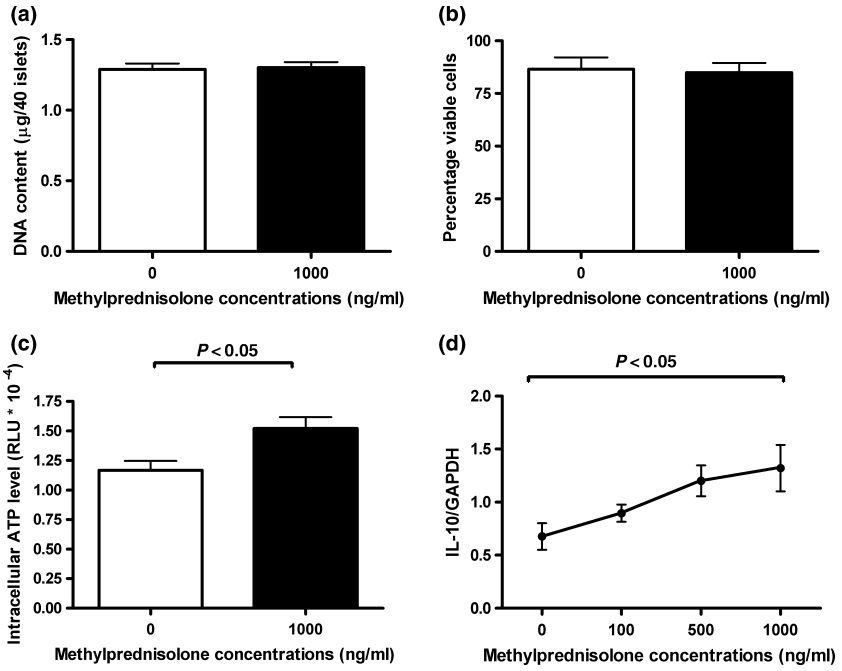

Figure 3 Effects of 48-h methylprednisolone exposure on islet viability. (a) DNA content of 40 control islets and 40 methylprednisolone (1000 ng/ml) exposed islets (n = 5). (b) Calculated percentage viable cells in aliquots (n = 7) of control islets and methylprednisolone (1000 ng/ml) exposed islets, by fluorescent microscopy using propidium iodide and fluorescein diacetate dyes. (c) ATP content in 30 handpicked and lyzed islets, measured by a bioassay (n = 4). (d) IL-10 expression, measured by RT-qPCR (n = 6). Data are presented as mean values ± SEM.

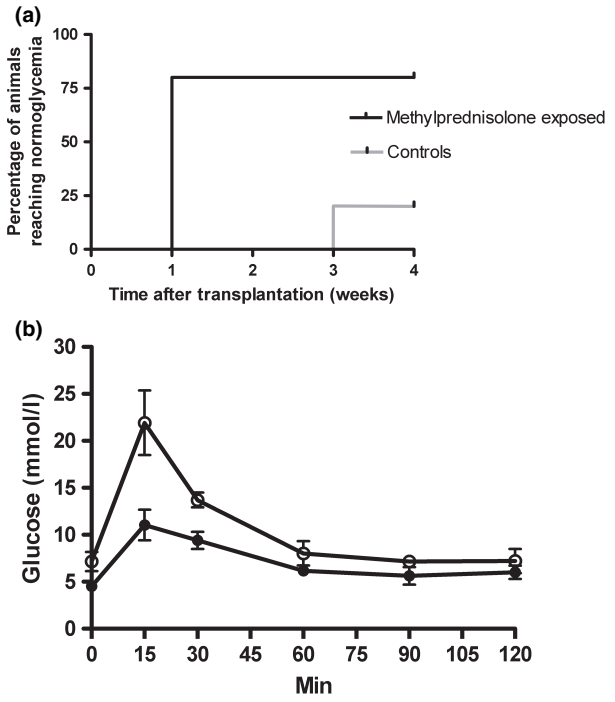

Figure 4 Graft function and IPGTT response of human islets (600 IEQ) transplanted under the left kidney capsule of diabetic SCID mice. (a) Kaplan–Meier analysis of cumulative proportion of animals reaching normoglycemia. Grey line: animals receiving control islets (n = 5), black line: animals receiving islets exposed to methylprednisolone (1000 ng/ml) (n = 5). (b) IPGTT (see Materials and methods) response of cured animals receiving methylprednisolone exposed islets (○) (n = 4), 4 weeks after transplantation compared to healthy, nontransplanted controls (•) (n = 4).

as brain death, hypoxia and islet isolation probably contribute to detrimental processes leading to islet loss and impaired post-transplant function. It is known that abnormal physiologic events and oxidative stress that occurs during brain death, organ retrieval, islet isolation and transplantation are detrimental to islet viability and function and several studies have shown that islets are particularly sensitive to pro-inflammatory cytokines [31,32]. Strategies capable of hindering such mechanisms will probably enhance islet quality and function.

The strong anti-inflammatory effects of glucocorticoids do have the potential to counteract inflammation and thus improve islet quality and function. However, glucocorticoids stimulate gluconeogenesis, promote peripheral insulin resistance and chronic administration is associated with diabetes and other side effects [21]. Therefore, logically, a key element for success of the Edmonton protocol was explained by avoidance of glucocorticoids as chronic immunosuppression [2].

Our data show that glucocorticoids added to human islets *in vitro* inhibits insulin secretion, but this inhibition was accompanied by an increase in the intracellular insulin content. This is consistent with the hypothesis that glucocorticoids predominantly affect secretion of insulin, rather than transcription [33]. In addition, the magnitude, but not the pattern of insulin secretion was affected by glucocorticoids. A gradual improvement in islet function measured by glucose-stimulated insulin secretion and stimulation index was observed over time.

After a total of 7 days in culture (5 days after discontinuation of glucocorticoids), islets that had been exposed to glucocorticoids performed significantly better than controls with respect to stimulated insulin secretion. Taken together, these findings may suggest that the glucocorticoid-induced reduction in insulin secretion does not represent a durable detrimental effect on insulin metabolism.

The study further shows that glucocorticoids added to human islets *in vitro* significantly suppress the expression of TF as well as MCP-1 and IL-8, on both mRNA and protein levels, illustrating a potent anti-inflammatory effect exerted by glucocorticoids on human islets. This anti-inflammatory effect was significant up to 3 days after discontinuation of glucocorticoid-exposure (using TF as a marker of inflammation).

We were not able to identify any detrimental effects of brief (48 h) glucocorticoid exposure on human islets *in vitro* after assessing caspase activity, DNA and ATP content, mitochondrial dehydrogenase activity and FDA/PI staining. This is in contrast with the work performed by Ranta *et al.* [34], where they found that dexamethasone induces apoptosis in murine islets. A possible explanation may be species specific differences in the affinity of the glucocorticoid receptor [35,36]. It has been shown that murine lymphocytes are more sensitive to prednisolone action than human [37]. Another possible explanation may be the different glucose concentrations utilized (5 mmol/l in our experiment, vs. 11 mmol/l in the work by Ranta *et al.*). In accordance with this, we found the anti-inflammatory effect of glucocorticoids to be abrogated by increasing the glucose concentration in the culture medium to 20 mmol/l.

In our hands, glucocorticoid exposure slightly increased the ATP content of human islets. We also observed a significant enhancement in the expression of the immunomodulatory cytokine IL-10. IL-10 is known to be secreted by macrophages, dendritic- and T-regulatory cells [38], and we speculate that the increase in IL-10 expression may originate from passenger leucocytes within the purified islet preparations, as previous studies have shown that glucocorticoids induce IL-10 production in leucocytes [39–41]. An important role for passenger leucocytes in islet transplantation has recently been suggested [42], and the glucocorticoid-induced IL-10 expression may, through induction of T-regulatory cells, have positive effects on the immune response induced by the transplanted islets [43,44].

We found that glucocorticoid exposed islets cured diabetes more effectively when transplanted under the kidney capsule of SCID mice. Four weeks after transplantation, 4/5 animals in the methylprednisolone were cured, compared to 1/5 in the control group. As we only observed modest positive effects on islet viability, we conclude that the indirect effects of glucocorticoids in reducing the production of inflammatory cytokines may be the main reason for the improved function *in vivo*. Given the decreased stimulated insulin secretion of methylprednisolone islets, this finding may seem puzzling. However, our data indicate that the methylprednisolone-induced reduction in insulin secretion is temporary: the stimulatory capacity was increased in the methylprednisolone group after 1 week in culture (5 days after steroid removal from culture medium). The data may even suggest that inhibition of insulin secretion from islets in the immediate post-transplant period, prior to revascularization at the implantation site may be beneficial.

It could be argued that methylprednisolone eliminates less viable islets, and therefore more potent islets were picked for transplantation in the methylprednisolone group. However, we find this unlikely for several reasons. First, methylprednisolone did not influence manual islet count or DNA content after culture for 48 h. Second, neither caspase 3, 7 or 8 activity was influenced by methylprednisolone. Third, FDA/PI staining showed similar amount of apoptotic islets in the groups. Fourth, we found a slightly elevated ATP content in methylprednisolone exposed islets.

In conclusion, these data may suggest that pretransplant glucocorticoid conditioning of human islets may represent a strategy for reducing inflammation in the transplant, with beneficial effects on engraftment.

As a consequence, we have introduced glucocorticoid preconditioning of the islet preparation prior to clinical transplantation in our center. In addition, currently, all organ donors are given 0.5 g methylprednisolone prior to procurement, and we are exploring single dose administration of glucocorticoids to the recipient at the time of islet transplantation. However, glucocorticoid administration to the recipient complicates glycemic control in the initial post-transplant phase (data not shown), which taken together with the observed initial inhibition of insulin secretion from islets and known unfavorable effects of glucocorticoids imply the necessity of further studies before glucocorticoids can be used in clinical islet transplantation.

Authorship

TL: designed, performed and analyzed the research, and wrote the paper. BF: performed research. OK: designed research, contributed reagents and helped write the paper. HS: performed research and analyzed data. AF: designed research and contributed substantially in writing the paper.

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