“Function of isolated pancreatic islets from patients at onset of type 1 diabetes; insulin secretion can be restored after some days in a non-diabetogenic environment in vitro. Results from the DiViD study”

Short running title: “In vitro studies of islets from type 1 diabetes patients”

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Word count, manuscript: 2024

Number of tables: 0 (1 online supplemental table)
Number of figures: 3 (1 online supplemental figure)
Abstract:

The understanding of the etiology of type 1 diabetes (T1D) remains limited. One objective of the Diabetes Virus Detection-study (DiViD) was to collect pancreatic tissue from living subjects shortly after the diagnosis of T1D. Here we report the insulin secretion ability by in vitro glucose perifusion and explore the expression of insulin pathway genes in isolated islets of Langerhans from these patients. Whole genome RNA sequencing was performed on islets from six DiViD patients and two organ donors that died at the onset of T1D and compared with findings in three non-diabetic organ donors. All human transcripts involved in the insulin pathway were present in the islets at onset of T1D. Glucose-induced insulin secretion was present in some patients at the onset of T1D, and a perfectly normalized biphasic insulin release was obtained after some days in a non-diabetogenic environment in vitro. This indicates that the potential for endogenous insulin production is good, which could be taken advantage of if the disease process was reversed at diagnosis.
Our understanding of the etiology of type 1 diabetes remains limited (1). Animal models show only partial similarities with the human disease (2), and there has been lack of well-preserved human tissue samples (3). The functionality of islets of Langerhans at onset of type 1 diabetes in humans remains poorly characterized. Previous in vitro studies (4;5) have shown function of islet cells obtained several months after diagnosis but so far there has been lack of in vitro access to isolated islets obtained from subjects at onset of type 1 diabetes required to obtain in-depth understanding. Studies demonstrating the gene expression profiles for the human pancreas and purified islets in type 1 diabetes have been published, providing interesting data supporting that type 1 diabetes is caused by a chronic inflammatory process with participation of innate immunity (6;7). The beta cells express and release cytokines and chemokines, providing a link between the beta cell and the immune system in early type 1 diabetes (8). However, these studies are based on few diseased cases, mainly including tissue from subjects with long-standing type 1 diabetes (6;7).

Insulin secretion from islets obtained from non-diabetic human subjects exhibits a typical bi-phasic pattern when stimulated with glucose (9). At diagnosis most patients with type 1 diabetes have significant, but insufficient insulin secretion, even though 40-50% of the β-cells may be present (10) suggesting beta cell dysfunction (11;12). Endoplasmic reticulum (ER) stress causes beta cell dysfunction, suggestively by entrapment of the protein Wolfram syndrome 1 (WFS1), inhibiting the synthesis of cAMP and thereby the secretion of insulin (13).
Here we report the ability of insulin secretion and explore the expression of insulin pathway genes in isolated islets of Langerhans obtained from subjects with recent onset type 1 diabetes and non-diabetic controls.

Materials and Methods

Patients and pancreas donors

Six patients (case 1-6), age 24-35 years who gave their written informed consent were recruited to the DiViD-study (14). In addition, the pancreases from two organ donors (case 7-8) who died at the onset of type 1 diabetes and from three organ donors (controls 1-3) without pancreatic disease were included in the study. Both donors with type 1 diabetes died of brain edema and total brain infarction described previously (15). Clinical data regarding cases and controls are shown in eTable 1. The Government’s Regional Ethics Committee in Norway and the Regional Ethics Committee in Uppsala approved the study. For details regarding the methods, see supplementary material.

Results

Islet function

The mean glucose-stimulated insulin secretion (GSIS) was reduced in islets from type 1 diabetes subjects (Fig. 1A). GSIS was lowest when islets were examined day 1 after isolation but seemed to increase after 3 and 6 days of in vitro culture (Fig. 1A). Islets from individual subjects with type 1 diabetes had varying levels of GSIS (Fig. 1B). When examined day 1 after isolation, islets from all subjects except case 6 had a very low or undetectable GSIS (Fig. 1B). After 3 and 6 days of culture, islets from two of the subjects (Case 1 and 2) secreted slightly increased amount of insulin upon glucose
stimulation, but did not display biphasic insulin secretion, whereas islets from case 4 that displayed a poor GSIS one day after isolation, recovered to a normal biphasic secretion after 3 and 6 days of culture. Case 6 displayed a close-to-normal GSIS already one day after isolation, and the insulin secretion levels increased further after 3 and 6 days of culture (Fig. 1B). For the rest of the cases (3, 5, 7 and 8) the insulin secretion remained low or undetectable after culturing. Islets from non-diabetic subjects responded with a biphasic insulin release already on day one and were not further stimulated.

*Whole transcriptome sequencing*

We generated a total of 362 million reads and mapped those to the human genome. Both cases and controls generated approximately the same amount of mapable reads. The full data set (reads) is openly available on doi: 10.17044/BILS/g000002.

When comparing expression similarities, using RPKM values, across all genes, islets from 5 of the 6 live subjects (Case 1-5, but not case 6) are grouped together and separate from the brain dead donors (case 7 and 8 and control 1-3), regardless of whether the islets are from type 1 diabetes or non-diabetic subjects (Fig. 2A), reflecting the major impact of brain death on the results obtained from the whole transcriptome analysis. Similarly, genes that are part of the complement system pathway also reflect the differences between islets from live and brain dead subjects (Fig. 2B). By contrast, the insulin secretion pathway groups islet samples by type 1 diabetes or not, notably resulting in longer branch lengths compared to the complement system pathway (Fig. 2C).
In all cases, except case 6, the genes that (according to KEGG database) is involved in the secretion of insulin, the insulin pathway, were less expressed when compared to the controls (Fig. 3). That includes all the genes in the insulin pathway, both the insulin gene \textit{INS} itself, and the genes involved in production and release of insulin. In case 6, all the genes in the pathway were up-regulated compared to the non-diabetic controls. \textit{INS} is about ten-fold lower expressed in all other diabetic samples, together with lowered expression of two upstream regulators, the pancreatic and duodenal homeobox 1 (\textit{PDX1}), and \textit{MAFA}, a transcription factor that binds \textit{RIPE3b}, a conserved enhancer element that regulates pancreatic beta cell-specific expression of \textit{INS} (16). Additional genes that are consistently lower in expression include (a) the adenylate cyclase activating polypeptide (\textit{PACAP}) and its receptor \textit{PACAPR}; (b) \textit{FFAR1}, a member of the GPR40 family of G protein-coupled receptors and may be involved in the metabolic regulation of insulin secretion; (c) G-protein controlled integral membrane protein and inward-rectifier type potassium channel (\textit{KCNJ11}); (d) ATP-binding cassette transporter of the MRP subfamily (\textit{ABCC8}), which is involved in multi-drug resistance but also functions as a modulator of ATP-sensitive potassium channels and insulin release; (e) calcium-activated nonselective ion channel that mediates transport of monovalent cations across membranes (\textit{TRPM4}); and (f) three major glucose transporters in the mammalian blood-brain barrier, which are found primarily in the cell membrane and on the cell surface, where they also function as receptors for human T-cell leukemia virus I and II (\textit{GLUT1/2/3}). By contrast, the muscarinic cholinergic receptor \textit{CHRM3}, and the G-protein coupled receptor \textit{CCKAR}, which binds non-sulfated members of the cholecystokinin family of peptide hormones and acts as a major physiologic mediator of pancreatic enzyme secretion, are unchanged in expression, or slightly more highly expressed.
Discussion

The results show that the expression of key regulatory elements, the insulin pathway and the GSIS were reduced in islets isolated from 7 of the 8 subjects with type 1 diabetes compared to islets from non-diabetic organ donors. However, in 1 of the 8 cases, the insulin pathway expression and the GSIS were remarkably preserved already at the day of isolation. The observed GSIS improvement after three and six days of culture in 4 of the 8 cases suggests that when the islets are removed from the ‘diabetic milieu’, remaining beta cells can recover. Soluble factors released within the vicinity of the islets could induce a functional impairment of the beta cells, which could be reversed after islet isolation and in vitro culture. However, a screening of 41 cytokines and chemokines in isolated islets from the six type 1 diabetes patients showed no difference from that in isolated islets from brain dead organ donors (data not shown).

It is known that prolonged exposure of human islets to high glucose impairs beta cell function (17) and since even short episodes of hyperglycemia dramatically affect glucose tolerance (18) it is possible that glucotoxicity could play a role in the reduced GSIS observed in islets from these patients. In 1940 Jackson described that intensive insulin therapy at diagnosis of T1D lead to a long partial remission of the disease (19). Later these findings were confirmed using C-peptide determinations (20). However, in an experimental transplantation model human islets were exposed 4-6 weeks in vivo to hyperglycemia followed by normoglycemia for 2 weeks (21). The results showed that even if the hyperglycemia induced impairment in glucose metabolism, depletion of insulin mRNA, decreased (pro)insulin biosynthesis, increased glycogen
accumulation and depletion of insulin content was reversed by the 2 week period of normoglycemia, the deleterious effects of the diabetic state on human islet insulin release remained.

Endoplasmatic reticulum (ER) stress has been proposed as an important contributor to beta cell dysfunction in type 1 diabetes (22). In this study, the gene expression of ER stress markers (BiP, CHOP, ATF4, and XBP1) was high in islets from all subjects with type 1 diabetes, but did not differ from the islets from the brain dead non-diabetic controls. The process of brain death has been shown to induce ER stress (23) and the finding of a similar level of expression of these genes in islets isolated from subjects with recent onset type 1 diabetes imply that the ER stress in these islets is a feature of type 1 diabetes. The expression of WFS1 was lower in islets from subjects with T1D (both brain-dead and live subjects) than in islets from the control donors, with the exception of case 6 from whom the islets with almost preserved GSIS were isolated. It may be speculated that ER stress and low expression of WFS1 contribute to the reduced beta cell function in T1D.

The level of transcription of the genes in the insulin pathway was reduced in most of the diabetic cases, compared to the non-diabetic controls. However, all genes in the insulin pathway were expressed, also in islets from the subjects with no or very low GSIS. This is in line with earlier studies, describing insulin synthesis and storage remaining in islets even many years after type 1 diabetes onset (6,24), and show that the destruction of the β-cells is a slow ongoing process (24). A limitation in all studies of transcriptome profiling of an unfractioned tissue is that any changes observed can be due to differences in either modulation of gene expression or from changes in its
cell composition. The current analysis was conducted on handpicked islets, thereby avoiding to a large extent the problem with analyzing pancreatic biopsies in which the islets constitute only about 1%. However, the insulin producing beta cells constitute only about 60% of the total cell number in human islets. A reduction in the number of insulin producing cells in the islets isolated from subjects with recent onset type 1 diabetes could be in agreement with the observation of a reduction in the insulin pathway.

The most obvious limitation of the present study is the small number of cases examined. The number of recruited patients was limited by the unexpectedly high frequency of complications arising from the biopsy procedure (14). Therefore we considered it unethical to continue to enrol patients. A further important consideration is the degree of matching between the tissues obtained from the patients with T1D and those used as controls. This study did not include pancreatic biopsies from healthy individuals and we therefore chose to employ tissue harvested by alternative methods. Non-diabetic organ donors were chosen as their clinical characteristics are well defined, all having normal HbA1c and being negative for 4 auto antibodies. The islets from the controls were not cultured and stimulated with glucose on day 3 and 6, but it has previously been shown that culturing of normal beta cells does not improve their insulin secretion ability (25). Due to the limited number of islets available from each of the cases, we chose to stimulate with glucose only, knowing that additional secretagogues would potentially provide more complete information.

In summary, our findings illustrate the importance of β-cell dysfunction, and not only loss of number of insulin producing cells, at onset of type 1 diabetes. The restoration
of specific function of the isolated islets removed from the diabetogenic milieu should encourage further characterization of the underlying mechanism(s) of this functional impairment. Hopefully, this would allow initiation of clinical intervention trials specifically aiming to restore beta cell function alone or combined with drugs targeting the injurious processes ongoing within the pancreas at onset of type 1 diabetes.

**Acknowledgements:** The project was founded by South-Eastern Norway Regional Health Authority (Grant to KDJ), The Novo Nordisk Foundation (Grant to KDJ and OK), through the PEVNET Study Group founded by the European Union's Seventh Framework Programme [FP7/2007-2013] under grant agreement n°261441 PEVNET (http://www.uta.fi/med/pevnet/publications.html), Barndiabetesfonden (Swedish Child Diabetes Foundation), the Swedish Medical Research Council (65X-12219-15-6, K2015-54X-12219-19-4), Diabetes Wellness Sweden, the Swedish Diabetes Foundation and the Family Ernfors Fund. OK’s position is in part supported by the National Institutes of Health (U01AI065192-06). Human pancreatic islets were obtained from the Nordic Network for Clinical Islet Transplantation supported by the Swedish national strategic research initiative EXODIAB (Excellence of Diabetes Research in Sweden) and the Juvenile Diabetes Research Foundation. The authors would like to acknowledge support of Uppsala Genome Center and UPPMAX for providing assistance in massive parallel sequencing and computational infrastructure. Work performed at Uppsala Genome Center has been funded by RFI/VR “SNISS” Swedish National Infrastructure for large Scale Sequencing and Science for Life Laboratory, Uppsala.
No potential conflict of interest relevant to this article was reported.

LK was responsible for clinical coordination and recruitment of patient, data collection, analysis and interpretation, and drafted the manuscript. OS performed the GSIS analyses, and analysed, interpreted and drafted the manuscript. GS performed the whole transcriptome sequencing analysis and interpretation and drafted the manuscript. BE and TB performed the surgery and participated in writing of the article. KFH, JL, MG and OK contributed to study design, data analysis, interpretation and writing of the manuscript. KDJ was the principal investigator of the study, had the initial idea of the DiViD study, and participated in study design, funding, regulatory issues, international collaboration, data collection, analysis and interpretation, and in writing of the manuscript. LK and KDJ are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

The authors thank specialist nurse Trine Roald, who has provided invaluable efforts in coordination of the study, Sofie Ingvast for excellent technical assistance, nurses and doctors at the local hospitals, providing contact with the patients, and finally the patients who participated in this study.
Figure legends

**FIG. 1. Glucose-stimulated insulin secretion by isolated islets.** Twenty handpicked islets were perifused with low glucose (1.67 mmol/L) for 42 min, high glucose (20 mmol/L) for 48 min, and then low glucose again as indicated. Fractions were collected at 6 min intervals and the secreted insulin was measured by ELISA. A: Mean insulin secretion from islets isolated from six type 1 diabetes patients (case 1-6) and cultured for 1 (open circles), 3 (black squares) or 6 days (open squares), and from islets isolated from 15 organ donors on day one without pancreatic disease (open triangles). B: Individual insulin secretion from islets from two organ donors with type 1 diabetes (case 7-8) and from six live patients with type 1 diabetes (case 1-6), cultured for 1 (open circles), 3 (black squares), or 6 days (open squares). Observational data.

**FIG. 2. Neighbor-joining trees based on expression similarity.** Shown are groupings for all genes (a), the complement system pathway (b), and the insulin secretion pathway (c). With the exception of sample case 6, live and brain dead donors cluster together in (a) and (b), while the insulin secretion pathway (c) splits diabetic from non-diabetic samples.

**FIG. 3. Insulin secretion pathway for all diabetic samples except case 6 (A) and for case 6 (B).** We normalized expression by three brain dead controls and show lower expression in blue and higher expression in red. With the exception of case 6, the majority of genes in the pathway are more lowly expressed in the diabetic samples. Similar figures for the rest of the cases separately are shown in eFigure 1.
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Ref Type: Conference Proceeding

Diabetes

a) All genes

b) Complement system pathway

c) Insulin secretion pathway
ONLINE SUPPLEMENTAL MATERIALS

Function of isolated pancreatic islets from patients at onset of type 1 diabetes; Insulin secretion can be restored after some days in a non-diabetogenic environment in vitro. Results from the DiViD study.

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Islet isolation
The most distal part (0.5-1 cm) of laparoscopic pancreatic tail resections performed at Oslo University Hospital was immediately shipped by air courier in cold organ preservation solution (Viaspan®, UW) to Uppsala University for islet isolation. The islets were isolated by a method based on the procedure used for clinical islet isolation that has been described previously (1). Basically, the pancreatic duct was located under a surgical microscope, cannulated with a fine catheter, and collagenase (Liberase®, Roche) was injected continuously. After about 30 min, the pancreatic tissue was cut into 3–4 pieces, transferred to two 15 mL glass tubes, and digested at 37°C with continuous shaking for 30 min. The digested pancreata was washed twice with cold culture media (CMRL-1066, ICN Biomedicals, Costa Mesa, CA supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 µg/mL gentamycin, 20 µg/mL ciprofloxacin 10 mM Nicotinamide, and 10% heat-inactivated fetal calf serum) and immediately transferred to culture dishes. 300-700 islets from each patient were handpicked from the digested tissue under a microscope by skilled islet technicians with multiple years of experience.
Islets from the brain-dead organ donors were isolated and cultured as described previously (1;2).

Islet function
Glucose-stimulated insulin-secretion (GSIS) was assessed in a dynamic perfusion system, Suprafusion 1000 (BRANDEL, Gaithersburg, MD). Twenty handpicked islets cultured for 1, 3, or 6 days were perifused with low glucose (1.67 mM) for 42 min, high glucose (20 mM) for 48 min, and then low glucose again. Fractions were collected at 6 min intervals and the secreted insulin was measured by ELISA.

Whole transcriptome sequencing and analyses
RNA was extracted with AllPrep DNA/RNA/Protein Mini Kit (Qiagen) from 50-100 islets per subject, immediately after handpicking from the digested pancreatic tail resections, or after storage of isolated islets from multi-organ donors (case 7 and 8, and controls 1-3) on day 1 after isolation at -80°C in RNAlater (Qiagen). The extracted RNA was of good quality (RIN values between 7.1 and 9.5) and sufficient quantity (>1 µg) for performing whole transcriptome sequencing. For details regarding the methods, see supplementary material. Total RNA samples were depleted from rRNA using the RiboMinus Eukaryote Kit for RNA and the RiboMinus Concentration Module. Total RNA was used to prepare a fragment library using the SOLiD Total RNA-seq Kit (Applied Biosystems). The libraries were sequenced using the AB SOLiD™ 5500xl-W system. The read length was 50 bp for all samples and directionality of RNA molecules was preserved in the sequencing. Reads were aligned to version GRCh37/hg19 of the human genome using version 1.1 of the Applied Biosystems whole transcriptome analysis tool (Uppsala genome Center). The neighbor-joining method, based on the normalized Euclidean distance of reads per kilobase of exon per million fragments mapped (RPKM) between samples was used to analyze differences in expression between all samples, using uniform gene expression to root the tree. Gene lists for the insulin secretion pathway and the complement system pathway were extracted from the KEGG database and relevant literature. RPKM values for all genes in the pathways were calculated (3) and each case was compared to the mean of the three controls in order to identify up- and down-regulated genes. Genes with RPKM values below 0.1 were considered non-expressed.
### Table 1. Demographic data, cases and controls.

| Category                        | No | Age (years) | Sex (M/F) | Time from T1D diagnosis (weeks) | HbA1c at biopsy (% mmol/mol) | Insulin (U/kg/day) | Anti-GAD (<0.08ai)* | Anti-insulin (<0.08ai)* | anti-ZnT8 (<0.12ai)* | Anti IA2 (<0.10ai)* |
|---------------------------------|----|-------------|-----------|--------------------------------|------------------------------|-------------------|--------------------|----------------------|----------------------|----------------------|---------------------|
| DiViD-cases, living newly diagnosed T1D patients | Case 1 | 25 | F | 4 | 6.7 (50) | 0.5 | 1.76 | 0.7 | 0.28 | 0.16 |
|                                 | Case 2 | 24 | M | 3 | 10.3 (89) | 0.35 | 0.79 | <0.01 | 0.44 | >3.00 |
|                                 | Case 3 | 34 | F | 9 | 7.1 (54) | 0.17 | 1.77 | <0.05 | 1.45 | >3.00 |
|                                 | Case 4 | 31 | M | 5 | 7.4 (57) | 0.4 | 0.77 | 0.1 | <0.01 | 2.54 |
|                                 | Case 5 | 24 | F | 5 | 7.4 (57) | 0.36 | 0.46 | 0.1 | 0.06 | >3 |
|                                 | Case 6 | 35 | M | 5 | 7.1 (54) | 0.52 | 1.85 | <0.05 | <0.01 | <0.04 |
| Organ-donors died at onset of T1D | Case 7 | 40 | M | 0 | nd | - | Neg. | Neg. | Neg. | Neg. |
| Organ-donors without T1D        | Control 1 | 22 | M | - | 5.5 (37) | - | Neg. | Neg. | Neg. | Neg. |

*Arbitrary units according to Diabetes Antibody Standardization Program (DASP) (4)*

**Legend eFig 1**

**Insulin secretion pathway for all diabetic cases (1-8) shown separately.** We normalized expression by three brain dead controls and show lower expression in blue and higher expression in red. With the exception of case 6, the majority of genes in the pathway are more lowly expressed in the diabetic samples.

**References**
