Published in final edited form as:

Acta Diabetol. 2014 April ; 51(2): 199–204. doi:10.1007/s00592-013-0479-5.

Gene Expression Analysis of Human Islets in a Subject at Onset of Type 1 Diabetes

Johan Hopfgarten[†], Per-Anton Stenwall[†], Anna Wiberg, Mahesh Anagandula, Sofie Ingvast, Therese Rosenling, Olle Korsgren, and Oskar Skog^{*}

Department of Immunology, Genetics and Pathology, Uppsala University, Uppsala, Sweden

Abstract

Swollen islet cells have been repeatedly described at onset of type 1 diabetes, but the underlying mechanism of this observation, termed hydropic degeneration, awaits characterization. In this study, laser capture microdissection was applied to extract the islets from an organ donor that died at onset of type 1 diabetes and from an organ donor without pancreatic disease. Morphologic analysis revealed extensive hydropic degeneration in 73% of the islets from the donor with type 1 diabetes. Expression levels of genes involved in apoptosis, ER stress, beta cell function, and inflammation were analyzed in isolated and laser-captured islets by qPCR. The chemokine MCP-1 was expressed in islets from the donor with type 1 diabetes while undetectable in the control donor. No other signs of inflammation were detected. There were no signs of apoptosis on the gene expression level, which was also confirmed by negative immunostaining for cleaved caspase-8. There was an increased expression of the transcription factor ATF4, involved in transcription of ER stress genes, in the diabetic islets but no further signs of ER stress were identified.

In summary, on the transcription level, islets at onset of type 1 diabetes in which many beta cells display hydropic degeneration show no obvious signs of apoptosis, ER stress or inflammation supporting the notion that these cells are responding normally to high glucose and eventually succumbing in beta cell exhaustion. Also, this study validates the feasibility of performing qPCR analysis of RNA extracted from islets from subjects with recent onset T1D and healthy controls by laser capture microdissection.

Keywords

Hydropic degeneration; type 1 diabetes; pathogenesis; etiology; laser capture microdissection

Introduction

Type 1 diabetes (T1D) is a disease emanating from the loss of the insulin-producing beta cells. Hydropic degeneration of the beta cells is considered a pathologic hallmark of the endocrine pancreas at onset of T1D. This extensive cellular edema has been repeatedly described in the scientific literature, i.e. in the early 20th century by A. Weichselbaum [1, 2], in the classic work from 1965 by W. Gepts [3], and in more recent reports [4, 5]. Hydropic degeneration has also been described in islets of rabbits treated with cortisone [6],

^{*}Address for correspondence: Oskar Skog, IGP, Rudbeck Laboratory C11, Uppsala University, 751 85 Uppsala, Sweden. Telephone: +46 18 611 00 00. oskar.skog@igp.uu.se.

[†]J.H. and P.A.S. contributed equally and are listed in alphabetical order.

Conflict of interest

None.

pancreatectomized dogs [7], in bank-voles infected with Ljungan virus [8], in domestic cats infused with glucose [9], and after transplantation of a suboptimal number of human islets in diabetic immunoincompetent mice [10, 11] suggesting that this beta cell phenotype might be the result of prolonged hyperglycemia. Hydropic degeneration of the beta cells is not due to intracellular accumulation of glycogen or fat, but rather attributed to an alteration in the electrolyte balance leading to an intracellular oedema [6].

In this study we had the unique possibility to study the islets from an organ donor that died at onset of T1D. The morphologic appearance of the islets was characterized and RNA was extracted from laser capture microdissected and isolated islets. A panel of genes involved in ER stress, inflammation, apoptosis and islet function was analyzed by qPCR with the aim to characterize the pancreatic islets at onset of T1D and gain understanding of the pathogenic processes causing the disease.

Materials and Methods

Human Pancreatic Specimens

A total of 4 human pancreases were included in the study. All were procured from heart beating multiorgan donors and transported to Uppsala in preservation solution minimizing post-mortal changes. Biopsies from the pancreas head were immediately fixed in 4% PFA or immersed in liquid nitrogen and subsequently stored at -80°C until further use. The size of the biopsies was roughly 0.5 cm^3 .

Pancreatic biopsies from one healthy control donor (HC) and one donor that died at onset of type 1 Diabetes (T1D) were selected for laser capture of islets and morphologic analyses. The donor with T1D was a 40-year-old, previously healthy man, without autoantibodies against islets antigens (IA2, GAD65, ZnT8), but with a history of increasing severe thirst and high diuresis during the past three weeks and clinical data indicative of T1D onset (described in detail in [12]). The HC donor was a 26-year-old man with a BMI of 26.3 kg/m^2 without any pancreatic disease.

Islet Isolation procedure

The isolation procedure was done according to a previously described protocol [13]. After isolation the islets were maintained in culture bags (Baxter Medical AB, Kista, Sweden) holding 200 mL CMRL-1066 (ICN Biomedicals, Costa Mesa, CA) supplemented with 10 mM HEPES, 2 mM L-glutamine, 50 mg/ml Gentamycin, (Gibco BRL, Invitrogen Ltd, Paisley, UK), 20 mg/mL Ciprofloxacin (Bayer Healthcare AG, Leverkusen, Germany), 10 mM nicotinamide, and 10% heat-inactivated human serum. The bags were retained at 37°C under a humidified atmosphere consisting of 5% CO_2 . The culture medium was changed day 1 after isolation and the islets were used for in vitro experiments on day 2.

IHC and morphologic analysis

Formalin-fixed and paraffin-embedded pancreatic biopsies were taken from the T1D and HC donor. Sections of 6 μm each were processed and labeled using a standard immunoperoxidase technique for paraffin sections. Antibodies against insulin and glucagon were purchased from BioGene and DAKO, respectively, and used according to the manufacturer's instructions without heat-induced epitope retrieval. Whole-slide high-resolution images of pancreatic specimens stained for insulin were created using an Aperio ScanScope system (Aperio Technologies) and viewed using ImageScope v10.2 software (Aperio Technologies). The fraction of insulin positivity was analyzed with the ImageScope Positive Pixel Count algorithm (version 9) using default settings. The number of pixels, defined by the software as positive or strongly positive, was divided by the total number of pixels to

receive the fraction of insulin-positivity in the section. Islets and swollen islet cells with a clear cytoplasm were counted manually by two independent observers.

Laser capture microdissection

Frozen tissue biopsies from the recent onset T1D and healthy control pancreata were prepared for laser capture microdissection (LCM), essentially as described [14, 15]. Immediately before LCM, the frozen tissue blocks were removed from the -80°C freezer, kept on dry ice and sectioned using a Leica CM 1950 cryostat at -22°C to the thickness of $10\text{ }\mu\text{m}$. The sections were mounted on Leica FrameSlides POL-Membranes ($0.9\text{ }\mu\text{m}$) by apposing the membrane directly against it, using a Leica Frame Support to support the membrane. Each membrane was kept at -22°C in the cryostat until it contained around 8 sections. Sections were then fixed in acetate for 2 minutes at -22°C and finally stored at -80°C until processed further.

Prior to staining, each membrane was removed from storage at -80°C and thawed at room temperature for 30 seconds. Hematoxylin-Eosin (HE) staining and dehydration of the tissue sections were performed with Arcturus® Histogene® Frozen Section Staining Kit (Applied Biosystems, Sweden) according to the manufacturer's instructions. The membrane was then immediately placed in the Leica LMD 6000 unit for laser microdissection of the tissue.

For selection and extraction of specimen, laser microdissection was performed, essentially as described [16], using a Leica LMD 6000, a Leica DFC300 FX camera and Leica Laser Microdissection v 7.4 software. Prior to microdissecting the tissue, the LCM apparatus was washed using Ambion® RNaseZAP® wipes (Invitrogen). Islets were identified and cut under $10\times$ magnification and collected in RNase free microfuge tubes containing 50 mL of Arcturus® PicoPure® Extraction Buffer, (Applied Biosystems, Sweden). When dissecting the tissue, the laser power setting was set at 25, the aperture setting at 20 and the speed setting at 15, with specimen balance at 8. After laser capturing the tissue was incubated at 42°C for 30 minutes in the extraction buffer. The sample was then frozen and stored at -80°C until extraction of RNA.

cDNA synthesis and qPCR analysis

RNA was extracted from the laser-captured tissue with Arcturus® PicoPure® RNA Isolation Kit (Applied Biosystems, Sweden) with on-column DNase treatment and from cultured islets with RNeasy Plus Mini kit (Qiagen AB, Sollentuna, Sweden). The quantity and quality of the extracted RNA were analyzed by the use of NanoDrop and Bioanalyzer (Agilent 2100). The quality of the RNA extracted from the laser-captured islets was not optimal (RIN values ranging from 1.5 to 3.5), but the Ct values of the reference genes were similar and satisfyingly low in all samples when 150 pg cDNA was analyzed (15 for 18S and 29 for GAPDH). The RNA was transcribed to cDNA by SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) according to the manufacturer's instructions. Real Time PCRs were run in triplicates with Power SYBR Green master mix (Applied Biosystems, Sweden) on a StepOnePlus™ Real-Time PCR system (Applied biosystems, Sweden). 150 pg cDNA was used for every reaction. Pre-designed gene-specific primer sets (QuantiTect® Primer Assays, Qiagen) were used for detection. The expression level of each gene was normalized against the geometric mean of the expression of the reference genes 18S and GAPDH ($2^{-\Delta\text{Ct}}$). For in vitro experiments the expression levels are presented as fold induction compared to untreated islets from the same donor ($2^{-\Delta\Delta\text{Ct}}$). Ct values >37 were regarded as negative. PCR specificity was verified by melt curve analysis of all PCR products.

Results

Morphology and insulin content of T1D and healthy control islets

Formalin-fixed paraffin-embedded pancreatic sections from the donor that died at onset of T1D and from the non-diabetic control donor were stained for insulin. The percent insulin positive area was only slightly decreased in the T1D sections ($0.36 \pm 0.13\%$) compared to the control ($0.6 \pm 0.24\%$). The intracellular insulin content, assessed by ELISA on homogenates of isolated islets, was only 0.260 ng/ng DNA in the islets from the subject with T1D, which should be compared to 2.418 ng/ng DNA in the islets from the healthy control.

A majority of the beta cells within the islets in the subject with T1D showed hydropic degeneration (multiple cells in 39 of 53 examined islets, 73%) (Fig. 1a). Cells with this morphology appeared only rarely in the sections from the non-diabetic control (1–2 cells in 4 of 50 examined islets, 8%)(Fig. 1b).

Expression analysis of T1D and healthy control islets

To characterize the islets from the donor that died at onset of T1D, the expression levels of a panel of genes involved in ER stress, apoptosis, inflammation, and islet function were studied and compared to the expression levels of the same genes in the HC islets.

The gene expression levels correlated well between cultured and laser captured islets. However, the expression of cytokines previously known to be affected by the islet isolation process, e.g. IL-8, MCP-1, CXCL2, were up-regulated in the cultured islets compared to laser-captured islets (Fig. 2). Of all the analyzed genes, only the expression of MCP-1 and MHC class I was increased in both cultured and laser captured T1D islets compared to the respective control (Fig. 3).

Analysis of genes involved in cell death (*BCL2*, *BAX*, *PTPN2*, *SURVIVIN*, and *MCL1*) provided no evidence of ongoing apoptosis in islets from the T1D donor (Fig. 3). The expression of *BAX* was increased about 4-fold in laser-captured T1D islets, but the expression of the other genes related to apoptosis was not different between T1D and control islets (Fig. 3). Staining of formalin-fixed and paraffin-embedded sections for cleaved caspase-8 also showed no signs of ongoing apoptosis in the islets from any of the two donors (data not shown).

The expression of ER stress genes *EIF2A*, *XBPI*, *CHOP*, and *BIP* were similar or lower in the islets from the T1D donor compared to the control (Fig. 3). However a fairly high expression of the transcription factor for *CHOP*, *ATF4*, was found in the laser captured islets from the T1D donor while it was below the detection limit in islets from the control.

There were few or no signs of ongoing inflammation when analyzing the expression of genes involved in inflammatory processes in laser-captured islets (Fig. 3). Expression of the genes encoding the cytokines IL-6 and IL-8, IFN β or OAS1 was not detected in islets from any of the two donors. Interestingly, transcription of the chemokine IP-10 was detected in islets from the control donor only, while the chemokine MCP-1 was only detected in islets from the T1D donor. There was no difference in the expression of the genes encoding CXCL2, or the Cocksackie-Adenovirus receptor (CAR) (Fig. 3).

The expression of the gene encoding insulin was reduced about 4-fold in islets from the donor with T1D, whereas the gene expression of glucagon was somewhat increased (Fig. 3). Interestingly, the expression of the gene encoding Glucose transporter 2 (GLUT2) was below the detection limit in islets from the T1D donor. Gene expression of Glucose transporter 1 (GLUT1), the transcription factor for glucagon (MAFB), and an insulin

transcription factor (MAFA) was similar in both donors. The expression of the gene encoding the beta cell specific transcription factor PDX1 was below the detection limit (Fig. 3).

Discussion

In this study we report the successful analysis of gene expression in islets acquired from a type 1 diabetic donor by laser capture micro-dissection and in isolated islets. The data presented reveals that even in islets with a large number of beta cells with hydropic degeneration there are no obvious signs of apoptosis, ER stress or inflammation. It is plausible that hydropic degeneration is an intracellular edema developing in response to prolonged exposure to high glucose resulting in beta cell exhaustion.

It is important to note that the morphology of the tissues examined in this study was very well preserved since the pancreata originated from heart-beating organ donors and the insulin staining was performed without heat-induced epitope retrieval. Heat treatment negatively affects tissue integrity and in our experience makes the swollen appearance of hydropic cells undetectable. Hydropic degeneration of beta cells was described more than 100 years ago in patients dying at onset of T1D [1], and has been reported in a number of different conditions of prolonged periods of hyperglycemia [6]. At onset of T1D large areas of the pancreas contain islets with a high number of remaining beta cells [17–19] and it is difficult to envision that the remaining beta cell mass would be unable to control the glucose metabolism, implying a severe functional impairment. Also, a large majority of T1D subjects have remaining c-peptide two years after diagnosis and in a significant fraction of these there is no decrease in c-peptide during the first two years [20]. Indeed, the beta cells presenting hydropic degeneration are likely capable of responding to high glucose but eventually succumb in beta cell exhaustion, whereas the cells with apparent normal granulation are functionally incompetent and unable to respond to glucose.

The only genes with higher expression in both cultured and laser-captured T1D islets, compared to islets from control donors, were those encoding MHC class I and MCP-1. Notably, the lack of alterations in the level of expression within T1D and healthy control islets could in part be due to the lack of appropriate control tissue, i.e. even perfectly preserved organs retrieved from non-diabetic subjects may have alterations in their gene expressions due to events prior to their death or by the processes induced during herniation of the brain, e.g. the release of large quantities of steroids and induction of inflammation [21–23]. MHC class I hyperexpression has been established as a common finding in islets from subjects with T1D [19, 24], and is confirmed by this data. MCP-1 is a monocyte-recruiting chemokine that is highly secreted by isolated islets, but this is the first time its expression is demonstrated in islets prior to islet isolation and culture.

Due to the abundant presence of RNases in pancreatic tissue, acquiring intact RNA from microdissected islets of Langerhans is particularly difficult. The present report shows that it is possible to perform expression analysis on islets extracted by laser capture, even on heavily degraded RNA. There was no significant difference in RNA integrity between the T1D and HC donor, allowing comparison of gene expression levels since the PCR primers were designed for amplification of short amplicons (~100 bp) and the reference genes can be expected to be equally degraded as the genes of interest [25]. These results are vital for future studies with refined protocols allowing expression analyses of micro-dissected subpopulations of cells within the pancreas of subjects with T1D.

In summary, islets at onset of T1D in which the majority of beta cells display hydropic degeneration show no obvious signs of apoptosis, ER stress or inflammation on the

transcriptional level, supporting the notion that these cells are responding normally to high glucose and eventually succumbing in beta cell exhaustion. Also, this study validates the feasibility of performing qPCR analysis of RNA extracted from islets from subjects with recent onset T1D and healthy controls by laser capture microdissection.

Acknowledgments

This study was supported by grants from the Swedish Medical Research Council (65X-12219-15-6) and EU-FP7-Health 2010 PEVNET 261441. OK's position is in part supported by the National Institutes of Health (2U01AI065192-06). 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.

References

1. Weichselbaum A, Stangl E. Zur Kenntniss der feineren Veränderungen des Pankreas bei Diabetes mellitus. Wiener Klinische Wochenschrift. 1901; 41:968–972.
2. Weichselbaum A. Über die Veränderungen des Pankreas bei Diabetes mellitus. Sitzungsberichte der kaiserlichen Akademie der Wissenschaften. 1910; 119:73–281.
3. Gepts W. Pathologic anatomy of the pancreas in juvenile diabetes mellitus. Diabetes. 1965; 14:619–633. [PubMed: 5318831]
4. Somoza N, Vargas F, Roura-Mir C, et al. Pancreas in recent onset insulin-dependent diabetes mellitus. Changes in HLA, adhesion molecules and autoantigens, restricted T cell receptor V beta usage, and cytokine profile. J Immunol. 1994; 153:1360–1377. [PubMed: 7913115]
5. Meier JJ, Bhushan A, Butler AE, Rizza RA, Butler PC. Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? Diabetologia. 2005; 48:2221–2228. [PubMed: 16205882]
6. Toreson WE. Glycogen infiltration (so-called hydropic degeneration) in the pancreas in human and experimental diabetes mellitus. Am J Pathol. 1951; 27:327–347. [PubMed: 14819264]
7. Volk BW, Lazarus SS. Ultramicroscopy of dog islets in growth hormone diabetes. Diabetes. 1962; 11:426–435. [PubMed: 13997764]
8. Blixt M, Niklasson B, Sandler S. Characterization of [beta]-cell function of pancreatic islets isolated from bank voles developing glucose intolerance/diabetes: An animal model showing features of both type 1 and type 2 diabetes mellitus, and a possible role of the Ljungan virus. General and Comparative Endocrinology. 2007; 154:41–47. [PubMed: 17686482]
9. Zini E, Osto M, Franchini M, et al. Hyperglycaemia but not hyperlipidaemia causes beta cell dysfunction and beta cell loss in the domestic cat. Diabetologia. 2009; 52:336–346. [PubMed: 19034421]
10. Jacobson S, Heuts F, Juarez J, et al. Alloreactivity but failure to reject human islet transplants by humanized Balb/c/Rag2gc mice. Scand J Immunol. 2009; 71:83–90. [PubMed: 20384859]
11. Olsson R, Carlsson PO. Better vascular engraftment and function in pancreatic islets transplanted without prior culture. Diabetologia. 2005; 48:469–476. [PubMed: 15696296]
12. Korsgren S, Molin Y, Salmela K, Lundgren T, Melhus A, Korsgren O. On the etiology of type 1 diabetes: a new animal model signifying a decisive role for bacteria eliciting an adverse innate immunity response. Am J Pathol. 181:1735–1748. [PubMed: 22944599]
13. Goto M, Eich TM, Felldin M, et al. Refinement of the automated method for human islet isolation and presentation of a closed system for in vitro islet culture. Transplantation. 2004; 78:1367–1375. [PubMed: 15548977]
14. Marselli L, Sgroi DC, Bonner-Weir S, Weir GC. Laser capture microdissection of human pancreatic beta-cells and RNA preparation for gene expression profiling. Methods Mol Biol. 2009; 560:87–98. [PubMed: 19504246]
15. Marselli L, Thorne J, Dahiya S, et al. Gene expression profiles of Beta-cell enriched tissue obtained by laser capture microdissection from subjects with type 2 diabetes. PLoS ONE. 2010; 5:e11499. [PubMed: 20644627]

16. Edwards RA. Laser capture microdissection of mammalian tissue. *J Vis Exp*. 2007:309. [PubMed: 18989416]
17. Gianani R, Campbell-Thompson M, Sarkar SA, et al. Dimorphic histopathology of long-standing childhood-onset diabetes. *Diabetologia*. 2010; 53:690–698. [PubMed: 20062967]
18. Atkinson MA, Rhodes CJ. Pancreatic regeneration in type 1 diabetes: dreams on a deserted islet? *Diabetologia*. 2005; 48:2200–2202. [PubMed: 16193285]
19. Coppieters KT, Dotta F, Amirian N, et al. Demonstration of islet-autoreactive CD8 T cells in insulinitic lesions from recent onset and long-term type 1 diabetes patients. *J Exp Med*. 2012; 209:51–60. [PubMed: 22213807]
20. Greenbaum CJ, Beam CA, Boulware D, et al. Fall in C-peptide during first 2 years from diagnosis: evidence of at least two distinct phases from composite Type 1 Diabetes TrialNet data. *Diabetes*. 2012; 61:2066–2073. [PubMed: 22688329]
21. Gramm HJ, Meinhold H, Bickel U, et al. Acute endocrine failure after brain death? Transplantation. 1992; 54:851–857. [PubMed: 1332223]
22. Contreras JL, Eckstein C, Smyth CA, et al. Brain death significantly reduces isolated pancreatic islet yields and functionality in vitro and in vivo after transplantation in rats. *Diabetes*. 2003; 52:2935–2942. [PubMed: 14633854]
23. Purins K, Sedigh A, Molnar C, et al. Standardized experimental brain death model for studies of intracranial dynamics, organ preservation, and organ transplantation in the pig. *Crit Care Med*. 2011; 39:512–517. [PubMed: 21187748]
24. Foulis AK, Farquharson MA, Hardman R. Aberrant expression of class II major histocompatibility complex molecules by B cells and hyperexpression of class I major histocompatibility complex molecules by insulin containing islets in type 1 (insulin-dependent) diabetes mellitus. *Diabetologia*. 1987; 30:333–343. [PubMed: 3301484]
25. Fleige S, Pfaffl MW. RNA integrity and the effect on the real-time qRT-PCR performance. *Mol Aspects Med*. 2006; 27:126–139. [PubMed: 16469371]

Fig. 1. Hydropic degeneration in islets from the donor that died at onset of T1D (A) and normal islets from the donor without pancreatic disease (B). Original magnifications $\times 40$

Fig. 2. Gene expression analysis of isolated vs. laser-captured islets from a donor with type 1 diabetes (left) and from a donor without pancreatic disease (right). The diagonal line represents equal expression levels in isolated and laser-captured islets. The dotted vertical and horizontal lines indicate the minimum detection level (Ct 37) for the data plotted on the respective axis. Genes marked with a red dot have expression levels in isolated islets that are below the detection limit in laser-captured islets, making relative comparison impossible

Fig. 3.

Gene expression analysis of islets from a donor with type 1 diabetes vs. islets from a donor without pancreatic disease, in isolated (left) and laser-captured islets (right). The diagonal line represents equal expression levels in diabetic and non-diabetic islets. The dotted vertical and horizontal lines indicate the minimum detection level (Ct 37) for the data plotted on the respective axis. Expression levels marked with a red dot are below the detection limit