Correlative microscopy reveals abnormalities in type 1 diabetes

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2018

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter 5

Advanced microscopy implementation in type 1 diabetes research

Chapter 5a

Large-scale digital electron microscopy resource for human type 1 diabetes

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\#Denotes equal contribution

\textit{Manuscript submitted}
Abstract

The cause of type 1 diabetes remains unknown. Here, we applied a recently developed microscopic imaging approach on a unique biobank of human islets of Langerhans to study ultrastructural morphology of islets in type 1 diabetes donors using electron microscopy (EM). To make human islet EM data of greatest value to multiple researchers, we created an online repository composed of large-scale EM images (termed ‘nanotomy’) that allows analysis of islets and surrounding exocrine tissues at macromolecule resolution. Donors include those without diabetes, type 1 and type 2 diabetes and donors with autoantibodies associated with type 1 diabetes. Using a web-based infrastructure, the equivalent of ~1 million traditional EM images is now available via www.nanotomy.org/nPOD. A first analysis based on morphology and subsequent ‘ColorEM’ reveals intermediate cells found in two donors with type 1 diabetes. Moreover, the specificity of leukocytes found in islet inflammation is observed as recently published by other methods. Thus, the EM islet nanotomy database reveals features from the macromolecular to the tissue level presented in an unbiased way. By providing open access to the repository, diabetes investigators worldwide can address countless research questions through the database.

Introduction

The underlying mechanism(s) initiating destruction of beta cells resulting in type 1 diabetes are still poorly understood (1), precluding curing the disease. Injectable insulin is the replacement treatment for type 1 diabetes since 1921 (2-4). The JDRF Network for Pancreatic Organ Donors with Diabetes (nPOD) provides high quality biospecimens to understand mechanisms leading to type 1 diabetes (5, 6). nPOD encourages investigators to work across multiple disciplines and implement new tools to study type 1 diabetes, as well as open-access publication of raw data that enables reuse and analysis by scientists worldwide to obtain new information from complex datasets (7, 8). Pancreases and other biospecimens are obtained from donors with type 1 diabetes as well as donors without diabetes (ND), type 2 diabetes, and type 1 diabetes-associated autoantibody-positive (AAb+) donors. AAb+ donors have one or more autoantibodies but no clinical diabetes symptoms. People with multiple AAb+ are at high risk to develop type 1 diabetes (9).

Here, we present a nanoscale data repository from nPOD organ donors of islets of Langerhans. Through large-scale electron microscopy (EM), termed ‘nanotomy’ for nano-anatomy (10-12), an extensive database of unbiased islet ultrastructure was established. Islets and surrounding exocrine tissue were imaged in fields of view up to 0.5 mm in diameter at 2.5 nm pixel size. From a web-based viewer, the entire tissue section can be analyzed from low to high resolution, ranging from structures as large as islets (sub-mm diameter) to single intra-islet cells and their organelles (μm- to nm-scale)(10). The parameters can be analyzed across an intact islet in near-molecular detail from a single dataset. Several relevant features were analyzed for this report including insulin granules, prevalence of mast cells and neutrophils near islets, and the presence of a rare type of intermediate cell seen only in donors with type 1 diabetes that contained both endocrine as well as zymogen secretory granules. Different granules were identified using a recently reported ‘ColorEM’ approach allowing label-free identification of elements in granules (13). The reported data are only a subset of features contained within the complex dataset. Data are open access available for reuse to analyze all ultrastructural information of specific nPOD donor samples to permit additional insights as well as in conjunction with findings using different techniques. This repository contains the most elaborate pancreatic EM data sets that is push-button available, bypassing laborious and expensive image-acquisition by individuals. The shared data is thus expected to greatly benefit the diabetes research community and additionally may boost open access image sharing of biobank material in other fields.
Research design and methods

Donors

Thirty-one EM islet datasets were created from 23 donors (ND (8), AAb+ (4), type 1 diabetes (8), type 2 diabetes (3)) and their nPOD CaseID with demographic details are listed in Table 1. Additional donor details can be obtained through the JDRF nPOD online pathology(14). Tissues were recovered following informed research consent from next of kin in the United States and shipped to the nPOD program at the University of Florida for processing as previously described (5, 6, 15). All experiments were conducted under the approval of the University of Florida Institutional Review Board and the current study fulfills all requirements for tests as approved by the medical ethical review board of the University Medical Center Groningen.

Figure 1 | nPOD nanotomy data generation workflow. Fixed pancreatic samples are received from nPOD. The tissue undergoes processing for scanning transmission electron microscopy (EM) as indicated followed by acquisition at 2.5 nm pixel size. The final stitched image is converted to html and uploaded to nanotomy.org.
Pancreas sample electron microscopy processing

Pancreas samples from the head, body, or tail regions (Table 1, S1) were fixed in cold, freshly prepared 2% paraformaldehyde-1% glutaraldehyde for 48 hours followed by transfer to phosphate-buffered saline for storage at 4°C before shipment to the EM laboratory in the Netherlands (15). Tissue vibratome sections (~50 µm; Microm HM 650V) were post-fixed in osmium tetroxide/potassium ferrocyanide, followed by dehydration and flat-embedding as previously reported (10).

Next, regions with islets were selected from toluidine stained 1 μm sections using light microscopy. Subsequent ultrathin (80 nm) sections were cut (UC7 ultramicrotome, Leica Microsystems, Vienna, Austria) and placed on formvar coated copper grids (Electron Microscopy Sciences, Hatfield, Pennsylvania). Finally, sections were contrasted with uranyl acetate followed by Reynold’s lead citrate (16) as previously described (10, 11).

EM acquisition and image processing (nanotomy)

Data were acquired on a Supra 55 scanning EM (SEM; Zeiss, Oberkochen, Germany) using a scanning transmission EM (STEM) detector at 28kV with 2.5 nm pixel size (2 nm pixel size for datasets 6098a and b, 6126a, 6197, 6087a, 6113, and 6198) using an external scan generator ATLAS 5 (Fibics, Ottawa, Canada) as previously described (11, 12). After tile stitching, data were exported as an html file and uploaded to the online image database (www.nanotomy.org) (Fig. 1).

Energy Dispersive X-Ray Analysis (EDX; ‘ColorEM’)

Energy dispersive x-ray analysis (EDX) imaging for element discrimination was performed essentially the same as recently described (13). Briefly, regions of interest were determined using the nanotomy maps. Next, subsequent sections of 100 nm were cut and placed on a formvar-coated single slot pyrolytic carbon grid (Ted Pella, INC., California, USA) followed by uranyl acetate staining. The selected regions of interest were imaged using an Oxford Instruments X-MaxN 150 mm² Silicon Drift Detector and AZtecEnergy software (Abingdon, UK) mounted on the Zeiss Supra SEM. EDX images were generated (sum of 30-40 frames) with 50 µs dwell time at 15kV acceleration voltage and 8.4 nA beam current. Image analysis and processing was done in Adobe Photoshop and included a Gaussian blur of 1.5 pixel radius to the raw elemental maps followed by adjustments of white points for each color channel in a level adjustment layer, followed by +25 brightness and +50 contrast adjustment layer. Raw data are shown in Figs. S1, 2.

Primary analysis of data

Complete nanotomy datasets were manually screened field by field for the presence of different cell types based on standard ultrastructural features (Table 1). The presence of each cell type and any abnormal cells, e.g. intermediate cells based on exocrine and endocrine vesicles as well as subtypes of mast cells which can be distinguished based on the morphology of granules: Tryptase mast cell granules have scrolls, whereas chymase-tryptase mast cells have more homogeneous granules (17).
Figure 2 | nPOD nanotomy repository allows zooming into islets up to macromolecular complexes. (A) nanotomy.org/nPOD web interface allows selection of islets based on nPOD caseID and subsequent analysis of the sample. (B) While analysis should be performed digital, one example is given. From left to right: Analyzing whole cross-sections of islets, part of an islet, several cells with subcellular details like the nucleus; organelles like mitochondria and secretory vesicles. Zooming and panning allows detailed analysis on small areas on a large variety of read outs while maintaining full context.
Chapter 5a

Results

nPOD nanotomy: Equivalent of ~1 million of regular EM images of islets of nPOD donors

The unique approach of the nPOD program (15) allowed systematic analysis of islets in pancreata of type 1 donors as well as controls and other donors (Fig. 1). The repository is organized by nPOD CaseID to permit linking other studies from the same donor (18) to these ultrastructural images and now contains 31 datasets of 23 donors (Fig. 2A). We set out to optimize a protocol for islet sample preparations and determined the ultrastructural quality of immersion fixed human donor pancreases. Strikingly, sample quality was deemed very high regardless of the duration of storage. Only 1 out of 24 donor samples received did not have useful morphology. As expected, islets of Langerhans were smaller and harder to find in type 1 diabetes donors. The nanotomy data acquired were uploaded to the nPOD nanotomy website (Fig. 2A). Images of complete cross sections of islets of Langerhans at macromolecular scale allow morphological analysis of complete islets, cells, organelles, and macromolecules (Fig. 2B) by simply zooming in at higher resolution at any region or feature of interest.

Label-free identification of multiple parameters in type 1 diabetes donors

Expert analysis allowed discrimination of different cell types present in the pancreas based on the morphology and grey levels of the secretory granules (Fig. 3). Islets were distinguishable by clustering of cells with lighter cytoplasm, smaller secretory granules, and less abundant ER than the acinar cells of the exocrine tissue. Islets from control donors contain the expected cell types and their various endocrine hormones (alpha, beta, delta, and pancreatic polypeptide (PP) cells) that have been characterized previously using traditional transmission EM analysis based on their distinct secretory vesicles (19-21). Glucagon-containing alpha cell granules (Fig. 3) are the most electron-dense, typically 200-250 nm in diameter, and often contain a thin halo between the membrane and the electron dense core. Insulin-containing beta cell granules (Fig. 3) are less electron-dense, typically 250-300 nm in diameter, and mature insulin granules have a prominent halo between the membrane and a crystalline core. Granules of delta and PP cells (Fig. 3) are of variable electron-density and are differentiated mainly by size with granules 200-350 nm and 120-160 nm in diameter, respectively (20). In the exocrine pancreas, acinar cells are characterized by abundant rough ER and larger zymogen-containing secretory granules (0.5-1.5 µm diameter; Fig. 3)(22). Mast cells are also frequently observed, with amorphous secretory granules (Fig. 3). For the mast cells which are of the tryptase+ subset, the granules also contain cylindrical clusters (Fig. 3)(23).
Figure 3 | Label-free identification of cells and organelles in nPOD nanotomy and ColorEM. Different pancreatic cell types are discriminated based on secretory granule appearance. Glucagon granules of the alpha cells often contain an electron dense core with a thin halo between the core and membrane. Insulin granules of the beta cells also contain a halo between core and membrane with a crystalline core in mature granules. Somatostatin granules of the delta cells and pancreatic polypeptide granules of the PP cells both contain relative small granules with variable grey values. Zymogen granules of exocrine acinar cells are relatively large (0.5-1 μm) and electron dense. Lastly, mast cells could be observed containing granules with cylindrical structures. Cells can also be distinguished by ColorEM via EDX as seen in the corresponding image to each cell type. EDX maps of phosphorus, nitrogen, and sulfur in green, red, and blue, respectively, are overlaid to show the relative difference in elemental composition of secretory granules of alpha, beta, delta, PP, acinar, and mast cells. Secretory granules of each cell type have high nitrogen (red) and cell types can be further distinguished based on enrichment of additional elements such as phosphorus (green) in alpha cell granules, displayed in orange, and sulfur (blue) in beta cell and mast cell granules, displayed in purple. Alpha, beta, delta, PP, acinar, and mast cell images are taken from nanotomy datasets from donors 6064, 6130, 6126, 6130, 6126, and 6087, respectively. ColorEM of donors 6126 (alpha, beta, delta, and acinar) and 6130 (PP and mast). Raw EDX data are shown in Fig. S1.
Although identification of endocrine cell types is feasible using these established granule morphological characteristics, elemental composition provides objective determination of cell types (13, 24). Elemental maps of phosphorus, nitrogen, and sulfur are overlaid to determine the variation of granule content within each endocrine cell type (Fig. 3, see Fig. S1 for raw element maps). The nuclei shown in the upper right corner of each image contain high amounts of nitrogen and phosphorus in the condensed heterochromatin, appearing yellow with the green and red overlay. All secretory granules have a high nitrogen content as expected from the high concentration of polypeptides. Additionally, granules of human beta and mast cells contain a prominent sulfur signal, and granules of alpha cells are enriched with phosphorus, in line with our earlier observations in rat islets (13). Thus, addition of a detector to the EM that allows elemental characterization by X-ray analysis allows differentiation of cell types and organelles by elemental content.

**Mast cell and neutrophil variation between donor groups**

Immune cells, including neutrophils and mast cells, are observed in the different datasets. Neutrophils are almost exclusively present in type 1 diabetes cases (Table 1), except for one case in each of the other groups, with the highest numbers in two type 1 diabetes patients who had acute pancreatitis (6064 and 6204; Supplementary Table 1). Neutrophils are found mainly in the exocrine tissue and not in the islets though some were found peri-islet (Fig. 4A, 8). Only one islet dataset from a type 2 diabetes donor (6133) exhibited a neutrophil inside the islet. While mast cells were observed in each of the four subgroups (Table 1), in type 1 diabetes they were exclusively seen in all donors with disease durations of 3 or more years (Fig. 4C-F).

Based on granular content, mast cells are subdivided into tryptase cells and chymase-tryptase cells. Tryptase mast cell granule content is characterized by well-defined scrolls (Fig. 4C,D), whereas chymase-tryptase mast cells have more homogeneous granules (Fig. 4E,F) (17). Mast cells were observed in 6 islet datasets from 5 type 1 donors, which were excluding one donor (6204) all of the tryptase subtype (Table 1).
Figure 4 | Identification of mast cells and neutrophils in type 1 diabetes donors. Neutrophils are present in type 1 diabetes samples predominantly located in the exocrine parenchyma (A) and some in the peri-islet region (B). Mast cells were present in both type 1 diabetes (C) and control samples (E). However, subtypes can be distinguished based on granular morphology, with prominent scrolls in tryptase-mast cells in type 1 diabetes (D) and homogenous granules in the tryptase-chymase-mast cells in controls (F). Bars: 5 μm (A-C,E) 0.5 μm (D,F). Donor 6064 (A-B), 6087 (C-D), and 6126 (E-F).
Intermediate cells observed in type 1 diabetes donor samples

The division of islets and acinar cell functions and topology of the pancreas is typically quite strict with hormones and digestive enzymes, respectively (20, 21). Furthermore, as pointed out above, the ultrastructure of both pancreatic regions are very different, e.g. looking at secretory granule morphology. However, unique cells that contained both zymogen and endocrine storage granules were identified (Fig. 5). Such intermediate cells were observed in 2 of 8 type 1 diabetes donors. In donor 6228 (Fig. 5A), the intermediate cells were at the periphery of the islet while intermediate cells observed in donor 6063 were localized more scattered throughout a remnant islet (Fig. 5B). Elemental analysis of serial sections to determine elemental composition of the granules showed high nitrogen content for both types of granules with an additional phosphorus signal in the endocrine granules in both donors, suggesting these contain glucagon (Fig. 5C, D). Therefore, both morphology and EDX analysis indicated that these cells contained glucagon as well as zymogen granules (Fig. 5, Fig. S2).

Discussion

We present the development of the first open-access online database of large field and depth of view islets from organ donors with type 1 diabetes in comparison to donors without diabetes. A disadvantage of traditional EM is due to the inherent laborious sample preparations, static imaging, often precluding dynamic physiologic data extraction, and limited fields of view with a lack of tissue context when acquiring data at high resolution. The latter problem has been overcome by our nanotomography approach as the complete ultrastructure of islet cross-sections are available. As nPOD samples are used to investigate different aspects of disease pathogenesis, these EM datasets can be useful in validating findings using different techniques. One example relates to abnormalities in mitochondria function that were found in other assays and the ultrastructural morphology now can be addressed online (Mathews, C, et al, personal communication). Open access nanotomography, like initiatives on DNA sequences, protein structures etc. allows the sharing of all acquired data for reuse in entirely different research questions. EM information is typically qualitative and could be best combined with more quantitative approaches, e.g. the analysis of many islets phenotypes. A disadvantage is the known heterogeneity of human islets. This counts for islet-to-islet variation, but also variation between humans. In addition, variation in organ donor studies increases because the unique material was acquired post-mortem. This makes these examples of nPOD nanotomography more representative of case studies. This extensive database will grow with the addition of prospective discoveries and inter-connections with other datasets, like nPOD DataShare (25).

ColorEM via EDX was found to be highly valuable in conclusive feature identification through elemental composition (13, 26). All types of secretory granules that contain peptides and proteins are rich in nitrogen. However, in addition to morphology, enrichment of specific elements within the different secretory granules can aid in discriminating cell types. As seen previously in beta cells of rat and mouse via EDX (13, 26) and electron energy loss spectroscopy (27), respectively, insulin-containing granules have a higher sulfur signal due to the relative abundance of cysteines (Fig. 3D)(20). Human glucagon granules are enriched with phosphorus, which is in line with glucagon granules in rodents (13, 26, 27). Furthermore, the granules in mast cell were found to be sulfur-rich, which may be explained by the presence of heparin, heparan sulfate, and relative sulfur-rich proteases (28, 29). Nanotomography and ColorEM was applied to the nPOD biobank to analyze islets of Langerhans in type 1 diabetes.
Type 1 diabetes is considered as a T-cell driven disease however in these donor islets, non-T cell immune cell infiltrates are observed in nPOD (Table 1). Neutrophils were observed in pancreases of 6 type 1 diabetes patients, and in one donor from each of the ND, AAb+, and type 2 diabetes subgroups. The neutrophils are typically found in the exocrine pancreas which is in accordance with similar observations made with both immunohistochemistry (30, 31) and EM (31). This supports the idea that not only islets of Langerhans, but also the exocrine pancreas, is affected during type 1 diabetes (32).

We did not find that mast cells are more prevalent in the type 1 diabetes case as reported recently (33). However, the granular morphology, as clearly present in the nanotomy data, allows for discrimination between two different mast cell subtypes. Tryptase mast cells are predominantly present in type 1 diabetes, whereas control, AAb+, and type 2 diabetes samples contained chymase-tryptase mast cells (Table1). Mast cells are classically known for their role in allergies, but a broader role for mast cells in physiology and immunity is being considered, including recruitment of neutrophils, and production of pro-inflammatory cytokines and chemokines (34).

The role of the exocrine pancreas has received variable attention as a component of type 1 diabetes (reviewed in (32)). Type 1 diabetes patients show a significant reduction in pancreas weight or volume at onset of diabetes and exocrine insufficiency has been reported (35-39). Other findings include alterations in exocrine proteomic profiles compared to healthy controls (40) as well as immunological alterations including increased incidence of various exocrine-specific autoantibodies (41-44), increased infiltration of immune cells (31, 45), and complement activation (46). Furthermore, maintained beta-cell mass (7, 47) but decreased pancreas weight in AAb+ donor samples indicates that exocrine tissue might be affected preceding changes of islets in type 1 diabetes (37). This raises the question of whether endocrine-exocrine interactions have a role in the pathogenesis of type 1 diabetes. Interestingly, we find mixed endocrine-exocrine cells only in type 1 diabetes donors. Similar intermediate cells were found in a diabetic prone rat (13). Since intermediate cells, although without the affected morphology, have been observed in non-diabetic and type 2 diabetes human islets albeit with insulin granules (48-50). Additional methods are needed to determine whether this phenomenon is specifically related to type 1 diabetes pathogenesis or diabetes in general.

nPOD nanotomy allows direct qualitative ultrastructural analysis to investigate subcellular alterations of islets of Langerhans in type 1 diabetes. While each dataset represents one entire cross section of an islet at nm-scale resolution, quantitative analysis with other approaches should be used in parallel. This holds especially true when findings are found in some islets but not in others, localized to certain regions, or are extremely rare. The expanding database of human pancreatic tissue EM marks a milestone in the sharing of raw, extensive, relevant, diabetes-specific data. A first analysis revealed the presence of unsuspected innate immune cells, and intermediate exocrine and endocrine cells were found only in donors with type 1 diabetes. Open access nPOD nanotomy, in conjunction with complementary studies, will expedite better insight into the pathogenesis.
Chapter 5a

Acknowledgements

We thank Johana Isaza-Correa and Ruby Kalicharan (UMCG) for technical assistance and Jacob Hoogenboom (Delft, The Netherlands) for discussions on EDX. We acknowledge pancreas EM samples from JDRF nPOD staff members and thank Organ Procurement Organizations that partner with nPOD to recover organ donors. This work was supported by the JDRF (6-2006-1140 25-2013-268 to MCT; 25-2012-770 to MCT and BNGG); The Netherlands organization for scientific research (ZonMW 91111.006; STW Microscopy Valley 12718 to BNGG) and the European Association for the Study of Diabetes (EASD; to BNGG).
Figure 5 | Endocrine-exocrine mixing in ‘intermediate cells’ in type 1 diabetes pancreas. Cells containing both exocrine and endocrine granules were observed in donor 6228 at the islet’s periphery (A) and 6063 throughout the islet with strong exocrine morphology based on the extensive ER (B). The intermediate cells contained both secretory granules resembling exocrine and glucagon granules based on morphology and elemental content using colorEM (C and D) with exocrine granules in red and glucagon granules in orange (see figure 3 for reference). Absence of uranyl staining in B and D explains the difference in contrast to A and C. Scale bars: 2 µm in overviews, 1 µm in boxed regions, and 1 µm in C and D. Raw EDX data are shown in Fig. S2.
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Table 1 | Mast cells and neutrophils enrichment in pancreas of nPOD donors. Donors are sorted by condition and case-ID, and information on pancreas donor region, type of autoantibodies present as measured by radioimmunoassay (RIA), age of demise, and disease duration is provided. The number of mast cells was recorded as well as the phenotype, tryptase (T), tryptase-chymase (TC), or indistinguishable (n/a). The neutrophils observed were mostly in the exocrine region except those marked (*) had one or more in and/or around the islet.
References


8. JDRF nPOD: Online pathology information for investigators - http://ahc-path-apr01.ahc.ufl.edu/Login.php.


Large-scale digital electron microscopy resource for human type 1 diabetes


25. JDRF nPOD: Online datashare for investigators - https://www.jdrfnpod.org/for-investigators/datashare/


Chapter 5a


**Figure S1 | Raw EDX data from different cell types.** Element specific images displaying nitrogen (N), phosphorus (P), sulfur (S), osmium (Os), uranium (U) are shown. See figure 3 for further details. Bar: 2 µm.
Figure S2 | Raw EDX data from intermediate cells. Element specific images displaying nitrogen (N), phosphorus (P), sulfur (S), osmium (Os), uranium (U) are shown. See figure 3 for further details. Bar: 1 μm.
Chapter 5b

Exocrine pancreas cell lysates specifically evoke beta cell stress

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Work in progress: The work in this chapter was proposed by PdB and BNGG and granted by the European association for the study of diabetes (EASD) based on our findings in chapter 5a.
Chapter 5b

Abstract

The trigger for type 1 diabetes (T1D) is unknown. Recent studies revealed that the exocrine pancreas is affected in T1D patients, which can already be observed before the onset of T1D. In chapters 4b and 5a rare intermediate endocrine and exocrine cells were observed in islets of ‘pre-diabetic’ rats and early onset T1D donors respectively. In this chapter, a functional follow-up pilot study is performed to elucidate whether lysates specifically of exocrine cells could induce beta cell stress. The pro-inflammatory C-X-C motif chemokine ligand 10 (CXCL-10) gene expression is increased in insulinoma cells upon exocrine cell lysate treatment. Thus far, only CXCL-10 was upregulated of the beta cell stress associated genes tested. Currently endoplasmic reticulum (ER) stress markers are being investigated. Taken together, exocrine cell damage might provoke a pro-inflammatory reaction by beta cells which should be further elucidated upon experimental model optimization as proposed in this chapter.

Introduction

Type 1 diabetes (T1D) is an autoimmune disorder in which the insulin producing beta cells from the islets of Langerhans in the pancreas are destroyed resulting in elevated and uncontrolled blood glucose with accompanying severe complications. Compiling evidence indicate that beta cell stress and defects precede the massive beta cell death upon T cell infiltration and clinical onset of T1D. However, underlying mechanism(s) including intrinsic or extrinsic triggers involved in beta cell dysfunction are not clear. Chapter 4b and 5a of this thesis describe the appearance of intermediate exocrine and endocrine cells displaying an affected ultrastructure in EM in ‘pre-diabetic’ BB rats and early onset T1D nPOD islets respectively. Subsequently, we proposed that exocrine cell damage triggers beta cell stress at the onset of T1D. This is in line with a current growing notion that the exocrine pancreas, and not only the islets of Langerhans, is affected during T1D.

Functional follow up experiments aim to address whether beta cells acquire a stressed phenotype specifically upon treatment with exocrine cell lysates. Hallmarks for early T1D associated beta cell stress include the production of pro-inflammatory mediators, human leukocyte antigen (HLA) class I hyperexpression, endoplasmic reticulum (ER) stress, and neoantigen formation. We find that the pro-inflammatory chemokine C-X-C motif chemokine 10 (CXCL-10) mRNA expression is most prominently increased in insulinoma cells upon exocrine lysate treatment. This might indicate that exocrine cell damage could evoke a pro-inflammatory response following beta cell stress.

Material and methods

Cell culturing

Rat insulinoma (RIN-m5F), and mouse macrophage (J774) cell lines were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco, 42430-025) supplemented with 1% penicillin/streptomycin (pen/strep; PAA), 10% fetal calf serum (FCS; Greiner Bio-one), and beta-mercaptoethanol (BME; Sigma-Aldrich) to a final concentration of 72 μM was added to RIN-m5F medium. The rat pancreatic exocrine cell line AR42J was cultured in DMEM (Gibco, 10-013-CVK) supplemented with 1% pen/strep and 20% FCS. Table 1 shows an overview of cell lines used in this study.
Lysate stimulation

Min6, RIN-m5F, and J774 cells were seeded in a 24 wells plate at a density of $1.0 \times 10^5$ cells/well and left to adhere for 24 hours. Upon trypsinization and centrifugation, non-seeded Min6, RIN-m5F and AR42J cells were resuspended in dH$_2$O in a concentration of $1.0 \times 10^5$ cells/100 μl and left for 15 minutes to induce osmotic cell lysis. Subsequently stimulation of the seeded cells was performed by adding 100 μl of the cell lysates, i.e. an equivalent of $1.0 \times 10^5$ cells, +/- the presence of lipopolysaccharide (LPS, 200 ng/ml). As a control 100 μl dH$_2$O +/- LPS was added to the seeded cells. Stimulated cells incubated for 24 hours before further processing.

mRNA extraction and real-time PCR

An RNeasy Mini kit (Qiagen) was for cell lyses of stimulated cells in accordance with the manufacturer’s instructions. To avoid RNA degradation by RNases, BME was added to the RTL lysis buffer as proposed in the manufacturer’s protocol. Reverse transcription of the mRNA for cDNA synthesis was performed with the iScript™ cDNA Synthesis Kit (Bio-Rad) according to the manufacturer’s manual. Quantitative real-time PCR was performed using SYBR Green (SsoAdvanced Universal™ SYBR Green Supermix; Bio-Rad) and a StepOnePlus Real-Time PCR system (ThermoFisher). Expression values were corrected by the housekeeping genes β-actin and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primer sequences are listed in supplementary table 1.

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<th>Cell line name</th>
<th>Cell type</th>
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<td>AR42J</td>
<td>Exocrine pancreas</td>
<td>Rat</td>
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<td>J774</td>
<td>Macrophages</td>
<td>mouse</td>
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Table 1 | Overview of cell lines used in this study

Results and discussion

Mechanisms and triggers leading to autoimmune attack against beta cells are poorly understood. In this thesis intermediate endocrine and exocrine cells with an affected ultrastructure in ‘pre-diabetic’ BB rats (chapter 4b) and early onset human T1D donor islets (chapter 5a) are described. Subsequently, we proposed that damaged exocrine cells could trigger beta cell stress at the onset of T1D, which is functionally addressed in this chapter.

Pro-inflammatory cytokine and chemokine production by beta cells is a sign of beta cell stress and might evoke a local immune response$^{5,6}$. Expression of the chemokine CXCL10 is increased in RIN-m5F cells upon stimulation with AR42J cell lysates, and not with RIN-m5F cell lysate stimulation (Figure 1). Furthermore LPS, used as co-stimulator$^{11}$ and as additional control for exocrine lysate specificity, did not evoke increased CXCL-10 expression. As a first indicator for beta cell specific CXCL-10 expression upon exocrine lysate stimulation, expression was not increased in J774 macrophages upon stimulation (Figure 1). Responsiveness of J774 cells was indicated by increased CXCL-10 expression upon LPS treatment. Expression of the pro-inflammatory cytokines interleukin 1 beta (IL-1β) and IL-6 was not increased by insulinoma cells upon exocrine lysate stimulation (Figure 2). These cytokines were upregulated in J774 macrophages upon LPS treatment, but not exocrine lysates, which shows LPS effectiveness (Figure 2). Ideally, other target cells should be stimulated with exocrine lysates to elucidate the beta cell specificity of the response, these would for example include alpha cells. CXCL-10 serum levels are specifically elevated during early onset T1D$^{12}$ and is
known as a potent chemoattractant for T cells\(^1\). Furthermore, histopathology showed that CXCL-10 is highly present in early onset T1D donor islets accompanied with infiltrated T cells expressing the CXCR-3 receptor for CXCL-10\(^2\). Moreover, beta cells have been identified as the resource for CXCL-10 in both animal models\(^3\) and recent onset T1D patient material\(^4\).

<table>
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<th>Abbreviation</th>
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<td>Chemoattractant for immune cells</td>
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<td>CXCR-3</td>
<td>C-X-C motif chemokine receptor 3</td>
<td>Receptor for CXCL-10 on target immune cells</td>
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<td>Interleukin 1 beta</td>
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<td>XBP-1</td>
<td>X-Box binding protein 1</td>
<td>ER stress: alternative mRNA splicing of XBP-1</td>
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<tr>
<td>BiP</td>
<td>binding immunoglobulin protein</td>
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<tr>
<td>LPS</td>
<td>Lipopolysacharide</td>
<td>Cell wall component of gram negative bacteria</td>
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Table 2 | Explanation of important molecules discussed in this chapter.

**Figure 1 | Exocrine lysates evoke CXCL-10 expression in RIN-m5F cells.** CXCL-10 mRNA expression by RIN-m5F cells is increased upon exocrine lysate stimulation compared to unstimulated controls (upper panel). Moreover, lysates from beta cells do not increase CXCL-10 expression. Furthermore, LPS has no effect on CXCL-10 expression from RIN-m5F cells. Only LPS was able to increase CXCL-10 expression by J774 macrophages, and not exocrine or beta cell lysates. N=3 except for the beta cell lysate treated J774 cells, which is n=1.
Exocrine pancreas cell lysates specifically evoke beta cell stress

Additional to assessing a pro-inflammatory response in beta cells, endoplasmic reticulum (ER) stress is a prominent hallmark for early T1D associated beta cell stress \(^8, 17\). Moreover, ER stress is considered as a driving force in neoantigen formation, which is increasingly considered as a link between beta cell stress and autoimmune reactivity\(^9\). Here, ER stress in beta cells, assessed by spliced X-Box binding protein 1 (XBP-1s) mRNA expression\(^17\), was not observed upon exocrine lysate stimulation (Figure S1). Next experiments will assess the involvement of other ER stress markers previously linked to stressed beta cells in T1D, including binding immunoglobulin protein (BiP)\(^17\) and C/EBP homologous protein (CHOP)\(^18\). Also, the degree of neoantigen formation could be investigated, for example through monitoring aberrant post-translation modifications\(^19\) or defective ribosomal products\(^20\).

**Figure 2 | No increase in pro-inflammatory cytokines by RIN-m5F cells upon exocrine lysate stimulation.** mRNA expression of the pro-inflammatory cytokines IL-1β and IL-1 by RIN-m5F cells are not increased upon stimulation of exocrine lysates nor by LPS stimulation (left). These pro-inflammatory cytokines are increased in J774 macrophages upon LPS treatment but also here exocrine lysate stimulation did not increase cytokine expression (right). N=3
Although CXCL-10 mRNA expression by beta cells upon exocrine lysate stimulation is promising, the model used is limited in reflecting the interaction between exocrine damage and beta cells in T1D. First, lysate creation through osmotic shock is a fairly uncontrolled process, and pancreatic protease activity could already break down exocrine mediators before addition to the target cells. Therefore, to avoid lysate transfer, the fluorescent photosensitizing protein supernova will be expressed in exocrine cells only in a co-culture with beta cells. Upon excitation with intense green light exocrine cells are selectively ablated in the vicinity with beta cells, followed by monitoring beta cell stress. First results on selective cell death through photosensitizing supernova tagged to either mitochondria or the plasma membrane look promising (data not shown).

A next limitation of the current model is that it is completely based on cell lines. Insulinoma cells do not have the real beta cell phenotype and can only be used for basic mechanistic studies. Therefore, the extend of exocrine damage in terms of beta cell stress on for example isolated islets or induced pluripotent stem cell (iPSC), possibly T1D patient, derived beta cells. Furthermore, the supernova based exocrine cell ablation could be translated to an in vivo model such as the zebrafish.

Conclusion

The trigger for T1D is unknown. Recent findings show that the exocrine pancreas is affected during T1D, which can already be observed before the onset of T1D. Our imaging studies revealed that intermediate exocrine-endocrine cells with stressed features have been observed in ‘pre-diabetic’ BB rats and early onset human T1D donor islets (Chapters 4b and 5a of this thesis). Results from a first functional follow-up pilot suggest that beta cells acquire a pro-inflammatory phenotype, by the increase of CXCL-10 expression, specifically upon stimulation with exocrine cell lysates. These first data encourage to further investigate a potential role for endocrine-exocrine interactions at the onset of T1D and subsequent model optimization would better reflect T1D associated beta cell stress upon exocrine cell damage.

Acknowledgments

Our work relevant to this chapter is supported by the Netherlands organization for scientific research (STW Microscopy Valley 12718), the Jan Kornelis de Cock Stichting and the European association for the study of diabetes (EASD).
Exocrine pancreas cell lysates specifically evoke beta cell stress

References


Supplementary information

Supplementary figure 1 | No increased spliced XBP-1 expression by RIN-m5F cells upon exocrine cell stimulation. No changes in the ER stress marker XBP-1s mRNA expression by RIN-m5F cells could be observed upon stimulation with exocrine and beta cell lysates or LPS treatment.

Supplementary table 1 | RT-qPCR primer sequences used for this study.

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