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Expression of Human Leukocyte Antigen Class I in Endocrine and Exocrine Pancreatic Tissue at Onset of Type 1 Diabetes

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The cause of type 1 diabetes remains unknown. To dissect the link between hyperexpression of human leukocyte antigen (HLA) class I on the islet cells, we examined its expression in subjects with recent-onset type 1 diabetes. IHC showed seemingly pronounced hyperexpression in subjects with recent-onset type 1 diabetes, as well as in some nondiabetic subjects. In all subjects, HLA class I expression on exocrine tissue was low. However, no difference in the level of HLA class I expression was found between islet and exocrine tissue using Western blot, flow cytometry, real-time quantitative PCR, or RNA sequencing analyses. Also, the level of HLA class I expression on the messenger level was not increased in islets from subjects with recent-onset type 1 diabetes compared with that in nondiabetic subjects. Consistently, the HLA class I specific enhanceosome (NLRC5) and related transcription factors, as well as interferons, were not enhanced in islets from recent-onset type 1 diabetic subjects. In conclusion, a discrepancy in HLA class I expression in islets assessed by IHC was observed compared with that using quantitative techniques showing similar expression of HLA class I in islets and exocrine tissue in subjects with recent-onset type 1 diabetes, nor could any differences be found between type 1 diabetic and nondiabetic subjects. Results presented provide important clues for a better understanding on how this complex disease develops. (*Am J Pathol* 2015, 185: 129–138; <http://dx.doi.org/10.1016/j.ajpath.2014.09.004>)

The cause of type 1 diabetes remains unknown. The disease seems to be a result of a complex interplay between genetic predisposition, the immune system, and environmental factors.¹

Pancreas is a difficult organ to study, and the methodological options in studying living islets are extremely limited. Most published studies describe pancreatic materials collected post-mortem. An updated review summarizes the present knowledge of the morphological characteristics and insulinitis in type 1 diabetes.² Although these studies have provided new insight in the inflammatory process in humans with type 1 diabetes, these materials have several disadvantages because of post-mortem autolysis, patient heterogeneity, and lack of clinical information. In The Diabetes Virus Detection (DiViD) Study, fresh pancreatic tissue was collected from living newly diagnosed type 1 diabetic patients shortly after diagnosis, and used in the present study.³

The focus of type 1 diabetes research has mainly been concentrated on the endocrine pancreas. However, recently, the exocrine pancreas has attained more interest.^{4–6} A

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reduction in total pancreatic volume seems present already at the time of diagnosis when compared with nondiabetic healthy volunteers,^{7–9} and a subclinical exocrine deficiency has also been reported.^{10–14} Morphological examinations of the pancreas from subjects with type 1 diabetes show considerable engagement of the exocrine pancreas.^{4–6} Collectively, available information demonstrates that type 1 diabetes in humans is a pancreatic disease, with its main clinical manifestations emanating from the loss of the insulin-producing cells.

Still, a consistently reported finding in subjects with type 1 diabetes is hyperexpression of human leukocyte antigen (HLA) class I in the islets of Langerhans, whereas this expression seems almost absent on the exocrine cells.^{6,15–24} Also, in the area of solid organ transplantation, similar

observations have been reported.²⁵ HLA class I molecules are ubiquitously expressed on the surface of almost all nucleated cells, and their surface density determines, to a large extent, the function and strength of the CD8⁺ T-cell–dependent immune surveillance of all cells within our bodies. Expression of HLA class I is regulated by cell type–specific factors,^{26–29} but can also be up-regulated by various cytokines [interferon (IFN) $\alpha\beta\gamma$ and tumor necrosis factor (TNF) α].^{30–32} In contrast, HLA class I down-regulation occurs via an array of mechanisms by viruses (adenovirus, cytomegalovirus, human papilloma virus, HIV, and hepatitis C virus) and constitutes a viable strategy to promote immune escape.^{33,34} However, this down-regulation is a double-edged sword because it may also trigger natural killer cell–mediated lysis of the infected cells.³⁵

Table 1 Donor Characteristics

Donor no.	T1D (weeks from diagnosis)	T2D	Autoantibodies	Age, years	BMI, kg/m ²	HbA1c, % (mmol/mol)	HLA exo*	HLA endo*
T1-1	Y (0)	N	Neg	40	27.2	ND	0	1–2
T1-2	Y (0)	N	Neg	29	24.2	10.4	1	2–4
T1-3	Y (4)	N	GAD/INS/ZnT8/IA2	25	21	6.7 (50)	0	4
T1-4	Y (3)	N	GAD/ZnT8/IA2	24	20.9	10.3 (89)	0	2–4
T1-5	Y (9)	N	GAD/ZnT8/IA2	34	23.7	7.1 (54)	2	4
T1-6	Y (5)	N	GAD/INS/IA2	31	25.6	7.4 (57)	0	2–4
T1-7	Y (5)	N	GAD/INS/IA2	24	28.6	7.4 (57)	0	1–4
T1-8	Y (5)	N	GAD	35	26.7	7.1 (54)	2	4
T2-1	N	Y	ND	54	35.1	ND	0	3–4
T2-2	N	Y	Neg	74	34.6	ND	0	2
T2-3	N	Y	ND	70	29.4	ND	0	2
T2-4	N	Y	Neg	16	29.2	ND	0	1–3
T2-5	N	Y	Neg	74	23.9	6.3 (45)	0–1	2–4
T2-6	N	Y	Neg	64	33.3	7.3 (56)	1	2–3
T2-7	N	Y	Neg	45	23.4	7.3 (56)	4	2
T2-8	N	Y	Neg	69	28.4	6.4 (46)	1–2	2
T2-9	N	Y	Neg	67	22.9	6.3 (45)	1–2	2–4
Ab-1	N	N	GAD/IA2	38	19.4	5.3 (34)	1	3–4
Ab-2	N	N	GAD/IA2	70	22.5	5.2 (33)	0–1	3–4
Ab-3	N	N	GAD	52	32.1	5.8 (40)	0–1	2–4
Ab-4	N	N	GAD	31	24.8	5.7 (39)	0–1	2–4
Ab-5	N	N	GAD	74	27.2	5.8 (40)	1–2	3–4
Ab-6	N	N	GAD	52	28.4	5.5 (37)	ND	ND
C-1	N	N	Neg	47	22.2	6.1 (43)	0–1	3–4
C-2	N	N	Neg	72	25.2	4.2 (22)	0–1	3–4
C-3	N	N	Neg	65	22.9	5.6 (38)	1–2	3
C-4	N	N	ND	66	42.2	ND	0	2–3
C-5	N	N	ND	54	41.8	ND	0	2
C-6	N	N	ND	56	40.7	ND	0–1	3–4
C-7	N	N	Neg	18	19.6	ND	0	1–2
C-8	N	N	ND	20	24.1	ND	0	3
C-9	N	N	ND	19	23.1	ND	0	2–4
C-10	N	N	ND	22	24.4	ND	0	2
C-11	N	N	Neg	8	20.4	5.2 (33)	1	2
C-12	N	N	Neg	22	28.9	5.5 (37)	2	3
C-13	N	N	Neg	25	22.9	5.9 (41)	0–1	3
C-14	N	N	Neg	20	27.8	5.8 (40)	0	2–3

*Intensity of staining was evaluated visually and graded from 0 (negative) to 4 (intense).

BMI, body mass index; endo, endocrine; exo, exocrine; GAD, glutamic acid decarboxylase; HbA1c, glycated hemoglobin; IA2, insulinoma associated protein 2; N, no; ND, not determined; Neg, negative; Y, yes.

Hyperexpression of HLA class I on the islet cells has so far only been reported using immunohistochemical (IHC) techniques. IHC is not a quantitative technique, and results obtained depend on several factors other than the actual level of expression of the targeted antigen.³⁶ Therefore, the aim of the present study was to examine the expression of HLA class I on the insulin-producing cells using quantitative techniques on both protein and messenger levels in eight subjects examined 1 to 60 days after diagnosis of type 1 diabetes and to compare obtained findings with those in the exocrine cells and with those in nondiabetic control islets.

Materials and Methods

Ethics Statement

All work involving human tissue was conducted according to the principles expressed in the Declaration of Helsinki and in the European Council's Convention on Human Rights and Biomedicine. Written informed consent was given by the patients with type 1 diabetes (T1D), and the DiViD Study (<http://www.clinicaltrials.gov>, study NCT01129232, last accessed August 28, 2014) was approved by the Governmental Regional Ethics Committee (Oslo, Norway). Consent for organ donation (for clinical transplantation and for use in research) was obtained verbally from the deceased's next of kin by the attending physician and documented in the medical records of the deceased in accordance with Swedish law and as approved by the Regional Ethics Committee. The study was approved by the Regional Ethics Committee (Uppsala, Sweden), according to the Act Concerning the Ethical Review of Research Involving Humans.

Human Pancreatic Specimens

Laparoscopic pancreatic tail resections were performed on six live patients with recent-onset T1D in the DiViD Study (donors T1-3 to T1-8) (Table 1). Biopsy specimens from the pancreatic tail resections and from the head region of the organ donor pancreases were immediately fixed in 4% paraformaldehyde or immersed in liquid nitrogen and subsequently stored at -80°C . The clinical characteristics of these patients have been described previously.³ Pancreatic specimens from multiorgan donors that died at onset of T1D ($n = 2$),³⁷ with type 2 diabetes (T2D) ($n = 9$), with autoantibodies against IA2 (insulinoma associated protein 2) and/or glutamic acid decarboxylase 65 ($n = 6$), and without pancreatic disease or diabetes-related autoantibodies ($n = 14$), were obtained through the Nordic Network for Clinical Islet Transplantation (Table 1). These cases were used for analysis of HLA class I protein expression by IHC. All cases with T1D and three nondiabetic controls were used for the analysis of HLA class I RNA expression in handpicked islets. Two T1D donors (cases T1 and T2) and four nondiabetic controls were used for analysis of HLA class I RNA expression in laser-captured islets. Five nondiabetic controls were used for analysis of HLA class I expression by flow cytometry.

Islet Isolation

The most distal part (0.5 to 1 cm) of laparoscopic pancreatic tail resections performed at Oslo University Hospital (Oslo, Norway) was shipped in cold organ preservation solution (Viaspan, UW solution; Bristol-Myers Squibb AB, Solna, Sweden) 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.³⁸ Basically, the pancreatic duct was located under a surgical microscope and cannulated with a fine catheter, and collagenase (Liberase; Roche, Indianapolis, IN) was injected and digested at 37°C for 30 minutes. Islets (300 to 700) from each patient were handpicked under a microscope.

Islets from the two brain-dead organ donors with acute-onset T1D and donors without pancreatic disease were isolated and cultured as described previously.^{37,38}

RNA Extraction and Whole Transcriptome Sequencing

RNA was extracted with the AllPrep DNA/RNA/Protein Mini Kit (Qiagen, Stockholm, Sweden) from 50 to 100 islets per subject, immediately after handpicking from the digested pancreatic tail resections, or after storage of isolated islets from the multiorgan donors on day 1 after isolation at -80°C in RNeasy Lysis Buffer (Qiagen, Stockholm, Sweden). The extracted RNA was of good quality (RNA Integrity Number values between 7.1 and 9.5) and enough quantity ($>1\ \mu\text{g}$) for performing whole transcriptome sequencing. Fragment library construction, sequencing on an AB SOLiD 5500xl-W system, and mapping of reads were performed by the Uppsala Genome Center (Rudbeck Laboratory, Uppsala, Sweden).³⁹ Data for *HLA-A*, *HLA-B*, *HLA-C*, and selected other genes are presented herein as reads per kilobase per million mapped reads. The entire data set will be published in a manuscript currently in preparation and made publicly available.

Expression levels for *HLA-A*, *HLA-B*, *HLA-C*, and selected other genes were extracted from the transcriptome data, acquired at the Human Protein Atlas (Uppsala, Sweden) by Illumina (San Diego, CA),⁴⁰ from exocrine and endocrine pancreatic tissue isolated from four subjects without pancreatic disease. The data are presented as fragments per kilobase per million mapped reads.

IHC Analysis

Sections (6 μm thick) from formalin-fixed, paraffin-embedded pancreatic biopsy specimens were processed and stained using a standard immunoperoxidase technique. After heat-induced epitope retrieval, the sections were stained with an anti-HLA class I ABC antibody (clone EMR8-5; dilution 1:300; Abcam, Cambridge, UK) visualized with Dako EnVision (Dako, Glostrup, Denmark). The dilution was optimized to minimize unspecific staining, and no staining was observed after removal of the primary antibody. The sections were counterstained with hematoxylin

Figure 1 staining for HLA class I on formalin-fixed pancreatic sections from patients with recent-onset type 1 diabetes (**A** and **B**), nondiabetic organ donors (**C**, **E**, and **F**), and an organ donor with type 2 diabetes (**D**) shows a distinct staining in islets and a relatively weak staining of exocrine cells, except in **D**. In frozen pancreatic sections, the expression of HLA class I is more evenly distributed between acinar and endocrine cells. **F**: From the same donor as in **E**.

and analyzed by light microscopy. Intensity of staining was evaluated visually and graded from 0 (negative) to 4 (intense) by a blinded investigator (O.S.). Consecutive sections (8 μ m thick) from frozen pancreatic biopsy specimens were treated and stained in the same way (but without heat-induced epitope retrieval) or mounted on PEN-membrane glass slides (Arcturus; Life Technologies, Paisley, UK) and used for laser-capture microdissection (LCM).

LCM and qPCR

LCM was performed using a protocol modified from Marselli et al.⁴¹ Briefly, frozen pancreatic sections were fixed in 70% ethanol for 30 seconds, rinsed by five dips in RNase-free water, stained with HistoGene Staining Solution (Life Technologies) containing 500 U/mL Superase (Life Technologies) for 1 minute, rinsed again in RNase-free water as above, dehydrated twice in 100% ethanol, and once in xylene for 5 minutes. After letting the slides air dry for 5 minutes, an Arcturus^{XT} microdissection instrument was used to microdissect islets and exocrine tissue. Microdissected tissue was extracted from the LCM cap by incubation in buffer RLT plus (Qiagen) with β -mercaptoethanol at 42°C for 30 minutes, followed by RNA extraction using an RNeasy Plus Microkit (Qiagen). The RNA was primed with random primers (Invitrogen, Carlsbad, CA) and transcribed to cDNA by SuperScript II RT (Invitrogen), according to the

manufacturer's instructions. Locus-specific quantitative real-time PCR (qPCR) for HLAs A, B, and C was performed, as described by Garcia-Ruano et al,⁴² with Power SYBR Green master mix (Life Technologies) on a Step One Plus Real-Time PCR system (Life Technologies). Predesigned gene-specific primer sets (QuantiTect Primer Assays; Qiagen) were used for detection of INS, RRN18S, and glyceraldehyde-3-phosphate dehydrogenase cDNA. PCR specificity was verified by melt curve analysis of all PCR products.

Western Blot Analysis

Islet preparations containing 35% to 85% endocrine cells (estimated by dithizone staining and computer-assisted digital image analysis⁴³), and exocrine preparations containing <5% endocrine cells, from four organ donors without pancreatic disease were lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich, St. Louis, MO) supplemented with protease inhibitors (Sigma-Aldrich) for 20 minutes at 4°C while rocking. Protein concentration was measured by Qubit (Invitrogen), and 30 μ g of each protein lysate was loaded onto a 10% to 20% Criterion Precast SDS-PAGE gel (BioRad, Hercules, CA). Proteins were then transferred to a polyvinylidene fluoride membrane (BioRad). The membrane was first incubated with a mouse anti-HLA class I ABC antibody (clone EMR8-5, 1:500; Abcam) and after stripping (0.2 mol/L NaOH for 5 minutes) with a rabbit anti-insulin/proinsulin antibody (HPA4932, dilution 1:500; Atlas Antibodies, Stockholm, Sweden). The primary antibodies were incubated at room temperature for 1 hour. After washing, horseradish peroxidase-conjugated secondary antibodies (anti-rabbit, dilution 1:3000, and anti-mouse, dilution 1:7000; Dako) were added for 1 hour. Immobilon Western chemiluminescent horseradish peroxidase substrate from Millipore (Billerica, MA) was used. Chemiluminescence was detected by a charge-

Figure 2 Expression of HLA class I in pancreatic cells was analyzed by flow cytometry using single cells from isolated human islets and exocrine tissue, cultured with or without the addition of IL-1 β /IFN- γ (IFNG). Expression of HLA class I in insulin-positive (beta cells) and insulin-negative (mainly exocrine cells) pancreatic cells is visualized in the histograms. Representative data from two of five analyzed nondiabetic donors are shown. FSC, forward scatter; SSC, side scatter.

Figure 3 Expression of HLA class I in pancreatic tissue enriched for endocrine (Endo) and exocrine (Exo) cells was analyzed in four organ donors without pancreatic disease. Extracted proteins were separated by SDS-PAGE, and HLA class I and (pro)insulin were detected by Western blot analysis. The same membrane was stained with amido black for total protein loading.

coupled device camera (BioRad). The membrane was also stained for total protein using amido black, confirming that similar amounts of protein were loaded from the endocrine and exocrine preparations from each donor and allowing a comparison of HLA class I expression between the two types of tissue. The intensities of the bands were quantified by densitometric scanning using Kodak Digital Science 1D software version 3.0 (Eastman Kodak, New Haven, CT).

In Vitro Experiments and Flow Cytometry

To test the effect of substances that could potentially be responsible for the up-regulation of HLA class I in T1D islets, isolated pancreatic tissue enriched for islets (50% to 70%) was cultured in 10-mL islet culture media³⁸ with the addition of 50 µg/mL poly(I:C) (Sigma-Aldrich), a combination of heat-inactivated Gram-positive and Gram-negative bacteria (10^6 colony-forming units/mL each of *Enterococcus faecalis* strain B1008314 and *Escherichia coli* strain S0705198),³⁷ endotoxin (lipoteichoic acid and lipopolysaccharide; 15 µg/mL; InvivoGen, Toulouse, France), inflammatory cytokines (IFN-γ and IL-1β; 20 ng/mL), or high glucose (20 mmol/L) for 24 hours. Islets cultured in 10 mL CMRL-1066 supplemented with 50 mg/mL gentamicin (Gibco BRL, Invitrogen Ltd, Paisley, UK), 20 mg/mL ciprofloxacin (Bayer Healthcare AG, Leverkusen, Germany), and 2% fetal bovine serum were infected for 72 hours with 1000 50% tissue culture infective dose (1000 times the dose that induces cytopathic effect in 50% of inoculated wells of green monkey kidney cells) of CBV3 strain Nancy or CBV4 strain JVB, as described previously.⁴⁴

After the *in vitro* experiments, the pancreatic islets and exocrine cell clusters were washed once in phosphate-buffered saline and dissociated by Accutase (Sigma-Aldrich) treatment in culture dishes for 10 minutes at 37°C, with occasional gentle pipetting and visual inspection under a light microscope. To ensure that HLA class I expression was not affected by this enzymatic treatment, peripheral blood mononuclear cells were subjected to the same handling and compared with untreated peripheral blood mononuclear cells. Single-cell suspensions were rinsed and incubated for 1 hour at 37°C in phosphate-buffered saline supplemented with 10% goat serum. Surface staining was conducted for 30 minutes at 4°C using fluorescently labeled (allophycocyanin) anti-HLA class I ABC (clone W6/32; BioLegend, San Diego, CA). After washing, permeabilization using FoxP3 permeabilization kit (eBiosciences, San Diego, CA) was performed according to the manufacturer's instructions. Intracellular insulin staining using anti-insulin-PE (R-phycoerythrin) antibody (Cell Signaling, Danvers, MA) was done for 50 minutes at room temperature. Cells were analyzed on a BD FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) using FACSDiva software version 6.0.

Figure 4 mRNA expression of the genes encoding HLA class I: *HLA-A* (black bars), *HLA-B* (gray bars), and *HLA-C* (white bars) extracted from whole transcriptome analysis of endocrine (endo) and exocrine (exo) pancreatic tissue from four donors without pancreatic disease (A) and of isolated islets from two organ donors that died at onset of type 1 diabetes, six patients 3 to 9 weeks after type 1 diabetes diagnosis, and three organ donors without pancreatic disease (B). FPKM, fragments per kilobase per million mapped reads; RPKM, reads per kilobase per million mapped reads.

Results

HLA Class I Protein Expression Assessed with IHC

In formalin-fixed, paraffin-embedded pancreatic sections labeled with an antibody against HLA class I, the endocrine tissue displayed moderate to intense staining, whereas the

exocrine tissue was negative or weakly stained in most subjects (Figure 1, A–C and E). Intensely stained islets were present in 7 of 8 subjects with recent-onset T1D (Figure 1, A and C, and Table 1), in 3 of 9 donors with T2D, in 5 of 6 donors with diabetes-related autoantibodies, and in 4 of 14 control donors. In the donors with T1D, most islets were stained intensely (Figure 1, A and C), but islets with weaker staining were also present (Figure 1B). In four of eight donors with T1D, acinar cells in close proximity to the islets were also positive (Figure 1A). This staining pattern was not found in any of the subjects without T1D. Intense staining of exocrine tissue was present in one donor with T2D (Figure 1D), but this was not a general finding. Frozen pancreatic sections stained with the same antibody against HLA class I displayed a more evenly distributed expression in endocrine and exocrine cells (Figure 1F).

HLA Class I Protein Expression Assessed with Flow Cytometry

CD3⁺ T cells examined with or without enzymatic and mechanical treatment showed the same level of expression of HLA class I on the cell surface. Flow cytometry analysis of HLA class I expression in single-cell suspensions of the pancreatic tissue revealed no hyperexpression of HLA class I in beta cells compared with other pancreatic cells (Figure 2). Twenty-four hour culture in the presence of IFN- γ /IL-1 β increased the expression of HLA class I in all pancreatic cells (Figure 2). Culture with bacteria, bacterial components, or double-stranded RNA had no effect on the HLA class I expression, whereas a slightly decreased expression was noted in beta cells infected with enterovirus (data not shown).

HLA Class I Protein Expression Assessed with Western Blot Analysis

Western blot analysis of isolated endocrine and exocrine pancreatic tissue from four donors without pancreatic disease revealed no difference in expression level of HLA class I between these tissues (Figure 3).

HLA Class I RNA Expression Assessed with RNA Sequencing

Analysis of data from RNA sequencing transcriptome analysis revealed no difference in the mRNA expression

Figure 5 mRNA expression of transcription factors and other regulators involved in expression of HLA class I genes and genes encoding TNF (*TNF*), type I IFN (*IFNA1*, *IFNA2*, *IFNA4*, *IFNA5*, *IFNA6*, *IFNA7*, *IFNA8*, *IFNA10*, *IFNA13*, *IFNA14*, *IFNA16*, *IFNA17*, *IFNA21*, *IFNB1*, *IFNK*, and *IFNW1*), and type II IFN (*IFNG*) in isolated endocrine (endo) and exocrine (exo) pancreatic tissue from four donors without pancreatic disease (A) and in isolated islets from two organ donors that died at onset of type 1 diabetes, six patients 3 to 9 weeks after type 1 diabetes diagnosis, and three organ donors without pancreatic disease (B). FPKM, fragments per kilobase per million mapped reads; RPKM, reads per kilobase per million mapped reads.

levels of HLA A, B, or C between endocrine and exocrine pancreatic tissue in nondiabetic controls (Figure 4A). Also, isolated pancreatic islets from two organ donors that died at onset of T1D, six live patients with recent-onset T1D, and three nondiabetic organ donors expressed comparable levels of HLAs A, B, and C, with a tendency of higher HLA expression in the nondiabetic subjects (Figure 4B). No difference in mRNA expression was found for the major histocompatibility complex class I—specific enhanceosome and related transcription factors in isolated islets when compared with exocrine tissue or between diabetic and nondiabetic subjects (Figure 5). Likewise, no difference in mRNA expression was found in the expression of cytokines known to up-regulate HLA expression in isolated islets when compared with exocrine tissue or between diabetic and nondiabetic subjects (Figure 5).

HLA Class I RNA Expression Assessed with LCM and qPCR

qPCR on RNA extracted by LCM from insulin-containing islets, showing distinct hyperexpression of HLA class I according to IHC analysis, revealed no increased mRNA expression of HLA A, B, or C compared with exocrine tissue from the same recent-onset type 1 diabetic donor or compared with islets or exocrine tissue from nondiabetic control donors (Figure 6).

Discussion

The present study confirms previous findings showing seemingly pronounced and distinct hyperexpression of HLA class I, assessed with IHC, on the islet cells in subjects with recent-onset type 1 diabetes.^{6,15–24} Also, a high expression of HLA class I on the islet cells was found in some subjects without type 1 diabetes. However, no evidence of hyperexpression of HLA class I on the islet cells was found using quantitative techniques when compared with that on the exocrine cells. Also, the level of HLA class I expression on islet cells in subjects with type 1 diabetes was not increased compared with that in nondiabetic subjects.

It is important to consider that the HLA class I protein expression is the result of multiple factors, and that the HLA class I RNA expression reflects the situation at the time of sampling but also during the time of handling and processing the samples. Herein, care was taken to immediately reduce biopsy temperature and thereby also the metabolism in the tissues to minimize this effect. The fact that mRNA and protein expression can diverge is well documented. This is a major criticism of chips and RNA assessments, where validation on the protein level is usually required. When analyzing isolated islets, it has to be considered that the process of isolation may alter the expression of multiple genes (including those encoding

HLA class I). Therefore, LCM of islets and exocrine tissue from frozen tissue sections was applied to verify the obtained findings.

IHC is the most commonly used method to display protein expression in tissues. However, the pattern of expression in composite tissues, such as the pancreas, should be interpreted with caution, because obtained results critically depend on the accessibility of the epitope(s) recognized by the primary antibody. Availability of epitopes on proteins in tissue sections varies markedly between tissues, type of fixation, and staining technique.³⁶ This can partly be circumvented by the use of several different primary antibodies. However, the primary antibody used herein is widely used and, by using IHC, we confirm the previously reported hyperexpression of HLA class I in islets.^{6,15–24} However, IHC on frozen sections provided an indication of a high expression of HLA class I also in the exocrine cells and conveyed a message that the previously reported hyperexpression of HLA class I on islet cells possibly could find its explanation in the formalin fixation technique used.^{16,45}

A high expression of HLA class I proteins on the cell surface may be due to a low turnover (ie, low internalization). This notion is interesting and would be an important field for future research. Another mechanism could be that stressed beta cells express cross-reactive proteins. The possibility to use fresh pancreatic tissues obtained during islet isolation offers several advantages, and Western blot analysis showed abundant and equal amounts of HLA class I molecules on purified islets and exocrine cells. Also, flow cytometry on islet single cells was applied to study the expression of HLA class I on the cell surface. To enable this, single cells had to be generated by mild enzymatic digestion, followed by gentle mechanical shear forces.^{46,47} CD3⁺ T cells were examined with or without combined enzymatic and mechanical treatment and showed no change in the level of expression of HLA class I on the cell surface.

Figure 6 mRNA expression of the genes encoding HLA class I: *HLA-A* (black bars), *HLA-B* (gray bars), and *HLA-C* (white bars) in laser-captured islets from four donors without pancreatic disease and in two patients with T1D. The islets captured from the patients with type 1 diabetes stained intensely for HLA class I with IHC on serial sections. The mRNA expression is presented relative to the expression of the reference genes (*18S* and *GAPDH*).

The technique to prepare single cells for flow cytometry was, therefore, judged suitable to study the surface expression of HLA class I on islet and exocrine cells. Moreover, the monoclonal antibody used to detect HLA class I expression was applied in excess to allow the intensity of the obtained signal to reflect the number of HLA molecules on the cell surface of each cell analyzed. Islet cells showed high surface expression of HLA class I that was not different from that of the exocrine cells and resting CD3⁺ T cells. Surface expression of HLA class I on islet cells was increased by culturing the islets overnight in a cytokine blend of IFN- γ and IL-1 β . Also, a slight decrease of HLA class I expression was found on islets 3 days after *in vitro* enterovirus infection. Obtained results show that the HLA expression on islet cells can be modified as expected,^{30–32} and also validate that the flow cytometry technique applied readily detects even minor changes of expression of HLA on the cell surface.

A limitation of this study is that quantitative techniques on the protein level (flow cytometry and Western blot analysis) were only applied on nondiabetic subjects, because of the lack of pancreases available from subjects with recent-onset type 1 diabetes within our islet isolation facility during the past few years. To enable studies of the HLA class I expression on the islet cells during development of type 1 diabetes, pancreatic sections and RNA from isolated islets obtained from eight subjects within 1 to 60 days of diagnosis were analyzed. Notably, tissue-specific regulation of HLA expression, as well as induced hyperexpression, mainly occurs at the level of the transcript, whereas induced down-regulation of HLA occurs at both the transcript and translational levels. Therefore, determination of the level of messenger should allow accurate determination of HLA hyperexpression within the islets. Results presented show a similar level of expression of mRNA for HLAs A, B, and C in isolated islets obtained from subjects with recent onset of T1D and nondiabetic controls. Likewise, no difference in mRNA expression in isolated islets was found for the major histocompatibility complex class I–specific enhanceosome (NLRC5; alias CITA; and related transcription factors), IFN- α , IFN- β , IFN- γ , and TNF- α (ie, the main regulators^{26–29} and cytokines^{30–32} associated with induced hyperexpression of HLA class I on islet cells *in vitro*). The laser-capture technique was applied to further reinforce the study and to allow specific analyses of islets showing distinct morphological evidence of hyperexpression of HLA class I using IHC. Again, no evidence of HLA class I mRNA hyperexpression in islet cells when compared with that in the exocrine cells from the same individual could be found, nor could any differences be found between type 1 diabetic and nondiabetic subjects.

Islet hyperexpression of HLA class I has been suggested to be an important step in T1D development by providing a so-called fertile field for pre-existing autoreactive T cells.⁴⁸ Our findings suggest that this model may need revision,

which should be based on quantitative molecular techniques in addition to IHC.

In conclusion, we observed a discrepancy between HLA class I expression in the islets, as evidenced by IHC and HLA class I expression using quantitative techniques. Also, by using these techniques in samples obtained shortly after diagnosis of type 1 diabetes in young adult patients, we found a similar expression of HLA class I in islets and in exocrine tissue and that the HLA class I–specific enhanceosome (NLRC5) and related transcription factors, as well as IFNs, were not switched on. Results presented provide important clues for a better understanding on how this complex disease develops.

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O.S. designed the study, acquired data, and wrote the manuscript; S.K., A.W., and A.D. acquired data and contributed to discussion and writing; B.E. and T.B. performed the surgery in the DiViD Study and participated in writing of the manuscript; L.K. and K.D.J. contributed to data analysis and interpretation and writing of the manuscript; K.D.J. is principle investigator of the DiViD Study; O.K. designed the study, interpreted data, wrote the manuscript, and is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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