Mast cells infiltrate pancreatic islets in human type 1 diabetes

Short title: Mast cells in human insulitis

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Abstract

Background and aims. Beta cell destruction in human type 1 diabetes occurs through the interplay of genetic and environmental factors and is mediated by immune cell infiltration of pancreatic islets. The aim of the present study was to investigate the role of different inflammatory cells in the type 1 diabetes insulitis scenario. **Materials and Methods**. Pancreatic samples were obtained from the glands of 7 T1D, 7 T2D and 7 ND organ donors, with similar clinical features, and processed by standard electron microscopy techniques. **Results.** A greater amounts of mast cells were found to infiltrate pancreatic islets in human type 1 diabetic samples, than in control and type 2 diabetic pancreases. Evidence of mast cell degranulation was shown, and the extent of the infiltration was correlated with beta cell damage. In addition, we observed that histamine, the amine highly expressed in mast cells, could directly contribute to beta cell apoptosis by a caspase-independent pathway. **Conclusion.** These findings suggest that mast cells may be responsible, at least in part, of the immune-mediated beta cell alterations in human type 1 diabetes. If so, protection of beta cells in type 1 diabetes might benefit from inhibition of mast cell activation and degranulation.

Introduction

The development and progression of type 1 diabetes results from the autoimmune destruction of pancreatic beta cells. Triggering of this autoimmune process occurs through the interaction between genetic and environmental factors (1,2), but the exact stimuli and cells participating in this process remain to be determined. This limited understanding of the ultimate triggers and effectors of beta cell death in type 1 diabetes hampers the ongoing efforts to prevent or cure the disease. In most patients the early stages of type 1 diabetes are associated with an immune cell infiltration specifically targeting the islets of Langerhans, which has been termed insulitis (1-3). Well defined histological criteria to detect insulitis have recently proposed, i.e. ≥15 CD45+ (leucocyte common antigen) cells in a minimum of three islets of approximately 150 μm in diameter (3). There is also agreement that the majority of infiltrating cells are of lymphocytic nature, with a predominance of $CD3^+CD8^+$ cells in recent-onset type 1 diabetes and an increasing contribution by B lymphocytes ($CD20^+$) at later stages (4,5). Innate immunity also plays a role in this process. Thus, significant numbers of islet-infiltrating macrophages were observed in cases of recent onset type 1 diabetes (4,6), becoming the most prominent infiltrating cell type in islets devoid of insulin positive cells (5). More recently, the presence of neutrophils has been observed by electron microscopy and immune histochemical analysis in the pancreas of subjects with type 1 diabetes, both at onset and at later stages of the disease (7). In the present study we show that mast cells are an additional actor in the human type 1 diabetes islet infiltrating cell scenario. These innate immunity cells are normally localized near body surfaces and are consequently considered as first-line defenders against several environmental insults (8). Following activation through the classical IgE-dependent or the alternative non-IgE-dependent [via the Toll-Like Receptors –TLRs - system pathway (9)], mast cells play a key role in allergic reactions (8,10). They possess chemotactic as well as immunodulatory properties (8,11). Interestingly, a role of mast cells in autoimmune disorders such as Crohn's disease, multiple sclerosis and rheumatoid arthritis has been proposed (12,13), and these cells may also contribute to autoimmune diabetes in experimental animal models, such as the

BioBreeding rat (14,15). We hereby report that mast cells infiltrate pancreatic islets during insulitis in human type 1 diabetes and that the extent of the infiltration correlates with beta cell damage; in addition, we show that histamine, the amine highly expressed in mast cells, can directly contribute to beta cell death via a caspase-independent apoptotic pathway.

Materials and Methods

Histology: Pancreatic samples from 7 non-diabetic, 7 type 1 and 7 type 2 diabetic multiorgan donors were studied (see **Table 1** for details) with the approval of the local ethics committee at the University of Pisa. Type of diabetes was identified based on medical records, status of anti-GAD autoantibodies and/or morphological as well as ultrastructural appearance of pancreatic islets (**Supplemental Figure 1**). Samples for optical and electron microscopy were prepared as previously detailed (16). When mast cells were studied in semithin sections, staining was perfomed with a 1:1 mixture of tolouidin blue (1% in bidistilled water) and methylen blue (1% in bidistilled water). Identification of mast cells by light microscopy was mainly based on metachromatic staining by tolouidine blue. In some cases mast cells identification was also confirmed in paraffin-embedded sections by the use of a mouse anti-human mast-cell triptase monoclonal antibody, clone G3 (Chemicon – Millipore, Billerica, MA, USA). In the electron microscopy studies, mast cell identification was based on their particular ultrastructural appearance, including the monolobed nucleus, the surface architecture composed of narrow, elongated folds, the presence of typical cytoplasmic granules and the absence of cytoplasmic glycogen aggregates (17). Cell counts per square millimeter were performed in semithin sections of pancreatic tissue. The same cells were counted at the electron microscope in consecutive ultrathin section, in order to allow the control of ultrastructural characteristics of the identified cells (**Figure** 1). The count of inflammatory cells infiltrating pancreatic islets was performed on tissue sections where recognizable islets were identified and was performed in all islets. All counts were independently performed by two

investigators in a blinded way (samples were identified only by randomly assigned numbers). Apoptotic beta cells were identified based on the presence of marked chromatin condensation and/or blebs (**Supplemental Figure 2**), as reported earlier by us and others (18,19).

Islet isolation and incubation: Isolated islets were prepared from the pancreas of 15 independent non-diabetic multi-organ donors (age: 69±16 yrs; gender: 8M/7F; body mass index: 26.1±3.0 kg/m^2; cause of death: 12 cardiovascular events and 3 trauma; duration of intensive care unit stay: 3±2 days; pancreas cold ischemia time: 18±6 hours) by collagenase digestion followed by density gradient purification, as previously reported (19). After isolation, islets were maintained for 2–3 days in M199 medium, containing 5.5 mmol/l glucose, supplemented with 10% serum and antibiotics. Then, batches of approximately 1,000 islets were cultured for 72h in M199 medium supplemented with 10% (vol/vol) serum and 100 U/ ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin and 750 ng/ml amphotericin B, and either with or without the addition of 100 μmol/l histamine dihydrochloride. All the compounds were from Sigma-Aldrich (St. Louis, MO). In some experiments, the effect of caspase inhibition by 20 μM Z-VAD-FMK (Promega, Madison, Wisconsin, USA) was also tested.

Western blot experiments: Isolated human islets were lysed in a buffer containing 40 mmol/l Tris, 4% CHAPS, 7 mol/l urea, 2 mol/L thiourea, 1% DTT and supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The lysates were centrifuged to remove the cellular debris and the protein amount evaluated by the Bradford method. Western blot assay of caspase 9 and 3 activation in human islets incubated for 24h with histamine (see above) was performed as previously described (20). Briefly, equal amounts of total protein were heated at 100°C for 5 min, resolved by electrophoresis in 14% SDS-polyacrylamide gel and electroblotted onto nitrocellulose membranes. Immunodetection was performed after overnight incubation with antibodies for cleaved caspase 9 and 3 (Cell Signaling, Danvers, USA), and α-tubulin (Cell Signaling, Danvers, USA) was used as the loading control.

Gene expression studies: Gene expression was determined as described (16,19). Briefly, total RNA was extracted using the Purelink™ RNA mini kit (Life Technologies, Carlsbad, CA, USA) and quantified by absorbance at A260/A280 nm (ratio >1.9) in a NanoDrop 2000C. RNA integrity was assessed with an Agilent 2100 Bioanalyzer. For quantitative PCR experiments, total RNA was reverse-transcripted from 1 μg with SuperScript® VILO™ cDNA Synthesis Kit (Life technologies). The oligonucleotides of interest were obtained from assay-on-demand gene expression products (Life Technologies). Messenger RNA levels were quantified and normalized for β-actin in a VIIA7 analyzer (Life Technologies).

Mitochondrial studies. The activity of mitochondrial complex I was assayed in control and histamine-treated (72h) INS-1E cells (kindly provided by prof. Claes Wollheim) by using commercially available kits (Mitochondrial Complex I Activity Assay Kit, Millipore, Germany) following the instructions provided by the manufacturer. INS-1E cells were cultured in a humidified atmosphere containing 5% CO_2 in complete medium composed of RPMI 1640 supplemented with 10% heat-inactivated fetal calf serum, 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin, and 100 μg/ml streptomycin (21). The mitochondrial membrane potential ($\Delta\psi_m$) in control and histamine-treated INS-1 cells was measured cytofluorimetrically as previously described (22) with a Becton-Dickinson Facscan equipped with a Cell Quest software (Becton Dickinson, Franklin Lakes, New Jersey).

Statistical analysis. Results are given as mean±SD, and differences between groups were assessed by the two-tailed Student's t-test or the ANOVA test plus the Bonferroni correction, as appropriate. Unilinear regression analysis was also performed to evaluate correlations between selected parameters. A p value of less than 0.05 was considered statistically significant.

Results

Figure 2A-F reports representative images of mast cells identified by different techniques, showing the presence of this cell type in the non-endocrine tissue as well as in both the peri-insular and the

intra-insular regions. Quantification of mast cells in the non-endocrine tissue was performed by optical microscopy, and the count of these cells at the periphery and inside the islets was performed on both toluidine stained samples and by electron microscopy analysis. There were 8.2±2.5 per mm^2 and 17.5±3.7 per mm^2 mast cells respectively in control and type 1 diabetic pancreatic samples ($p < 0.05$), whereas the number of mast cells in type 2 diabetic specimens (6.6±1.3 per mm^2) was similar to that of controls. In agreement with previous reports (4,5,23,24) our data also showed that lymphocyte number was significantly ($p<0.05$) increased only in type 1 diabetic samples, whereas macrophages significantly ($p<0.05$) increased in both type 1 and type 2 diabetic samples with respect to controls (Lymphocites: ND 2.8±1.3; T1D 33.2±7.3; T2d 2.9±1.0 cells per mm^2. Macrophages: ND 5.2±1.8; T1D 12.4±1.3; T2D 18.1±3.0 cells per mm^2). To assess detailed features of islet cells and the presence of islet infiltrating mast cells, electron microscopy was used (**Figure 1**). The number of beta cells counted was 484, 447 and 469 respectively in non-diabetic, type 1 and type 2 diabetic pancreases. Cells with signs of apoptosis were 0.5±0.4% in control islets, 6.5±3.8% in type 1 diabetic islets and 4.9±1.5% in type 2 diabetic islets (both $p < 0.05$ vs controls). The proportion of islets with at least 1 mast cell was 27, 86 and 33% respectively in control, type 1 ($p < 0.05$) and type 2 diabetic cases (non-significant vs controls). Mast cell number per islet was 0.6±0.2 in control samples, 2.1±0.4 in type 1 diabetic specimens ($p < 0.05$), and 0.6±0.3 in type 2 cases. In addition, in control samples all the mast cells were located at the periphery of the islets, whereas in type 1 diabetes cases 44% of the mast cells were within the islets. Several mast cells in type 1 diabetes samples appeared to be at least partially degranulated (**Figure 3A-F**). In type 1 diabetic samples, number of mast cells per mm^2 of pancreatic tissue was inversely correlated with the duration of diabetes ($r = 0.82$, $p < 0.05$), indicating attenuation of the infiltration over time; however, the number of islet infiltrating mast cells was not affected by duration of the disease ($r = 0.21$). Interestingly, the amount of beta cells with signs of apoptosis was positively correlated with the number of mast cells around and in the islets ($r = 0.62$, $p < 0.05$).

Since histamine is one of the most abundant compounds stored by mast cells (8,13), we next tested if direct exposure of isolated non-diabetic human islets to this amine affects beta cell survival. After 72h incubation the proportion of beta cells with signs of apoptosis was 1.0±0.6% in control islets (out of 257 beta cells counted by electron microscopy) and 6.1±2.0% (out of 306 counted beta cells) in islets incubated with 100 μmol/l histamine ($p < 0.01$). In order to investigate the mechanisms involved, we tested the activation of caspases 9 and 3 by Western blotting. As shown in **Figure 4A**, no difference in cleaved caspases was observed in islets exposed or not to histamine. In line with these observations, the presence of a caspase inhibitor did not reduce the extent of beta cell apoptosis following histamine exposure, as assessed by electron microscopy (**Figure 4B**). Of note, an intrinsic form of caspase-independent apoptosis has been described (25). In this case, molecules such as the apoptosis-inducing factor (AIF) are released from the mitochondria, translocate to the nucleus and mediate DNA fragmentation (25). Intriguingly, we observed a significantly increased expression of AIF in human islets exposed to histamine (**Figure 4C).** The impact of histamine on mitochondria was confirmed by experiments performed in insulin-producing INS-1E cells. Thus, we noticed that a 72h incubation with 100 μmol/l histamine caused inhibition of mitochondrial complex I enzyme activity (**Figure 4D**) ($p < 0.05$) and depolarization of the mitochondrial membrane potential as assayed by cytofluorometry (**Figure 4E**) ($p < 0.01$).

Discussion

To the best of our knowledge, mast cell infiltration of human pancreatic islets has not been previously described in type 1 diabetic patients. Here we show that the number of these innate immune system cells is increased in type 1 diabetic islets, even in patients with long-term disease (> 20 years of diabetes), and correlates with the extent of beta cells with signs of apoptosis. These findings can not be explained by differences in cause of death, duration of intensive care unit stay, or pancreas cold ischemia time, that may affect the degree and composition of pancreas infiltration (26), since these variables were similar between non-diabetic and type 1 diabetic donors

(Supplemental Table 1). In addition, mast cell infiltration was not seen in islets of type 2 diabetic donors, suggesting that this feature is peculiar of type 1 diabetes. Finally, we demonstrate that histamine, the amine largely present in mast cells, can induce beta cell apoptosis, possibly by activating an intrinsic, caspase-independent apoptotic machinery. This pathway is characterized by mitochondrial dysfunction and release of mitochondrial proteins such as AIF, that can directly fragment the DNA without the need of caspase activation (25). In line with this possibility, we observed that histamine upregulates AIF and that the use of a caspase inhibitor does not prevent beta cell apoptosis. Overall, the present results are coherent with the observation of human islet infiltration by other cells of the innate immune system (such as neutrophils) (7) and with the concept that mast cells may be involved in autoimmune processes, including experimental diabetes (12,14,15). It is noteworthy that mast cells are present in islets obtained from long-term diabetic individuals (present findings), suggesting that components of the innate immune response continue to contribute for beta cell dysfunction and death even after putative full activation of the adaptive immune response.

At the moment, it is not clear why mast cells move into the islets in type 1 diabetes. These cells are actively involved in the recognition of viruses and viral products (8). Since viral infections have been implicated in the pathogenesis of beta cell damage in type 1 diabetes (27), it is conceivable that the presence of a viral infection and local inflammation in the islets attract these cells. In line with this, mast cells are attracted to tissues expressing the chemokines CCL2, CCL3, CCL5, CXCL2 and CXCL 4, among others (28), and human islets exposed to viral infection (29) or to pro-inflammatory cytokines (30,31) express high levels of these chemokines.

Intriguingly, electron microscopy examination showed that at least some of islet-infiltrating mast cells are partially degranulated, indicating functional activation. Mast cells can be activated through the classical IgE-dependent or the alternative non-IgE-dependent activation via Toll-Like Receptors (TLRs) (9). In preliminary immunohistochemical experiments we have observed that the mast cells in the pancreatic tissue of type 1 diabetic patients are anti-IgE negative (LM and MM, personal

communication), favoring the view of an activation triggered via TLRs (9). Once activated, mast cells can recruit other inflammatory and immune cells (32) and/or directly damage beta cells by releasing histamine (as suggested by our results), both effects potentially contributing to beta cell dysfunction and death. In line with this hypothesis, histamine receptors are expressed in mouse and human pancreatic beta cells (33), and both pizotifen, an inhibitor of histamine action, and disodium-cromoglycate, an inhibitor of mast cell degranulation, suppress diabetes development in C57B1/6J mice treated with multiple low doses of streptozotocin (34).

In conclusion, the present observations suggest that mast cells are putative contributory cells for immune-mediated beta cell dysfunction and death in type 1 diabetes. In case these observations are confirmed by additional studies with larger number of samples, it will suggest that inhibitors of histamine action or mast cell degranulation might be considered as adjuvant therapies to protect beta cells in type 1 diabetes.

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AUTHOR CONTRIBUTIONS

LM, MM, DEL, PM and VDT conceived the study, designed the experiments, analyzed and interpreted data, and wrote the manuscript. UB, FF, MB, LM, MS and FS collected pancreas samples from organ donors and prepared isolated islets. LM, MM, DC and FD provided ultrastructural and morphometric data. LM, MB and TCN performed molecular studies. VDT 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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Legend to Figures

Figure 1. Consecutive semithin and ultrathin sections of a type 1 diabetic patient sample showing a pancreatic islets surrounded by an infiltrate containing different inflammatory cells (Mst = mast cell; Mc = macrophage; L = lymphocyte). The comparison between semithin and ultrathin consecutive sections illustrates how the identification of the different types of inflammatory cells at the optical microscope was further confirmed by their observation at the electron microscope.

Figure 2. Microphotographs obtained in type 1 diabetic pancreatic samples after immunocytochemistry (A and B), semithin section analysis (C) or electron microscopy (D-F). Mast cells are shown in the non-endocrine tissue (A: arrows, and D: indicated as MC), in the peri-insular region (B: arrows, and E: indicated as MC), and within islets (C: arrows, and F: indicated as MC)

Figure 3. Microphotographs obtained from type 1 diabetic pancreatic samples after semithin section analysis (A) or electron microscopy (B-C) to evaluate morphological evidence of mast cell activation. A: A degranulated mast cell in the peri-insular region; B: Mast cells showing signs of degranulation (arrows indicate mast cell granules and hollow granules in the cytoplasm of a mast cell); C: free mast cell granules (arrows) in close proximity of a beta cell.

Figure 4. Effects of histamine exposure (100 μmol/l for 72h) on isolated human islets (A-C) and INS-1E cells (D,E). A: No difference in the expression of cleaved (active) caspases 9 and 3 in human islets either not exposed (Control) or exposed to histamine; B: Proportion of beta cells with signs of apoptosis in control human islets and human islets exposed to caspase inhibitor alone, histamine alone or caspase inhibitor plus histamine; *: $p < 0.05$ (Bonferroni corrected) vs control islets and islets exposed to caspase inhibitor alone; C: Gene expression by qPCR of apoptosis induced factor (AIF; expression corrected for β-actin) in control islets and islets exposed to histamine; *: $p < 0.05$ by the Student's t-test; D: Mitochondrial complex 1 enzymatic activity in INS-1E cells exposed or not to histamine; *: $p < 0.05$ by the Student's t-test; E: Cytofluorimetric analysis of the mitochondrial membrane potential in INS-1E cells exposed or not to histamine.