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SHORT COMMUNICATION

Are we overestimating the loss of beta cells in type 2 diabetes?

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Abstract
Aims/hypothesis Previous work has demonstrated that beta cell amount (whether measured as beta cell mass, beta cell volume or insulin-positive area) is decreased in type 2 diabetes; however, recent findings suggest that mechanisms other than death may contribute to beta cell failure in this disease. To better characterise beta cell mass and function in type 2 diabetes, we performed morphological, ultra-structural and functional studies using histological samples and isolated islets.
Methods Pancreases from ten non-diabetic (ND) and ten matched type 2 diabetic organ donors were studied by insulin, glucagon and chromogranin A immunocytochemistry and electron microscopy (EM). Glucose-stimulated insulin secretion was assessed using isolated islets and studies were performed using independent ND islet preparations after 24 h exposure to 22.2 mmol/l glucose.
Results Immunocytochemistry showed that the fractional islet insulin-positive area was lower in type 2 diabetic islets (54.9±6.3% vs 72.1±8.7%, $p<0.01$), whereas glucagon (23.3±5.4% vs 20.2±5.3%) and chromogranin A (86.4±6.1% vs 89.0±5.5%) staining was similar between the two groups. EM showed that the proportion of beta cells in type 2 diabetic islets was only marginally decreased; marked beta cell degranulation was found in diabetic beta cells; these findings were all reproduced after exposing isolated ND islets to high glucose. Glucose-stimulated insulin secretion was 40–50% lower from type 2 diabetic islets ($p<0.01$), which again was mimicked by culturing non-diabetic islets in high glucose.
Conclusions/interpretation These results suggest that, at least in subgroups of type 2 diabetic patients, the loss of beta cells as assessed so far might be overestimated, possibly due to changes in beta cell phenotype other than death, also contributing to beta cell failure in type 2 diabetes.

Keywords Beta cells · Chromogranin A · Electron microscopy · Glucagon · Insulin · Insulin granules

Abbreviation
EM Electron microscopy

Introduction

Decreased beta cell functional mass is a hallmark of type 2 diabetes [1, 2]. Studies with pancreatic samples obtained at autopsy, from organ donors or after pancreatectomy have shown that beta cell mass is significantly reduced in type 2 diabetic individuals [2–6]. However, beta cell functional defects also play a major role in the pathophysiology of the disease [7], and improvements at this level may better explain, for instance, why 30–100% of patients who have undergone bariatric surgery may show diabetes remission within a few days after surgery and only a 1–2% weight loss [8]. In addition,

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recent work has shown that beta cell dedifferentiation may also contribute to beta cell failure in type 2 diabetes [9]. We therefore hypothesised that beta cell loss may be overestimated in type 2 diabetes, and performed morphological, morphometric, ultrastructural and functional studies with pancreatic tissue samples and isolated islets from non-diabetic and type 2 diabetic individuals to explore this possibility.

Methods

Cases Pancreases were obtained from heart-beating organ donors and handled with the approval of the local ethics committee. Pancreases were from ten non-diabetic (age: 68±4 years; sex: five men, five women; BMI: 26.3±1.6 kg/m^2) and ten type 2 diabetic (age: 65±7 years; sex: four men, six women; BMI: 26.7±2.3 kg/m^2; known duration of diabetes: 3–15 years; pharmacological treatment: metformin alone in five patients, metformin plus sulfonylurea in three patients, insulin in two patients) individuals. Non-diabetic donors had no history of diabetes; the mean plasma glucose value during their intensive care unit stay was 8.0±1.9 mmol/l (range: 4.9–10.6 mmol/l) and the mean fructosamine concentration was 244±23 μmol/l (range: 211–277 μmol/l). Islet preparations from three independent pancreases of non-diabetic donors (age: 65±4 years; sex: one man, two women; BMI: 25.3±1.9 kg/m^2) were also studied in selected experiments (see below).

Immunocytochemistry and electron microscopy Samples were taken at the level of the pancreatic neck to perform immunocytochemistry [10, 11] and electron microscopy (EM) [12] evaluations. For immunocytochemistry, sequential 4 μm sections were stained for insulin (guinea pig anti-insulin antibody, 1:100; Invitrogen, Carlsbad, CA, USA), glucagon (polyclonal rabbit anti-human glucagon antibody, 1:3,000 (Dako, Carpinteria, CA, USA) and chromogranin A (mouse monoclonal anti-chromogranin A antibody, 1 μg/ml; Ventana, Oro Valley, AZ, USA). The anti-insulin and the anti-glucagon primary antibodies were also tested at 1:50, 1:200 and 1:300, and at 1:1,000, 1:2,000 and 1:4,000 dilution, respectively, and the working dilution was chosen based on the balance between signal intensity and non-specific background staining (electronic supplementary material [ESM] Fig. 1). Biotinylated secondary antibody, which reacts with mouse, rabbit, guinea pig and rat primary antibodies, was purchased as Histostain-Plus kits (Invitrogen).

Morphometric analysis was performed by measuring insulin, glucagon and chromogranin A areas in the whole pancreatic tissue sections and in the islets (more than four insulin-positive cells). Since the weight of pancreas specimens was not known, mass values (pancreatic weight × fractional area positive for a given hormone) could not be determined and, in line with previous studies in humans [10, 11], the measurement of area was used as surrogate marker for cell mass. To perform islet count, the entire pancreatic sections were imaged using a Leica DM5500B microscope (Leica, Wetzlar, Germany) equipped with a motorised stage ×50 magnification (×5 objective lens). A tile image of the pancreatic section was generated by using Leica MetaMorph AF software, version 1.8, counts were performed and, in the case of borderline sizes, were confirmed at a magnification of ×400 (×40 objective lens; to discriminate between islets and clusters). The areas of islets and clusters stained positive for insulin, glucagon or chromogranin A were manually delimited by the operators at a magnification of ×400 by the use of the Trace Region Leica tool, values were obtained through the Region Measurement tool and calculations finally performed by Excel Office suite (Microsoft, Redmond, WA, USA) to determine the fractional areas. EM assessment [12] was performed on 5,311 islet cells in total to evaluate the proportion of beta cells; insulin granules morphometric analysis was performed with 661 beta cells in non-diabetic and 598 beta cells in diabetic samples.

Islet isolation and functional studies Isolated islets were prepared as described previously [12, 13], and insulin secretion determined by batch incubation (45 min) in response to 3.3 and 16.7 mmol/l glucose [12]. In addition, morphological, functional and ultra-structural studies were performed with islets from three independent non-diabetic donors after 24 h incubation with 22.2 mmol/l glucose.

Statistical analysis: Results are expressed as mean ± SD. Differences between groups were assessed by the two-tailed or paired Student's *t* test, as appropriate.

Results

The area of the pancreatic sections ranged from 63.1 to 139.2 mm^2. In accordance with previous studies [13, 14], light microscopy revealed that the islets varied in terms of size, insulin- and glucagon-positive areas, and they either retained a normal architecture or instead displayed sparse architecture (ESM Fig. 2). Overall, the number of islets trended lower in diabetic (10.9±2.6 per 10 mm^2) compared with non-diabetic (14.4±11.5 per 10 mm^2) samples. Average islet diameter did not differ between diabetic (101.4±42.0 μm) and non-diabetic (108.2±48.7 μm) samples. Fractional pancreatic insulin-positive area (0.47±0.13% vs 1.02±0.43) and fractional islet insulin-positive area (54.9±6.3% vs 72.1±8.7%, see representative images in Fig. 1a, e), were significantly lower (both $p<0.01$) in type 2 diabetic samples. Altogether, although obtained from a relatively small number of samples, these results largely confirm previous work reporting a decreased amount of beta cells in type 2 diabetes [2–6]. However, the main aim of the present study was to evaluate if this apparent

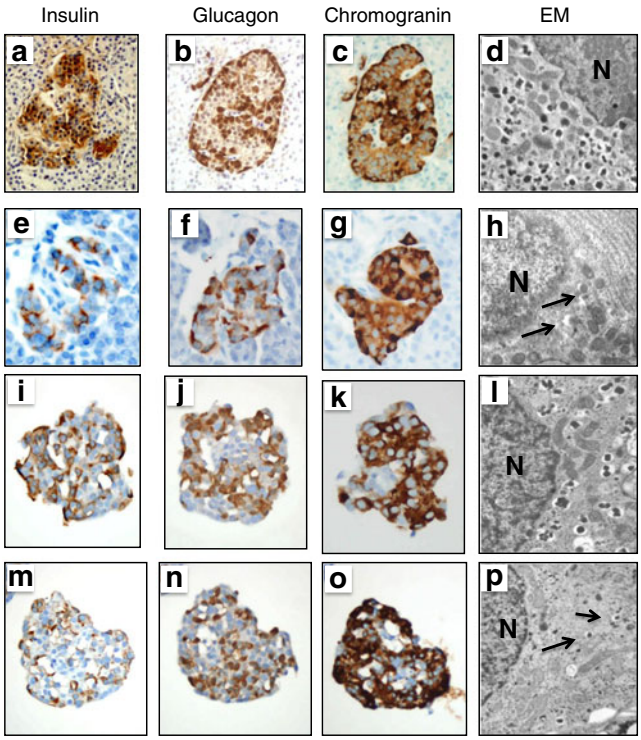

Fig. 1 Representative images of insulin, glucagon and chromogranin A immunostaining in: non-diabetic islet cells (**a**, **b** and **c**); type 2 diabetic islet cells (**e**, **f** and **g**); non-diabetic islet cells pre-exposed to normal glucose (5.5 mmol/l) (**i**, **j** and **k**); non-diabetic islet cells pre-exposed to high glucose (22.2 mmol/l) (**m**, **n** and **o**). Representative electron microscopy (EM) images of a non-diabetic normal beta cell (**d**), a type 2 diabetic beta cell (**h**), a non-diabetic beta cell pre-exposed to normal glucose (**l**) and a non-diabetic beta cell pre-exposed to high glucose (**p**); note the low number of insulin granules (arrows) in h and p. N, nucleus

loss of beta cells may be overestimated. Indeed, when beta cells were counted by EM (Fig. 1d, h), the approximately 10% decrease in type 2 diabetic specimens (63.0±8.6% vs 70.1±6.4%) was barely significant ($p=0.051$). In addition, beta cell proportion as measured by light and EM differed significantly in samples from the diabetic ($p=0.027$) but not from the non-diabetic ($p=0.56$) group. There were significantly ($p<0.01$) fewer insulin granules (Fig. 1d, h) in diabetic beta cells (3.1±0.2 vs 5.6±0.3 ml%). Glucagon-containing cells (Fig. 1b, f) were similarly represented in non-diabetic (light microscopy: 20.2±5.3%; EM: 21.3±6.6%) and diabetic (23.3±5.4% and 21.6±6.9%) samples. Intriguingly, chromogranin A (a marker of endocrine cells) expression was detected in a similar proportion of non-diabetic (89.0±5.5%) and type 2 diabetic (86.4±6.1%) islets, including cells negative for insulin (Fig. 1c, g).

When isolated non-diabetic islets were studied, 24 h exposure to high glucose reduced the insulin-positive area as assessed by light microscopy (Fig. 1i, m) (treated vs untreated: 49±11% vs 74±8%; $p=0.03$) but not as assessed by EM (67±5% vs 71±7%; $p=0.5$) (Fig. 1l, p). A marked reduction in insulin granules (Fig. 1p) was observed in beta cells from islets exposed to high-glucose. There was no change in glucagon staining (Fig. 1j, n) or chromogranin A staining (Fig. 1k, o) in response to high glucose exposure.

Insulin release (pmol/islet/min) at 3.3 mmol/l glucose did not differ significantly between ND (0.21±0.07) and type 2 diabetic (0.19±0.06) islets. However, insulin secretion at 16.7 mmol/l glucose was ~50% lower from the diabetic cells (0.24±0.03 vs 0.52±0.33, $p=0.04$); after pre-culture with high glucose, a decrease in glucose-stimulated insulin release (0.28±0.15 vs 0.61±0.13, $p=0.04$) was found, similar to that of type 2 diabetic islets.

Conclusions

Previous work has unequivocably revealed that the beta cell amount in human type 2 diabetes is reduced, mainly due to increased apoptosis, and the implications of this have been widely discussed [2–6, 15, 16]. In addition, in carefully performed examinations, morphological variations in type 2 diabetic islets have also been reported [13, 14]. The present study confirms the presence of such alterations by the use of insulin immunocytochemistry and the measurement of fractional pancreatic beta cell area (a surrogate of beta cell mass) [10]. More importantly, our results, based on parallel light and electron microscopy examinations of pancreatic samples from non-diabetic and diabetic donors, suggest that a proportion of beta cells in type 2 diabetic islets may not be detectable by standard immunohistochemistry staining, possibly due to insulin degranulation, potentially leading to an overestimation of beta cell loss, in at least some type 2 diabetic pancreases or islets. However, we confirmed the marked loss of glucose-stimulated insulin secretion function in type 2 diabetic islets [7, 17]. Interestingly, these results were largely mimicked by pre-exposure of non-diabetic islets to high glucose concentrations. Although the experimental condition we used (24 h incubation at 22.2 mmol/l glucose) is a simplistic attempt to reproduce what may happen to the beta cells in clinical diabetes, by doing so we were nevertheless able to show that the beta cell count was decreased as assessed by insulin staining but not as assessed by EM examination, and glucose-stimulated insulin secretion was blunted. This indicates that glucose-stimulated insulin secretion from human islets may be reduced without an actual loss of beta cells.

Although a relatively low number of pancreases were included in our study, the results are overall in agreement with recent work showing that changes in beta cell phenotype, including de-differentiation [9], may contribute to beta cell failure in type 2 diabetes in addition to apoptosis. On the whole, these findings support the concept that rescuing a beta cell functional phenotype could represent a major focus in the treatment of type 2 diabetic patients.

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Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement LM, MM and PM were involved in the conception and design of the study; LM, MS, MM, DC, MB, FSy, LM, DF, FSc, FO, FF, UB and PM were involved in the acquisition, analysis and interpretation of data; PMas was involved in the interpretation of data; LM and PM drafted the article; MS, MM, DC, MB, FSy, LM, DF, FSc, FO, FF, PMas and UB critically revised the article; all authors gave final approval of the version to be published.

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