Cytohistologic analyses of β cell dedifferentiation induced by inflammation in human islets

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Abstract
β cell dedifferentiation is a key mechanism for β cell dysfunction in type 2 diabetes mellitus (T2DM). Although it has been indicated in previous studies that β cell dedifferentiation could be induced by inflammation, the cytohistologic analyses of inflammation-induced β cell dedifferentiation in human islets is lacking. The present study aims to cytohistologically characterize the β cell dedifferentiation of human islets treated by proinflammatory cytokines Interleukin-1β/Tumor necrosis factor-α/Interferon-γ (IL-1β/TNF-α/IFN-γ), which is a frequently-used method to mimic the islet inflammation in previous studies. The loss of cytosolic FOXO1 expression, the loss of nucleic NKX6.1 expression, and the gain of ALDH1A3 expression in β cells are proclaimed as marking events for β cell dedifferentiation. Taking advantages of islets from organ donors and the immunofluorescence staining methods, the present study visualized the β cell dedifferentiation events marked by different markers, and quantified the frequency of each event as well. We successfully captured and described the characteristics of the differentiating/differentiated β cells. We found that dedifferentiated β cells were increased in the cytokines treated islets, evidenced by the increase of β cells with FOXO1 translocated to the nucleus (INS-FOXOnuc), β cells with NKX6.1 exported from the nucleus (INS+NKX6.1cyt), and β cells loss of NKX6.1 expression (INS−NKX6.1−), and β cells with dual expression of insulin and progenitor marker ALDH1A3. Consistently, we found that proinflammatory cytokines IL-1β/TNF-α/IFN-γ treatment reduced the mRNA expression of key β cell markers, but elevated the expression of progenitor marker genes. This study gives the most direct evidence for inflammation-induced β cell dedifferentiation in human islets, and supports the concept that anti-inflammation treatments may facilitate alleviating the β cell dedifferentiation in human T2DM islets.

Keywords
β cell dedifferentiation, IFN-γ, IL-1β, inflammation, TNF-α

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Introduction

Diabetes is characterized by hyperglycemia and insufficient insulin secretion from pancreatic \(\beta\) cells. \(\beta\) cell mass loss and functional failure are the root causes for the inadequate insulin production and the consequent diabetes onset.\(^1\)\(^-\)\(^3\) Our earlier and others’ studies have reported significant \(\beta\) cell mass loss in T2DM subjects.\(^4\)\(^-\)\(^9\) Although apoptosis is observed in diabetic islets in human pancreatic sections, the number of apoptotic cells did not match the function degradation of diabetic islets. In the last decade, several studies have discovered \(\beta\) cell dedifferentiation in both diabetic animals and human beings.\(^10\)\(^-\)\(^11\) \(\beta\) cell dedifferentiation has been proposed as a key mechanism for the \(\beta\) cell dysfunction, as well as functional mass loss, in diabetic islets.\(^12\)\(^-\)\(^16\) \(\beta\) cell dedifferentiation is manifested by the loss of expressions of \(\beta\) cell functionality and identity genes, and achieving the expression of pancreatic or endocrine progenitor cell markers.\(^12\)\(^-\)\(^16\) Previous studies have disclosed some pathological factors that cause \(\beta\) cell dedifferentiation. Our group previously found that hypoxic signaling played a role in \(\beta\) cell dedifferentiation through inducing the expression of mature \(\beta\) cell disallowed genes in human islets, such as \textit{ALDH1A3} and \textit{LDHA}.\(^17\) Nordmann et al. investigated the role of inflammation in \(\beta\) cell dedifferentiation and found that IL-1\(\beta\) can reduce the expression of the functional gene of beta cells in mice islets, as well as human islets, but increase the expression of progenitor cell marker gene.\(^13\) However, in that study only the mRNA expression change was presented, which failed to demonstrate the imaging of dedifferentiated \(\beta\) cells with the dis-location or loss of important markers for \(\beta\) cell identity and the re-expression of progenitor cell markers. IL-1\(\beta\) antibodies failed to prevent the progression from prediabetes to diabetes in CNATOS clinical trial,\(^18\) suggesting that IL-1\(\beta\) is not the only important factor. Inflammatory response is a series of complex reactions involving not only IL-1\(\beta\), but also other inflammatory factors such as TNF-\(\alpha\), IFN-\(\gamma\), and so on. This study puts forward the hypothesis that IL-1\(\beta\), TNF-\(\alpha\), IFN-\(\gamma\) jointly induce \(\beta\) cells to dedifferentiate. In this study, the isolated human islets were treated with three proinflammatory cytokines, and then their function and dedifferentiation level were examined, to prove the important function of the pro-inflammatory factor in the process of \(\beta\) cell dedifferentiation.

| Table 1. Donor information. |
|---------------------------|
| **Case** | **Age (Y)** | **Sex (M/F)** | **BMI (Kg/m²)** | **HbA1c (%)** |
| H092 | N/A | M | 32.7 | N/A |
| H095 | 55 | F | 23.9 | 5.6 |
| H110 | 30 | M | 26.8 | 5.4 |
| H113 | 40 | M | 29.3 | N/A |
| H114 | 45 | M | 24.5 | 5.5 |

Materials and methods

Organ donors information

Human pancreases were obtained during the period between December 2016 to December 2018 from 5 ND organ donors with written informed consents for research. Donor information were provided in Table 1. All protocols were approved by the Medical Ethical Committee of Tianjin First Central Hospital (Review number: 2016N077KY). This study is not clinical trials, but laboratory study on human islets.

Islet isolation

Islets were isolated by Collagenase NB1 (SERVA, Heidelberg, Germany) and Neutral Protease NB (SERVA, Heidelberg, Germany) digestion followed by continuous density purification. Islets with a purity over 90% were collected and cultured on CMRL-1066 medium (Corning, Manassas, VA, USA), supplemented with 10% Human Serum Albumin (Baxter, Vienna, Austria), 100 U/mL penicillin and 100 mg/mL streptomycin at 37°C in 5% CO2.

Immunohistochemistry and morphometric analysis

Human islets were fixed in 10% formalin, embedded in paraffin and sliced into consecutive 3-μm-thick sections. After deparaffinization, sections were treated with EDTA antigen retrieval solution (Solarbio, Beijing, China) in microwave oven, washed with PBS, permeabilized with 0.1% Triton-100, and blocked with 5% BSA, followed by incubation with anti-insulin (1:200, Abcam, Cambridge, MA, USA), anti-NKX6.1 (1:500, Novus, CO, USA), anti-FOXO1 (1:100, LSBio, WA, USA) anti-ALDH1A3 (1:500, Novus, CO, USA), primary antibodies, and then with secondary antibodies (Jackson Immunoresearch laboratories and Molecular Probes). Sections were
counterstained with DAPI. The stained sections were scanned by Pannoramic MIDI and images were captured by Pannoramic Viewer (3DHistech, Budapest, Hungary). Quantification analysis was performed in a blinded fashion using the CytoNuclear count of Image Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). Islets containing at least one interest protein positive stained cell were scored. Meanwhile, only cells that had a clearly labeled nucleus were counted and positive cells for each marker or showing colocalization of different markers were included only if they had a strongly stained cytoplasm or nuclei in islet.

**Cytokines treatment**

Human islets were treated with recombinant human cytokines, IL-1β (Peprotech, NJ, USA), TNF-α (Peprotech, NJ, USA), and IFN-γ (Peprotech, NJ, USA) for 48 h. The working concentration of these cytokines were 10 ng/mL, 25 ng/mL, and 100 ng/mL, respectively, according to our preliminary experiments.

**mRNA expression analysis**

RNA extraction and cDNA synthesis was performed using the RNeasy Mini Kit (QIAGEN, Dusseldorf, Germany) and PrimeScript RT reagent Kit with GDNA Eraser (Takara, Kohoku-cho, Kusatsu, Japan) respectively. Quantitative real-time qPCR was measured with SYBR Premix ExTaq II (Takara, Kohoku-cho, Kusatsu, Japan) using LightCycler96 System (Roche, Basel, Switzerland). Relative mRNA expression of different treatments was calculated by the 2^−ΔΔCT method. The primer sequences are shown in Table 2.

| Genes       | Primers(5′-3′)                  |
|-------------|---------------------------------|
| NEUROD1-F   | ATTGCACCCAGCCCTTCTTTGAT         |
| NEUROD1-R   | ACTCGGCGAGGCTGTGTTT            |
| MAF A-F     | GCTGTCAGGTTGCGACCTCT           |
| MAF A-R     | CTTCAAGCACAGGAGGGCTGCA         |
| ALDH1A3-F   | ATGTGGGAAAACCCCCCTGTG          |
| ALDH1A3-R   | GAATGCTGCCACCTTCACT            |
| NGN3-F      | CCTAAAGCCGAGGTTGCGACTGA        |
| NGN3-R      | AGTGGCCAGGGTTGGTGTC            |
| PDX1-F      | CGGAACCTTTTCTTTTTTGAGTGTG     |
| PDX1-R      | AAGATGTTGAAGGCTACCTGCGGCTC    |
| NNX6.1-F    | GGGCTCGTTGGCCTATTCGTT         |
| NNX6.1-R    | CCACCTTGCTCGCCGCGTTCT         |
| FOXO1-F     | TGACATGCTCAGCAGACATC           |
| FOXO1-R     | TTGGCTAGGCGGTTCA              |
| β-actin-F   | CGTACATCAAGGAGAACTG           |
| β-actin-R   | CTAGAACATTTGCCGGTGAC         |

The expression of β cell functional genes were examined by q-RT-PCR in human islets treated with or without the three cytokines. The results showed that the key β cell markers such as genes encoding major transcription factors, PDX1, NEUROD1, FOXO1, MAF A, and NNX6.1, were reduced in the cytokines treated islets compared to the control islets (Figure 1(a)–(e)). Meanwhile, we found that the markers of progenitor cells, including NGN3 and ALDH1A3 were upregulated in cytokines treated islets (Figure 1(f) and (g)). These results suggested that the maturity of β cells in the cytokines treated islets might be impaired.

**2. Proinflammatory cytokines treatment induces β cell dedifferentiation of human islets**

To test whether β cells were undergoing β cell dedifferentiation in cytokines treated β cells, the expression of dedifferentiation markers was examined by immunofluorescence staining followed by microscopic analysis. Firstly, the expression of FOXO1 was analyzed. In the untreated control islets, FOXO1 protein was mainly expressed in the cytoplasm of β cells, and few cells were found with nucleic FOXO1 expression (Figure 2(a) and (b)). However, in the cytokines treated islets, β cells with FOXO1 dislocation increased to 50.8% in all β cells per islet (Figure 2(a) and (b)). The expression of NNX6.1, another β cell specific transcription factor, were analyzed. In the
control islets, NKX6.1 was primarily expressed in the nucleus of β cells. However, in the cytokines treated islets, we detected an increased number of β cells with the cytosolic expression of NKX6.1 (Figure 3(a), white arrows) or loss of NKX6.1 expression (Figure 3(a), yellow arrowheads). These cells accounted for 5.89% (Figure 3(b)) and 29.78% (Figure 3(c)) of all β cells per islet in cytokines-treated islets, but only for 0 (Figure 3(b)) and 10.75% in control islets (Figure 3(c)).

We then examined the expression of ALDH1A3, which is a marker for progenitor cells and also a marker for β cell dedifferentiation. ALDH1A3 is a mature β cell disallowed gene, but its expression in β cells was increased in the cytokines-treated islets, and the β cells with ALDH1A3 expression (INS+ALDH1A3+) were increased (Figure 4(a)). Overall, the β cells expressing ALDH1A3 accounted for 36% in all β cells in the cytokines-treated islets, though the percentage in control islets were only 12.35% (INS+ALDH1A3+/INS+, Figure 4(b)). These results suggested that proinflammatory cytokines induced the dedifferentiation of β cells.

**Discussion**

T2DM is an inflammation-related disease. We previously found that the islet inflammation in T2DM islets was significantly higher compared
A lot of anti-inflammation drugs have been tested for their efficacy in treating diabetes, such as IL-1β antibody Canakinumab, TNF-α antagonist CDP571 and NF-kB inhibitor Salsalate, as well as mesenchymal stem cell-based therapies. In this study, to test whether the systematic inflammation is further amplified in the islet, we treated the purified human islets in vitro with three common proinflammatory cytokines in diabetes, that is, IL-1β, TNF-α, and IFN-γ, and analyzed the influence of inflammation on β cell dedifferentiation.

Figure 2. FOXO1 expression in human islets with or without proinflammatory cytokines treatment: (a) immunofluorescence staining of FOXO1 and insulin in human islets with or without cytokines treatment. Arrows: cells with FOXO1 translocation to nucleus. Scale bar: 20 μm and (b) quantification of cells with FOXO1 translocation. N = 3. At least five islets were quantified in each pancreatic section.

***p < 0.001.
We found that cytokines treatment decreased the expression of β cell functional genes, such as β cell-specific transcription factors FOXO1, MAF A, PDX1, and NKX6.1, and glucose sensing gene GLUT2, but induced the mRNA expression of β cell dedifferentiation marker ALDH1A3 and progenitor marker NGN3 (Figure 1), indicating that inflammation might have changed the β cell maturation states. This is consistent with the previous study by Nordmann et al.13

The dislocation or loss of expression of important transcription factors in β cells or re-expression
of β cell progenitor cell markers indicated that these β cells are functionally compromised and losing their differentiated identity. Therefore immunofluorescence staining analyses can provide stronger imaging evidence for the β cell dedifferentiation induced by cytokines in human islets. In our study, we found that FOXO1 protein is located in the cytoplasm and NKX6.1 in the nucleus in normal human β cells. However, in the cytokine-treated islets, FOXO1 translocated to nucleus (Figure 3), which has been suggested as an initiating event for β cell dedifferentiation. Meanwhile, NKX6.1 was expelled from nucleus to cytoplasm and finically disappeared, indicating inactivated

Figure 4. ALDH1A3 expression in human islets with or without proinflammatory cytokines treatment: (a) immunofluorescence staining of ALDH1A3 and insulin in human islets with or without proinflammatory cytokines treatment. White arrows: β cells with ALDH1A3 expression (INS⁺/ALDH1A3⁺ cells). Scale bar: 20 μm and (b) quantification of β cells with ALDH1A3 expression (INS⁺/ALDH1A3⁺/INS⁺). N=3. At least five islets were quantified in each pancreatic section. ***p < 0.001.
transcription of INS gene. Importantly, we detected the re-expression of pancreatic progenitor cell markers in β cells, evidenced by the increase of ALDH1A3 positive β cells (Figure 4). ALDH1A3 has a very low expression in normal β cells. However, in the cytokines-treated β cells, a number of β cells achieved the expression of ALDH1A3 in cytoplasm (Figure 4). These results suggested that the cytokines can induce the β cell dedifferentiation. Inflammation has long been recognized as a culprit for β cell dysfunction. Our earlier study also demonstrated that MSCs can ameliorate β cell dedifferentiation in T2DM islets by regulating islet inflammation. Here this study provided the cyto-histologic evidence for inflammation-induced β cell dedifferentiation in human islets, and deepened the understanding on the mechanism for inflammation-induced β cell dysfunction in human islets.

There are some limitations on this study. First, only in vitro experiments were involved in the present study, and in vivo studies needs to be designed and carried out to further validate the findings here in future. Secondly, the phenotype change of other cell types, such as α cells and delta cells, were not investigated in this study, which we believe is worth investigating and will be characterized in our future studies.

Conclusion

In conclusion, we provided the most direct evidence for the β cell dedifferentiation induced by islet inflammation in human islets, and suggested that anti-inflammation therapies may benefit to ameliorating the β cell dedifferentiation in human T2DM islets.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Ethics approval

Ethical approval for this study was obtained from Medical Ethical Committee of Tianjin First Central Hospital (Review number: 2016N077KY).

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Informed consent

Written informed consent was obtained from all subjects before the study.

Trial registration

Not applicable.

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