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An Increase in Chromogranin A-Positive, Hormone-Negative Endocrine Cells in Pancreas in Cystic Fibrosis

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We sought to establish whether an increase in chromogranin A-positive, hormone-negative (CPHN) endocrine cells occurs in the pancreas of patients with cystic fibrosis (CF), as potential evidence of neogenesis.

Pancreata were obtained at autopsy from nondiabetic patients with CF (n = 12) and age-matched nondiabetic control subject (CS) individuals without CF (n = 12). In addition, pancreas from three diabetic patients with CF was obtained. Pancreas sections were stained for chromogranin A, insulin, and a cocktail of glucagon, somatostatin, pancreatic polypeptide, and ghrelin and evaluated for the frequency of CPHN cells.

There was a higher frequency of CPHN cells in islets of the patients with CF compared with the CS group. Moreover, CPHN cells occurring as single cells or clusters scattered in the exocrine pancreas were also more frequent in patients with CF.

The increased frequency of CPHN cells in pancreas of patients with CF may indicate an attempt at endocrine cell regeneration.

Freeform/Key Words: β cell, cystic fibrosis, inflammation

Cystic fibrosis (CF) is an autosomal recessive genetic disorder in which there are mutations in both copies of the gene encoding the CF transmembrane conductance regulator protein [1, 2]. Mutations of the CF transmembrane conductance regulator protein affect chloride ion channel function [3], causing abnormally high secretory viscosity. These hyperviscous secretions result in functional and pathological damage to numerous organs, notably the lungs, liver, kidney, and pancreas, via chronic secretory outflow obstruction.

Most patients with CF have extensive pancreatic fibrosis and fatty infiltration with destruction of the exocrine pancreas, leading to exocrine insufficiency [4]. In addition, the endocrine component of pancreas is affected, with glucose intolerance reported in 50% to 70% of adult patients [5, 6] and frank diabetes affecting ~40% of adults aged >30 years [7]. Diabetes occurring in the setting of CF is designated CF-related diabetes (CFRD) [8], an entity

Abbreviations: CF, cystic fibrosis; CF-D, cystic fibrosis with diabetes; CFRD, cystic fibrosis–related diabetes; CPHN, chromogranin A-positive, hormone-negative; CS, control subject; NS, not significant; T1D, type 1 diabetes; T2D, type 2 diabetes.

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distinct from either type 1 diabetes (T1D) or type 2 diabetes (T2D) and associated with worse outcomes [9]. Unlike T1D, CFRD does not result from autoimmune attack on β-cells [10]. Several publications have reported an ~50% decrease in insulin-expressing β-cells within islets in CFRD compared with both patients with non-CFRD CF and the control subject (CS) group without CF [11–15]; this is comparable to the deficit of islet β-cells in T2D, and also, CFRD islets are characterized by deposition of amyloid [16]. Although this evidence suggests that CFRD is more akin to T2D, key differences in clinical features and islet function indicate that CFRD is a distinct disease entity, with islet morphology and function being compromised secondarily as a consequence of exocrine pathology [17, 18]. A recent study with detailed characterization of increased inflammatory mediators (including IL6, IL1B, CXCL10, TNFα, and IFNγ) within the human CF islet demonstrated that CFRD is caused by islet loss and inflammation [19]. Islet-derived chemokine CXCL10 and its receptor CXCR3 interaction was also reported to be involved in β-cell *trans*-differentiation in humans with chronic pancreatitis [20]. We have recently reported that, in the setting of both T1D and T2D, there is an increase in pancreatic nonhormone-expressing endocrine cells [21–23]. We interpreted this as an endogenous attempt, albeit insufficient, to regenerate the β-cell complement lost to disease, as the increased frequency of these chromogranin A-positive, hormone-negative (CPHN) cells recapitulates the scenario of early human development [21]. Our recent data also demonstrated the abundance of CPHN cells in chronic pancreatitis, suggesting the damage in the exocrine pancreas might also lead to morphological changes in the endocrine pancreas [20]. Therefore, in this study, we sought to determine whether there was an increase in CPHN cells in the pancreas of patients with CF.

1. Materials and Methods

A. Autopsy Cases

Human pancreatic tissue was obtained at autopsy from 12 nondiabetic individuals with documented CF during life, 3 diabetic individuals with CF (CF-D), and 12 nondiabetic, age-matched control individuals without CF (CS) (Supplemental Table 1 and Supplemental Fig. 1A). The patients with CF-D, patients with CF [16], and CS group [24, 25] have been included in previous publications. Patients with CF were identified by retrospective analysis of the autopsy database (A.S.M.M.) held at the University of Minnesota. CS individuals were identified from the Mayo Clinic autopsy database (R.A.R.). For inclusion in the study, a full autopsy had to have been performed within 24 hours of death and a sample of pancreatic tissue of adequate size and quality stored. Patients were excluded if the pancreas sections were compromised, either by autolysis or acute pancreatitis. None of the nondiabetic patients, with or without CF, selected for inclusion in the study had a history of diabetes. Patients with CF-D had a documented history of diabetes and were on insulin therapy. The patient characteristics and diagnoses leading to death are presented in Supplemental Table 1. Institutional review board approval was obtained from the Mayo Clinic, the University of Minnesota, and the University of California, Los Angeles. Fasting blood glucose values were unavailable. The determination of nondiabetic status in patients with CF and CS individuals was based on an absence of a history of diabetes in the medical record. A further three patients with CF and documented diabetes were identified in the University of Minnesota autopsy database (Supplemental Table 1).

B. Pancreatic Tissue Processing

All autopsies were performed at the University of Minnesota (patients with CF) or the Mayo Clinic (CS group). At both institutions, a sample of tail of pancreas measuring approximately $2.0 \times 1.0 \times 0.5$ cm in size was resected per routine protocol and, together with a sample of spleen, was fixed in formaldehyde prior to being embedded in paraffin. The 4-μm sections were obtained from these tissue blocks.

C. Immunofluorescence Staining and Quantification of CPHN Cells

The 4-μm sections were stained for chromogranin A, insulin, glucagon, somatostatin, pancreatic polypeptide, and ghrelin to detect CPHN cells as described previously [21, 22]. For the determination of pancreatic endocrine cells in the CS individuals and patients with CF, 50 islets, clusters of endocrine cells and single endocrine cells per sample, were imaged at ×20 magnification using a Leica DM6000 microscope (Leica Microsystems, Buffalo Grove, IL) with a Hamamatsu Orca-ER camera (C4742-80-12AG; Indigo Scientific, Niagara Falls, NY) and Openlab software (Improvision, Santa Clara, CA). An islet was defined as a grouping of four or more chromogranin A–positive cells. A cluster was defined as a grouping of three or fewer chromogranin A-positive cells. Within the fields imaged to obtain the 50 islets per patient, all clusters of endocrine cells (one, two, or three adjacent endocrine cells) were counted and recorded. Each field of view was calculated to be 0.292 mm^2. Endocrine cells as well as CPHN cells were quantified and analyzed according to our previous reports [21, 22].

Analysis was performed in a blinded fashion (M.C.), and all CPHN cells were independently confirmed by two other observers (A.S.M.M. and A.E.B.). The endocrine cells contained within each islet were manually counted and the following data recorded: (1) the number of cells staining only for chromogranin A, (2) the number of cells costaining for the endocrine hormone cocktail and chromogranin A, (3) the number of cells costaining for insulin and chromogranin A, and (4) the number of cells staining for insulin, hormone cocktail, and chromogranin A (polyhormonal cells).

The mean number of endocrine cells counted within islets for the nondiabetic CF and CS groups was 2039 ± 258 and 2526 ± 220 cells per individual, respectively. The mean number of endocrine cells counted in clusters in the nondiabetic CF and CS groups was 84 ± 9 and 122 ± 35 cells per individual, respectively. The mean number of CPHN cells per individual identified in islets was 25.7 ± 9.6 cells per individual from the nondiabetic patients with CF and 6.9 ± 2.5 cells per individual from the CS group. The mean number of CPHN cells per individual counted in clusters was 14.8 ± 3.2 per individual for the nondiabetic CF group and 7.7 ± 1.8 cells per individual for the CS group.

For the three patients in the diabetic CF group, the mean number of endocrine cells counted within islets was 2286 ± 285 cells per patient, the mean number of endocrine cells in clusters was 86 ± 40 cells per patient, the mean number of CPHN cells identified in islets was 26.0 ± 7.4 cells per patient, and the mean number of CPHN cells found as clusters was 17.7 ± 9.7 cells per patient.

The denominator used for the percentage of CPHN cells in islets was the total number endocrine cells counted in islets (number of CPHN cells counted in total 50 islets of one patient/number of total endocrine cells counted in 50 islets of one patient) ×100.

D. Statistical Analysis

Data are presented as means ± SEMs or ANOVA, as appropriate. Statistical calculations were performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

2. Results

A. Pancreas Pathology

Sections of pancreas from all the patients with CF demonstrated the classical features of the disease with destruction of the exocrine pancreas, extensive fibrosis, and fatty infiltration [14].

B. Composition of Endocrine Cells in Islets and Scattered Cells in Patients With CF Compared With the CS Group

There was no change in islet composition in nondiabetic CF in terms of the total number of endocrine cells per islet cross section compared with the CS group [40.8 ± 5.1 vs 50.5 ± 4.4

total endocrine cells/islet section, CF vs CS, P = not significant (NS)] (Fig. 1A). There was also no difference in the number of endocrine cocktail cells (cells that express all the pancreatic hormones except insulin) per islet section (18.6 ± 3.1 vs 21.2 ± 2.4 endocrine cocktail cells/islet cross section, CF vs CS, P = NS) (Fig. 1B). Interestingly, however, there was a decrease in the number of β-cells per islet cross section in the patients with CF, even in the absence of diabetes (19.1 ± 2.0 vs 28.0 ± 2.7 β-cells/islet cross section, CF vs CS, $P < 0.01$) (Fig. 1C). The mean number of endocrine cells found in clusters as well as single cells was no different between the CF and CS groups (23.1 ± 3.4 vs 18.7 ± 6.1 clustered endocrine cells/mm^2, CF vs CS, P = NS and 13.0 ± 2.0 vs 12.2 ± 3.4 single endocrine cells/mm^2, CF vs CS, P = NS) (Supplemental Fig. 1B and 1C; Table 1). There was no difference between the CF and CS groups in terms of the percentage of polyhormonal cells present within islets (0.02% ± 0.02% vs 0.00% ± 0.00%, CF vs CS, P = NS); however, more polyhormonal cells were identified in the single cells and clusters in the CF cohort (1.9% ± 0.8% vs 0.1% ± 0.1%, CF vs CS, $P < 0.01$) (Table 2) (Supplemental Figs. 2 and 3).

C. Comparison of CPHN Cells in Islets and Scattered Cells in Patients With CF Compared With the CS Group

Consistent with our previous findings, CPHN cells were detected in both the CS group (Fig. 2A, Supplemental Fig. 4) and patients with CF (Fig. 2B) or CF-D (Supplemental Fig. 5). We found there was a significant increase in CPHN cells in all compartments (in islets, as clustered cells or as single cells) of nondiabetic patients with CF compared with the CS group (0.4 ± 0.1 vs 0.1 ± 0.04 CPHN cells/islet section, CF vs CS, $P < 0.05$; 4.3 ± 1.0 vs 1.1 ± 0.2 clustered CPHN cells/mm^2, CF vs CS, $P < 0.01$; 3.6 ± 0.9 vs 1.0 ± 0.2 single CPHN cells/mm^2, CF vs CS, $P < 0.01$) (Fig. 2D–2F).

D. Pancreas From Patients With CF-D Resembles Nondiabetic CF Both in Terms of Islet Cross-Sectional Composition and Frequency of CPHN Cells

In the three patients with CF-D, there was no change in islet composition in terms of the total number of endocrine cells per islet cross section compared with either nondiabetic patients with CF or the CS group (45.7 ± 5.7 vs 40.8 ± 5.1 vs 50.5 ± 4.4 total endocrine cells/islet section, CF-D vs CF vs CS, P = NS). The mean number of endocrine cells found in clusters as well as single cells was no different between the CF-D or CS groups (17.8 ± 12.3 vs 18.7 ± 6.1

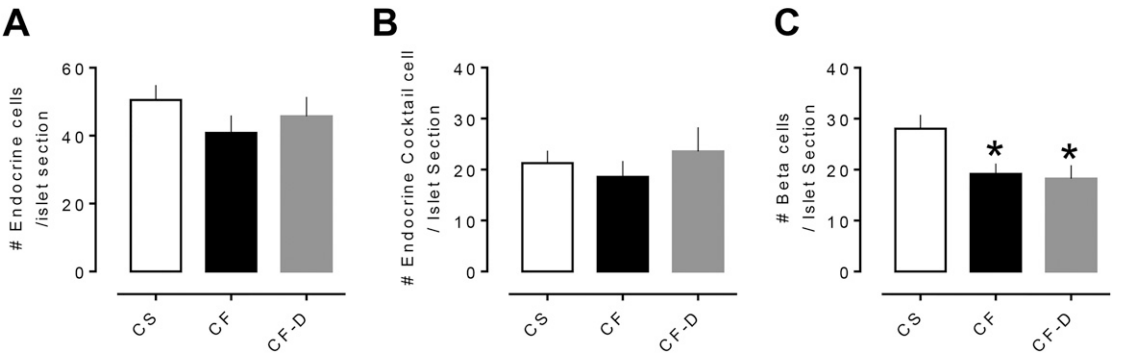

Figure 1. Islet endocrine compositions and frequency of CPHN cells in patients with CF and CF-D compared with the CS group. There was no change in islet composition in nondiabetic CF in terms of the total number of (A) endocrine cells per islet cross section (45.7 ± 5.7 vs 40.8 ± 5.1 vs 50.5 ± 4.4 total endocrine cells/islet section, CF-D vs CF vs CS, P = NS) and (B) endocrine cocktail cells (23.6 ± 4.8 vs 18.6 ± 3.1 vs 21.2 ± 2.4 endocrine cocktail cells/islet cross section, CF-D vs CF vs CS, P = NS). (C) There was, however, a decrease in the number of β cells per islet cross section in both diabetic and nondiabetic patients with CF (19.2 ± 2.1 vs 28.0 ± 2.7 β-cells/islet cross section, CF vs CS, $P < 0.01$ and 18.2 ± 2.6 vs 28.0 ± 2.7 β-cells/islet cross section, CF-D vs CS, $P < 0.05$). *$P < 0.05$, n = 12 (for CS and CF) and n = 3 (for CF-D).

Table 1. Composition of Endocrine Cells in Islets and Scattered Cells in Patients With CF or CF-D Compared With the CS Group

Characteristic	Total Endocrine Cells /Islet Section	β-Cells/Islet Section	Endocrine Cocktail Cells/Islet Section	Cluster Endocrine Cells/mm^2	Single Endocrine Cells/mm^2
CS	50.5 ± 4.4	28.0 ± 2.7	21.2 ± 2.4	18.7 ± 6.1	12.2 ± 3.4
CF	40.8 ± 5.1	19.1 ± 2.0[a]	18.6 ± 3.1	23.1 ± 3.4	13.0 ± 2.0
CF-D	45.7 ± 5.7	18.2 ± 2.6[a]	23.6 ± 4.8	17.8 ± 12.3	10.7 ± 6.0

Values are presented as mean ± SEM.
[a] $P < 0.05$ (compared with CS).

clustered endocrine cells/mm^2, CF-D vs CS, P = NS and 10.7 ± 6.0 vs 12.2 ± 3.4 single endocrine cells/mm^2, CF-D vs CS, P = NS) (Supplemental Fig. 1B and 1C). The number of β-cells per islet cross section in the patients with CF-D was comparable to the nondiabetic patients with CF (18.2 ± 2.6 vs 19.2 ± 2.1 β-cells/islet cross section, CF-D vs CF, P = NS) and was therefore also decreased in comparison with the CS group (18.2 ± 2.6 vs 28.0 ± 2.7 β-cells/islet cross section, CF-D vs CS, $P < 0.05$) (Fig. 1C). There was no difference in the number of endocrine cocktail cells per islet cross section (23.6 ± 4.8 vs 18.6 ± 3.1 vs 20.3 ± 2.5 endocrine cells/islet cross section, CF-D vs CF vs CS, P = NS) (Table 1). The number of CPHN cells per islet cross section in patients with CF-D was comparable to nondiabetic patients with CF (0.5 ± 0.1 vs 0.4 ± 0.1 CPHN cells/islet cross section, CF-D vs CF, P = NS) and was therefore increased in comparison with the CS group (0.5 ± 0.1 vs 0.1 ± 0.04 CPHN cells/islet section, CF-D vs CS, $P < 0.05$) (Fig. 2C). Likewise, the number of clustered or single CPHN cells in CF-D cases was also comparable to that of nondiabetic CF cases (3.2 ± 1.8 vs 4.3 ± 1.0 cluster CPHN cells/mm^2, CF-D vs CF, P = NS and 3.2 ± 1.8 vs 3.6 ± 0.9 single CPHN/mm^2, P = NS) and therefore also increased in comparison with the CS group (3.2 ± 1.8 vs 1.1 ± 0.2 cluster CPHN cells/mm^2, CF-D vs CS, $P < 0.05$ and 3.2 ± 1.8 vs 0.9 ± 0.2 single CPHN cells/mm^2, CF-D vs CS, $P < 0.05$) (Fig. 2D and 2E). Thus, there was no additive effect of diabetes on the pancreatic changes already observed in CF using these analytic parameters. The three CF-D cases had an increased percentage of polyhormonal cells within islets (0.11% ± 0.06% vs 0.02% ± 0.02%, CF-D vs CF, $P < 0.05$). In the patients with CF-D, the percentage of polyhormonal cells found in scattered cells was comparable to that of the nondiabetic CF cohort (1.3% ± 0.7% vs 1.9% ± 0.8%, CF-D vs CF, P = NS) (Table 2).

3. Discussion

CPHN cells are found in large numbers in normal human pancreas during development [21], and a resurgence is seen in both T1D [23] and T2D [21, 22] where there is a deficit in the endocrine and, more specifically, the β-cell complement [26, 27]. On that basis, we have

Table 2. Comparison of CPHN Cells in Islets and Scattered Cells in Patients With CF or CF-D Compared With the CS Group

Characteristic	CPHN Cells/Islet Section	Cluster CPHN Cells/mm^2	Single CPHN Cells/mm^2	% Polyhormonal Cells/Islet Section	% Scattered Polyhormonal Cells/mm^2
CS	0.1 ± 0.04	1.1 ± 0.2	1.0 ± 0.2	0.00 ± 0.00	0.1 ± 0.1
CF	0.4 ± 0.1[a]	4.3 ± 1.0[b]	3.6 ± 0.9[b]	0.02 ± 0.02	1.9 ± 0.8[a]
CF-D	0.5 ± 0.1[a]	3.2 ± 1.8[a]	3.2 ± 1.8[a]	0.11 ± 0.06[c]	1.3 ± 0.7[a]

Values are presented as mean ± SEM.
[a] $P < 0.05$.
[b] $P < 0.01$ (compared with CS).
[c] $P < 0.05$ (compared with CF).

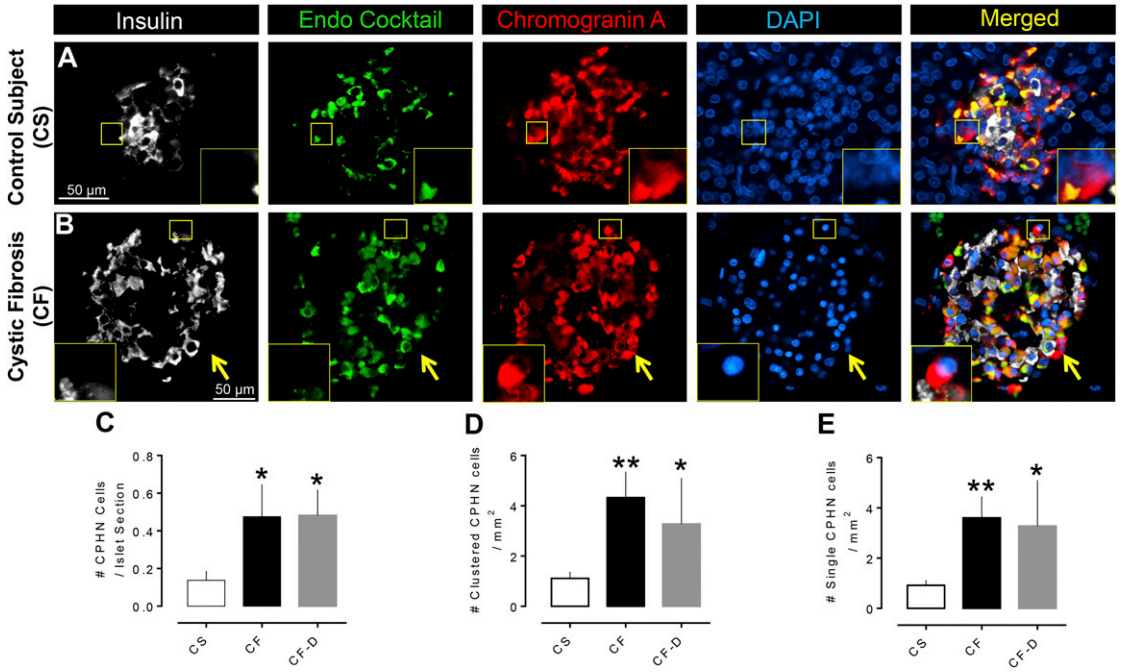

Figure 2. Example of a CPHN cell in the pancreas from (A) a nondiabetic CS and (B) a patient with CF. Individual layers, stained for insulin (white), endocrine cocktail (glucagon, somatostatin, pancreatic polypeptide, and ghrelin) (green), chromogranin A (red), and 4′,6-diamidino-2-phenylindole (DAPI) (blue), are shown along with the merged image. Insets show magnified images of the desired area (indicated by yellow squares) of low-power images to clearly locate the CPHN cells. Yellow arrows indicate the additional CPHN cells in CF cases. Scale bars, 50 µm. (C) The frequency of CPHN cells was increased in islets in patients with CF-D or CF compared with the nondiabetic CS group (0.4 ± 0.1 vs 0.1 ± 0.04 CPHN cells/islet section, CF vs CS, $P < 0.05$ and 0.5 ± 0.1 vs 0.1 ± 0.04 CPHN cells/islet section, CF-D vs CS, $P < 0.05$). The frequency of CPHN cells was also increased as (D) clustered cells (4.3 ± 1.0 vs 1.1 ± 0.2 clustered CPHN cells/mm^2, CF vs CS, $P < 0.01$ and 3.2 ± 1.8 vs 1.1 ± 0.2 cluster CPHN cells/mm^2, CF-D vs CS, $P < 0.05$) or as (E) single cells (3.6 ± 0.9 vs 1.0 ± 0.2 single CPHN cells/mm^2, CF vs CS, $P < 0.01$ and 3.2 ± 1.8 vs 0.9 ± 0.2 single CPHN cells/mm^2, CF-D vs CS, $P < 0.05$). *$P < 0.05$. **$P < 0.01$, n = 12 (for CS and CF) and n = 3 (for CF-D).

proposed that CPHN cells represent an immature endocrine cell type and that, in the setting of a pathological β-cell deficiency, they represent an attempted regeneration in an effort to restore the β-cell complement. Therefore, in the current study, we sought to determine whether an increase in CPHN cells would be found in the setting of nondiabetic CF, where the spatial proximity of the inflammation and fibrosis in the exocrine compartment affects the endocrine while avoiding the confounding effects of diabetes.

We detected a threefold increase in CPHN cells in both islets and cells scattered throughout the remaining exocrine pancreas in the pancreas of patients with CF. However, the percentage of CPHN cells occurring as scattered cells is much higher than that in islets. This is in keeping with our previous findings of CPHN cells being most commonly found as scattered cells, rather than in established islets, and also lends weight to the supposition that they are newly formed endocrine cells that have yet to mature into hormone-secreting cells. In the three patients with CFRD included here, the frequency and distribution of CPHN cells closely paralleled that in the nondiabetic patients with CF, so there does not appear to be an additive effect of diabetes, at least in this small cohort.

Of note, we found an increased number of polyhormonal cells within scattered endocrine cells in the patients with CF compared with controls, indicating possible disruption of endocrine cellular identity. This also raises the alternative possibility that the increased frequency of CPHN cells in patients with CF may be a reflection of changes in cell identity due to inflammation rather than neogenesis. In yet another potential scenario, the newly forming cells may not be able to fully differentiate in the inflammatory milieu.

Whether there is any regenerative capacity in the endocrine pancreas in humans has long been questioned, is a controversial issue, and is one that our group has tried to address from a number of different angles, looking at circumstances where there is known β-cell compromise (T1D and T2D) or generalized inflammation of the pancreas (chronic pancreatitis).

Pancreas from patients with CF is another pathological scenario in which to look for evidence of endocrine cell regeneration, and herein we aimed to study this in patients with CF but without the confounding influence of diabetes.

Although we believe that the population of CPHN cells could represent newly forming endocrine cells that have not yet matured into hormone-expressing cells, their numbers are too small to effectively replace endocrine cells lost to disease, and therefore it would appear that the regenerative capacity in the endocrine compartment is limited and insufficient.

In CF, exocrine tissue is destroyed specifically because mutant CF (delta508) leads to failure to secrete a protective mucinous layer to protect ducts, leading to a low-grade pancreatitis and gradual destruction of the exocrine pancreas. Finally, another possible explanation for increased CPHN cells in CF is that selective tissue loss has caused a relative increase in clustered CPHN cells per unit area. The compared measurement of the CPHN cells/mm^2 between individuals with and without CF in this article assumes that the inflammation-induced loss of exocrine tissue in CF does not selectively spare CPHN cells.

It is notable that in islets of nondiabetic patients with CF, we found a 1.5-fold decrease in β-cells per islet cross section compared with islets from the CS group. This is in keeping with prior published studies, where Soejima and Landing [13] reported a reduced percentage (43%) of β-cells in nondiabetic CF pancreas, Löhr *et al.* [15] reported a 50% decrease in β-cells in patients with advanced CF (21 nondiabetic, 1 glucose intolerant, and 1 with overt diabetes), and Abdul-Karim *et al.* [12] reported a tendency toward a reduced β-cell area percentage (46.7%) despite a small number of patients. It is therefore likely that the β-cell mass is progressively and negatively affected by collateral damage inflicted via the ongoing exocrine inflammation and is indirectly at least supportive of the assumption that the relative CPHN increase is not due to selective exocrine cell loss. This concept is underscored by the recent identification of pancreatic insufficiency in a cohort of patients with CF despite normal glucose tolerance according to Cystic Fibrosis Foundation criteria [17, 28, 29].

Human autopsy studies have inherent limitations. Due to the postmortem delay to autopsy, the tissue is often less well preserved than that obtained either surgically or from brain-dead organ donors. In any autopsy study where, by definition, each patient can be studied at only a single time point, it is impossible to prove or refute the concept that the cell population of interest, in this case the CPHN population, is dynamic and whether the cells present are newly forming of have been present since early development. Only lineage-tracing studies can definitively answer that question, and those cannot be performed in humans. So, we are left with observational human studies, where inferences must be made based on the collective evidence. In the case of CPHN cells, the evidence shows that they are found in high frequency in early life and that their numbers decrease rapidly, so that they are present, but infrequent, in normal adult pancreas. However, in the setting of disease, specifically in circumstances where there is known β-cell compromise (T1D and T2D) [21–23] or generalized inflammation of the pancreas (chronic pancreatitis) (20), there is an increase in the frequency of CPHN cells. Therefore, on this basis, we propose that this resurgence of CPHN cells represents an attempt, albeit insufficient, at endocrine cell regeneration.

4. Conclusion

Our data show that pancreatic endocrine cells in CF display changes in endocrine identity suggestive of attempted regeneration.

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