Persistent hyperinsulinemic hypoglycemia of infancy: constitutive activation of the mTOR pathway with associated exocrine-islet transdifferentiation and therapeutic implications

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Abstract: Background: Amino-acids stimulate the mammalian target of rapamycin complex (mTORC1); mTORC1 integrates amino-acid and energy-sensing pathways in beta-cells. Rapamycin inhibits mTORC1. We examined the mTOR pathway and cell cycle data in the exocrine pancreas in diffuse persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Design: Tissues from two diffuse PHHI cases, one pediatric control and from adult pancreatic tissue microarray were analyzed. The case studies are newborns of non-diabetic mothers, one with SUR1 mutation, and the other with a family history of PHHI. Immunostaining for (p)-mTOR(Ser2448), phospholipase(PLD)1, cell cycle analytes (Ki-67, Skp2, p27Kip1), and insulin were performed. Cell cycle analytes were assessed by automated cellular imaging or visual quantification. Multispectral imaging of double immunostaining for insulin/p-mTOR and transmission electron microscopy (TEM) were performed. Results: Hematoxylin-eosin and insulin-staining showed beta-cell hyperplasia in the exocrine pancreas, without mass effect. Overexpression of (p)-mTOR on the plasmalemmal, but not nuclear compartment, consistent with mTORC1, was noted in acinar elements. Residual expression was noted in islets. Double immunostaining revealed occasional exocrine cells co-expressing mTOR and insulin. No such co-expressions were seen in the control. TEM showed acinar cells containing zymogen and hormone-secreting granules. No nuclear Skp2 was noted. Conversely, p27Kip1 was expressed. Mitotic index was 1/40 (0.25/10) HPF. Conclusion: Morphoprotoeinic, histopathologic and morphometric findings in this study of diffuse PHHI coincide with existing genomic and signal transduction data in: 1) supporting a role for a constitutively activated and overexpressed mTORC1 pathway in the acinar pancreas in its pathogenesis; 2) reaffirming transdifferentiation of acinar-to-islet cells; 3) raising the possibility of rapamycin as a therapeutic option in PHHI.

Keywords: mTOR, PHHI, transdifferentiation, rapamycin

Introduction

Persistent hyperinsulinemic hypoglycemia of infancy (PHHI) has an incidence of 1/50,000 live births and is considered the most common cause of severe hypoglycemia in infants[1]. Clinically, it is manifested by marked hyperinsulinemia and severe hypoglycemia with its associated systemic complications and notably, by the absence of ketosis (ketonemia and ketonuria). Most authors would classify PHHI morphologically into two forms, focal adenomatous hyperplasia and a diffuse abnormality of the islets, respectively [2; 3]. To expand on this, in the focal form the histopathologic abnormalities are limited to one or several regions with the rest of the pancreas showing no pathologic changes. In the diffuse form of PHHI, the beta-cells are not only increased in number but are abnormal with some having hyperchromatic and hypertrophied nuclei that are conventionally accepted to be 3 times larger than nuclei of surrounding beta cells [4]. Furthermore, in the diffuse form, insulin-producing cells can also be seen within acini, outside any well-defined islet, and ductal to islet cell transformation (nesidioblastosis) is also present. In addition to the histopathologic differences, the clinical scientific literature provides genomic and clinical correlates that serve to characterize the two types. Specifically, the dif-
fuse form is associated with recessive mutations in SUR1 or KCNJ11 genes [5]. The focal type is associated with a mutation in the paternal allele of the SUR1 gene with loss of maternal allele of the KCNJ11 gene [6]. Clinically, the diffuse form of PHHI is managed with continuous feedings and medical therapies that include a potassium channel activator, glucose infusion and replacement of pancreatic enzymes. Moreover, surgery is used in an attempt to physically remove the mass of insulin-secreting cells. However, even with surgical removal of 95-98% of their pancreas, the children with the diffuse form develop hypoglycemic episodes or struggle with diabetes mellitus at one point in their lives. A better understanding of the pathobiology of the diffuse form is needed so that therapies that target and interrupt key pathways in the pathogenetic sequence can be applied in the hopes of controlling and managing the disease process.

In this context, and because there is a body of literature that implicates the mammalian target of rapamycin (mTOR) pathway in insulin secretion (vide infra); we studied the mTOR pathway in two cases with the diffuse form of PHHI. The specific objectives and design of this study were threefold and sequenced as follows: first, to assess components of the mTOR pathway and their state of activation using a morphoproteomic approach [7] and to compare and contrast the findings with those in control pancreases from adult and pediatric case material; second, to consider the possibility that exocrine to islet transdifferentiation, in association with the activation of the mTOR pathway is involved in the histogenesis of the islet cell mass both by looking for transition forms using dual immunostaining, multispectral imaging and transmission electron microscopic techniques, and by employing cell cycle analysis; and third, to integrate our findings regarding the mTOR pathway with the genomic and clinical data into a pathogenetic sequence that allows for targeted therapeutic intervention in the diffuse form of PHHI.

Materials and methods

Case and control selection

Formalin-fixed, paraffin-embedded blocks of pancreatic tissue from two cases of diffuse variant of persistent hyperinsulinemic hypoglycemia of infancy were retrieved (case 1 and case 2) from Texas Children’s Hospital. The infants were males, born at term to non-diabetic mothers. One infant had a birth weight of 3760 grams, and he had no genetic anomalies. However, his brother had a history of diffuse PHHI. The other infant had a birth weight of 5164.2 grams, and the birth was complicated by shoulder dystocia. He also had SUR1 gene mutation, and both parents were heterozygous for the mutation. Both infants had very low glucose levels immediately after birth, and they did not respond to conservative treatment. Subtotal pancreatectomy was performed, with 95% of the pancreas being removed from the first child, and 98% from the second child, after the intraoperative pathology consult showed diffuse nesidoblastosis. A detailed gestational and perinatal clinical history, as well as the initial presentation of the patients, and their follow up, is presented in Table 1.

A paraffin block of pancreas (incidental pancreatectomy from a 3-year-old trauma child) with no pathologic changes was used as control (case 3). A tissue microarray of pancreas from adult patients was also used as control.

We examined tissue cut 4 microns thick and stained with hematoxylin-eosin (H&E) from the two cases of PHHI, and also from the control pediatric case. Sections cut 4 microns in thickness were used for immunohistochemistry staining and transmission electron microscopy.

Insulin and p-mTOR Immunohistochemistry

Sequential double staining for insulin and p-mTOR was performed on 4 microns sections. The tissue was deparaffinized and rehydrated before antigen retrieval. Overnight incubation at 4 Celsius degrees was performed, and 1:200 rabbit monoclonal antibody against phosphorylated (p)-mammalian target of rapamycin (mTOR) at serine (Ser) 2448, (Cell Signaling Technology 2971) was applied. A secondary antibody (pk 6101 from Vectra kit) was applied. The tissue was then incubated with anti-insulin antibody (DAKO rabbit polyclonal 10564) 1:50 for 60 minutes at room temperature, and its expression was enhanced using red chromogen (Vulcan kit). The compartmental distribution of the brown chromogen (mTOR) and red chromogen (insulin) were enhanced using a multispectral imaging device (Nuance Multispectral Imaging Systems, CRi).
Phospholipase D1 Immunohistochemistry

IgG mouse monoclonal antibodies were used against phospholipase D1 (1:100, Santa Cruz). Then anti-insulin antibody was applied using the same technique described above.

Cell Cycle Data

To analyze the cell cycle progression and associated rate of proliferation, we applied monoclonal antibodies to S phase kinase-associated protein 2 (Skp2) (1:100, Santa Cruz), p27Kip1 (1:50, Novacastra), and Ki67 (1:500, DAKO).

We determined the percentage and intensity of Ki67 using an automated cellular imaging system (ACIS, DAKO® Corporation). The nuclear expression of Skp2 was quantified by counting the positive nuclei in per 100 cells in each of 10 high power fields. Similarly the percentage of p27Kip1 nuclear expression was determined by counting the number of positive nuclei per 100 insulin-expressing cells in each of 10 high power fields in the admixed exocrine-endocrine pancreas. A mitotic index was derived by counting the number of mitotic figures in 10 high power fields (4 sets of 10 were counted in each case and divided by 4).

Interpretation of the Immunohistochemistry

The slides were screened by three pathologists and the expression of protein analytes in the acinar, ductal and beta-islet cells of the cases with PHHI and the control case were then analyzed utilizing a bright field microscope with respect to the following: (1) presence or absence of expression of various analytes, and (2) localization of the protein analytes in the subcellular compartments namely cytoplasmic, plasmalemmal (cell membrane) and/or nuclear.

Table 1. Features of PHHI cases

<table>
<thead>
<tr>
<th>Feature</th>
<th>Case 1</th>
<th>Case 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gestational age at delivery</td>
<td>36 weeks, SVD</td>
<td>38 4/7 weeks, SVD</td>
</tr>
<tr>
<td>Mother’s history</td>
<td>G2P2, Caucasian, non-diabetic</td>
<td>G3P2, African-American, non-diabetic</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Weight</td>
<td>5014 grams</td>
<td>3660 grams</td>
</tr>
<tr>
<td>Genetic studies</td>
<td>SUR1 gene mutation</td>
<td>-</td>
</tr>
<tr>
<td>Family history</td>
<td>No diabetes; healthy brother</td>
<td>Brother with PHHI</td>
</tr>
<tr>
<td></td>
<td>Grandparent with diabetes</td>
<td></td>
</tr>
<tr>
<td>Glucose levels at presentation</td>
<td>“in the teens” (by surgeon)</td>
<td>&lt;40</td>
</tr>
<tr>
<td>Neurologic symptoms</td>
<td>Seizures</td>
<td></td>
</tr>
<tr>
<td>Preoperative treatment</td>
<td>Dextrose infusion</td>
<td>Dextrose infusion</td>
</tr>
<tr>
<td></td>
<td>Continuous feedings and dextrose infusion</td>
<td></td>
</tr>
<tr>
<td>Intra-operative consult</td>
<td>Diffuse changes</td>
<td>Diffuse changes</td>
</tr>
<tr>
<td>Operative procedure</td>
<td>98% pancreatectomy</td>
<td>95% pancreatectomy</td>
</tr>
<tr>
<td>Postoperative treatment</td>
<td>Cornstarch supplement</td>
<td>Insulin drip for two weeks</td>
</tr>
<tr>
<td></td>
<td>Gastrostomy tube</td>
<td>Gastrostomy tube</td>
</tr>
<tr>
<td></td>
<td>Octreotide</td>
<td>Octreotide</td>
</tr>
<tr>
<td></td>
<td>Diazoxide</td>
<td>Pancrealipase</td>
</tr>
<tr>
<td>Postoperative glucose level</td>
<td>&lt;50; controlled with medication</td>
<td>High for the first two weeks</td>
</tr>
<tr>
<td>Postoperative course</td>
<td>Weaned off diazoxide at 5 months</td>
<td>Discharged at 3 months</td>
</tr>
<tr>
<td></td>
<td>Weaned off octreotide at 30 months</td>
<td>Feedings and octreotide</td>
</tr>
<tr>
<td></td>
<td>G-tube removed at 3 years of age</td>
<td>Stable at 4 ½ months</td>
</tr>
<tr>
<td></td>
<td>Stable off meds at 4 years of age</td>
<td>Increased octreotide at 6 months</td>
</tr>
</tbody>
</table>

PHHI: mTOR activation and transdifferentiation
Any degree of antibody expression in various cellular compartments of the acini, ducts, islets and interstitium was analyzed, and its significance was established. Positive and negative controls, were run concurrently and the pathologic findings of PHHI were compared against them.

**Transmission Electron Microscopy (TEM)**

The only available tissue we had was embedded in paraffin. We deparaffinized the sections and treated them on the slide by rehydration with descending alcohols, rinsed the sections with Millonings Sodium Phosphate Buffer, fixed with 4% gluteraldehyde. 2% osmium was used for post-fixation. The tissue was then re-dehydrated with a graded series of ethanol and propylene oxide, and infiltrated with LX-112. A thin layer of resin (1mm) was left on the slide, and it was baked overnight at 60 Celsius degrees. After polymerization, the glass slide and layer of resin were warmed in a beaker of water that had been heated to boiling and removed from the burner. The tissue was pulled from the slide while warm. Different areas of the tissue were cut using a razor blade and glued onto a blank polymerized epoxy block for ultrathin sectioning and analysing under electron microscope [8].

**Results**

**Histologic evaluation of the two cases of diffuse PHHI and evaluation of insulin immunohistochemistry stain**

Microscopic examination of the H&E and insulin stains of the two cases of diffuse PHHI showed beta-cells organized in islets, and also in isolated groups scattered in the exocrine component throughout the tissue examined (Figure 1A). Some of these islet-cells showed karyomegaly with hyperchromasia (1B, arrow). Islet-cells budding from the ductal epithelium are also seen (1C, x200, arrows). Insulin immunohistochemistry highlights the diffuse nature of the pathologic process in PHHI and confirms their beta-cell nature (1D, x100).

**Constitutive activation and overexpression of mTORC1 pathway in the acinar cells, and activation in the ductal cells**

Microscopic examination of PHHI cases showed
plasmalemmal expression of p-mTOR (moderate intensity) in the acinar cells (Figures 2A, 2B). Immunohistochemical variability was noted in the expression of plasmalemmal p-mTOR between the two cases: case 1 had stronger and more uniform expression than case 2 (Figures 2A, 2B). p-mTOR was expressed on the plasmalemmal aspect in the ductal and intercalated cells in the concurrently run pediatric control case and in a subsequently assessed tissue microarray of adult exocrine pancreases (2D, original magnification x400), where the plasmalemmal expression is confined largely to centroacinar and intercalated duct cells (see arrows DC). There is no nuclear expression of p-mTOR in the exocrine pancreas and in the context of plasmalemmal expression is consistent with activation of mTORC1 (p-mTOR, Raptor, mLST8) in PHHI.

For completeness, we also compared our PHHI
cases with the pattern of distribution of p-mTOR in adult pancreatic tissue microarray (Figure 2D), and it showed similar results with the pediatric control case.

Lack of expression of phospholipase D1 in the acinar cells

Microscopic examination of the PHHI cases showed moderate plasmalemmal expression of PLD1 in the ductal cells and lack of expression on the plasmalemmal aspect of acinar cells (Figure 4). Only some of the centroacinar/intercalated duct cells had expression of PLD1.

Transmission electron microscopy

Transmission electron microscopy evaluation of pancreatic tissue from the two PHHI cases showed transition-type cells with rough endoplasmic reticulum in which zymogen granules, and endocrine granules are seen intermingled in the cytosol of the same acinar cell (Figure 5). Such cells have been variously labelled as acinar-islet or intermediate cells [12].

Expression of cell cycle markers and proliferation markers

The percentage of Ki67 in the population of cells with admixed exocrine and endocrine components was determined using the Automate-
Cell Imaging System (ACIS, DAKO® Corporate) and a mean positive nuclear score of 30.4% for case 1, and 28.6% for case 2 were established (Figure 6A). There was evidence of proliferation only in the interstitium, and focally in the islands of Langerhans in the control case (18%) (Figure 6B).

We quantified the nuclear expression of Skp2, by counting the number of positive nuclei per 100 cells in each of 10 HPF. The percentage of Skp2 positive nuclei for the control case was similar to that in the PHHI cases (0.12% versus 0.18%) (Figure 6C). This result suggests that the insulin-secreting cells that are arising in association with the mature acini and ducts do not reach S phase of the cell cycle [11].

Contrastively, p27Kip1 was diffusely expressed in the majority of the nuclei of insulin-expressing cells within the admixed endocrine and exocrine component (Figure 6D, red chromogen- insulin, brown chromogen- p27Kip1). Nuclear expression of p27Kip1 in the pattern described above suggests that the insulin-secreting cells do not progress into cell cycle beyond the Cyclin E/Cdk2-dependent G1 phase. Furthermore, this inverse relationship of p27Kip1 with Skp2 nuclear expression accords with observations in the literature [12].

The calculated mitotic index for our PHHI cases was 0.25 mitotic figures /10 HPF, and 0 for the pediatric control case.

A summary of the cell cycle data is incorporated

Table 2. Cell Cycle Parameters in PHHI

<table>
<thead>
<tr>
<th>Cell Cycle Data</th>
<th>Case 1</th>
<th>Case 2</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ki67</td>
<td>30.1%</td>
<td>28.6%</td>
<td>18%</td>
</tr>
<tr>
<td>p27Kip1</td>
<td>*%</td>
<td>*%</td>
<td>Only in few islet cells</td>
</tr>
<tr>
<td>Skp2</td>
<td>0.18%</td>
<td>0.18%</td>
<td>0.12%</td>
</tr>
<tr>
<td>Mitotic index</td>
<td>0.25/10 HPF</td>
<td>0.25/10 HPF</td>
<td>0/10 HPF</td>
</tr>
</tbody>
</table>

* Positive in the majority of nuclei in insulin-expressing cells within the admixed endocrine and exocrine component (See figure 6D).
Discussions

The findings of this study on two cases of diffuse PHHI will be discussed in three parts. Firstly, we review the histologic, immunohistochemical, ultrastructural, and morphoproteomic evidence that the pathogenesis of diffuse variant of PHHI involves G0/G1 cell cycle arrest in insulin-producing islet cells, and that these cells form through transdifferentiation of mature acinar and ductal elements. These are considered in the context of the National Library of Medicine's MEDLINE database. Secondly, we discuss our novel finding of the constitutive activation with overexpression of the mTORC1 pathway in the acinar cells. In conjunction with this, we analyze the expression of PLD1 in the exocrine and endocrine pancreas and observe that the acinar cells overexpressing p-mTOR do not express PLD1. Finally, we consider the known effect of rapamycin on mTORC1, and also on beta cells in the context of both our morphoproteomic findings and the genomic aspects of diffuse PHHI and its potential therapeutic application in such patients.

G0/G1 cell cycle arrest in insulin-producing islet cells of persistent hyperinsulinemic hypoglycemia of infancy (PHHI): evidence for islet neoformation from transdifferentiation of acinar and ductal cells

Persistent hyperinsulinemic hypoglycemia of infancy is associated with a proliferation of insulin-producing (beta-type) islet cells of the pancreas and increased secretion of insulin into the
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System circulation. In general, such a proliferation could involve one or more mechanisms to include cell cycle progression in pre-existing beta cells, neoformation of beta cells from pluripotential stem/progenitor cells and/or transdifferentiation of mature acinar and ductal cells of the exocrine pancreas into beta-cells. Similarly, the hyperinsulinemia could involve one or more pathogenetic factors such as a mass action effect consequent to an expanded population of beta cells, genetic hyper-responsiveness to agents such as amino acids, and/or constitutive activation of molecular signal transduction pathways involved in promoting the synthesis and release of insulin.

In our study of two cases of diffuse variant of PHHI, we observed histologic, immunohistochemical, ultrastructural and molecular/signal transduction pathway evidence that support the theory of transdifferentiation of mature acinar and ductal elements into insulin-secreting cells. H&E and immunohistochemical staining of the tissue for insulin showed endocrine cells organized in islets, but also in small clusters and single cells. These cells were diffusely present throughout the pancreas, some of them occupying acini and budding from ducts. Even with a very prominent increase in number of insulin-secreting cells, there was not a “mass effect” seen in the pancreas. It appears that the beta-cells were occupying the exocrine pancreas by replacement. TEM confirmed the existence of cells with admixed, endocrine and exocrine, component (transition or intermediate forms). Moreover, cell cycle analysis revealed a G0/G1 phase arrest for the cells with admixed endocrine and exocrine components by virtue of the following patterns: moderately elevated Ki-67 (which reflects the G1, S, G2, and M phases), at 30.4 % for case 1 and 28.6% for case 2 respectively, a low S phase kinase-associated protein (Skp2) percentage coinciding with a high percentage of islet and exocrine nuclei expressing p27Kip1, an inhibitor of Cyclin E/Cdk2 complex [12]. There was also a low mitotic index (0.25 mitotic figures /10 high power fields). Such cell cycle data corroborate the evidence from the literature as summarized below and coincide with the existence of transi-
Our findings regarding cell cycle arrest and acinar and ductal transdifferentiation into insulin-secreting cells are complemented and supported by the clinical and preclinical studies of others. Sempoux et al. [13], in a study of 18 cases of PHHI (11 focal and 7 diffuse forms), observed the proliferation rate of the beta-cells by virtue of Ki67 immunohistochemistry. They observed that these cells, in diffuse PHHI, do not have a significant increase in the proliferation rate compared with the control cases used (29.4% versus 19.6% in aged-matched controls). They concluded back in 2002 that these cells do not increase in number through proliferation. In a most recent publication, Lovisolo and co-workers [14] showed that there was an increase in the mean Ki-67 labeling index in the beta cells of the islets in the diffuse form of congenital hyperinsulinism versus the age-matched controls at 2.41% versus 1.87%; and although this small difference was statistically significant, it could simply reflect a G1 phase expression consequent to mTORC1 influence on G1 phase in transdifferentiated cells, as discussed above (also see Figures 3, 6 and 7). Kushner JA. [15] analyzed the beta-cell replication in mice of different ages by the use of 5-bromo-2-deoxyuridine (BrdU), a DNA precursor analogue that is faithfully incorporated in the dividing cells instead of thymidine, and can be detected with the use of specific monoclonal antisera. He concluded that beta-cell proliferation in 3-month-old wild type mice was only 0.2% following a 6 hour label. The proliferation rate decreased even more in older mice. Since he was aware of a possible toxic effect of BrdU on the beta-cells, he measured the apoptosis with TUNEL stain. Only very few cells were TUNEL stain positive. In the same paper, he studied the dependency of beta-cell growth on cyclinD2/Cdk4 activity, and one of his observations is that it is still unknown how much replication is needed to maintain the mass of beta-cells, and that these cells could conceivably live for the life of the organism.

In further support of transdifferentiation from the exocrine pancreas, Song et al. [16], in their research for alternatives for diabetic patients needing beta-cell transplantation, observed that pancreatic acini from 7 to 8-weeks-old male Sprague-Dawley rats, if isolated and cultured in suspension, will lose amylase expression, and will convert to cells with a duct-like phenotype. Insulin-positive cells were also observed at the periphery of the acini-derived spheroids. There were a few insulin-positive cells coexpressing cytokeratins, suggesting that a spontaneous acinar to ductal cell transdifferentiation process was further going on towards insulin-secreting cells. Moreover, Bouwens L. [17] studied the process of regeneration of insulin producing beta-cells after pancreatic injury on rodents. He concluded that these cells regenerate via neogenesis from pancreatic exocrine epithelial cells. Using immunohistochemistry for PDX1 (which is first expressed in all cells in pancreas, but it restricts to beta-cells in the adult pancreas), glut-2 (first expressed in all cells and then only in the insulin secreting cells) and vimentin (which is present in the stem cells) Bouwen concluded that stem cells should express all the three markers, and he did not observe cells with such traits in the pancreases examined. There are no dormant stem cells that would transform into hormone-producing cells in case of pancreatic injury. Finally, Bani and co-workers [12] described the presence of nesidioblastosis and intermediate cells (acinar-islet cells) scattered in the acinar tissue in three patients with hyperinsulinemic hypoglycemia, two adults with insulinoma and one child born to a diabetic mother. Such intermediate cells in their study were characterized by TEM as acinar-alpha or acinar-alpha-beta or acinar-beta types. Neoformation of islets from ductal elements similar to our two cases was also noted [10]. Most recently and in a related sense, it has been reported by Thorel and associates [18] showed in a preclinical study that alpha islet cells can convert (transform) into beta cells in response to near-total beta-cell ablation.

**Constitutive activation and overexpression of mTORC1 pathway in the acinar cells and constitutive activation of mTORC1 in the ductal cells**

Previous extensive research revealed that the genetic defect in the diffuse variant of PHHI is inactivating mutations in SUR1 and KCNJ11 genes [1], which lead to inactivation of ATP-dependent potassium channel. As a secondary effect, due to accumulation of extracellular potassium, the cells will depolarize and the calcium channels will become activated. Calcium will accumulate inside the cells at high concen-
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Our cases expressed p-mTOR (Ser 2448) in a very interesting pattern; we noticed expression of p-mTOR on the plasmalemmal aspect of ductal cells, and its constitutive activation and overexpression on the plasmalemmal aspect of acinar cells relative to the controls (Figure 2A, 2B). Only residual plasmalemmal p-mTOR was present in the well formed Langerhans islets in diffuse PHHI (Figure 3C). In the control case, plasmalemmal p-mTOR was expressed only in some centroacinar/intercalated duct cells (Figure 2C). Identical results with the ones observed in the control case were seen in the adult pancreatic tissue microarray examined.
The double immunostaining for p-mTOR – insulin (Figures 3A and 3B) showed the diffuse distribution of insulin-secreting cells throughout the pancreas, with some acinar and ductal cells expressing both, p-mTOR and endocrine granules. Some beta-cells were observed budding from mature ductal epithelium. For completeness, we analyzed the immunohistochemistry results with a multispectral pseudofluorescence device (Figure 3B), and performed transmission electron microscopy also (Figure 5), to demonstrate the presence of transition forms that contain zymogen granules and endocrine granules. Insulin secreting granules were present only in the Langerhans islets in the control case.

**Figure 9.** Considering the peculiar pattern of distribution of phospholipase D1 – whose product, phosphatidic acid is a rapamycin inhibitor – in diffuse PHHI (absent from the plasmalemmal aspect of the acinar cells with admixed endocrine and exocrine components, residual expression in the beta-islets and expression on the plasmalemmal aspect of mature ductal cells), we believe that rapamycin would inhibit the expression of m-TORC1 in the acinar, and possibly to some extent in ductal cells undergoing transdifferentiation, decreasing insulin synthesis in diffuse variant of PHHI.
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from the plasmalemmal aspect of acinar cells (Figure 4). It was moderately present in some centroacinar cells, and also in ductal cells. This pattern of distribution of phospholipase D1 made us believe that rapamycin would act on the acinar cells undergoing transdifferentiation to islet cells, and possible on the ducts. Our correlations show that calcium channel blockers combined with rapamycin [28-31], would be a great addition to the treatment of this entity, and would control the concentration of intracytosolic calcium. They could potentially inhibit the activation of p-mTORC1, and the process of transdifferentiation, by controlling the concentration of intracytosolic calcium. A normal level of calcium inside the cells would stop reg I protein from becoming over expressed, and the process of transdifferentiation of endocrine cells from acinar and ductal cells. With the addition of a calcium channel blocker and rapamycin to the current treatment, the expected effects would be decreased insulin secretion by decreased viability and potency, and also by stimulating autophagy of already formed beta-cells. Bas et al. [32] described three cases of PHHI that failed to respond to diazoxide and somatostatin, but were successfully controlled with nifedipine. The patients had good control of hypoglycemia even after 12 months of use of this calcium channel blocker, and there were no side effects associated with the treatment.

There are recent experimental studies that show the possible effect of rapamycin on beta-islets. Bussiere et al. [29] cultured human ductal cells (HDC) and neonatal porcine islets (NPI) with Rapamycin, and saw that there is a 50% decrease in HDC, and a 28% decrease in NPI after 24 hours. A negative TUNNEL stain made him conclude that the mechanism through which these cells are disappearing is not apoptosis.

Tanemura et al. [28] used pancreatic tissue from male BL6 mice, to isolate beta-islets. He incubated fresh islets for 24 hours in culture medium, in the presence or absence of Rapamycin, either 1 or 10 ng/mL. Western blot analysis showed accumulation of membrane bound LC3-II, which is an early marker of autophagy. The viability of islets incubated with rapamycin was also analyzed, and there was a 43% decrease in the viability of islets treated with 1 ng/mL rapamycin, and a 51% decrease when the islets were incubated with 10ng/mL rapamycin. It has been shown that activation of mammalian target of rapamycin complex 1 (mTORC1) in the pancreas leads to insulin synthesis by its proliferative and transcriptional effects. Bourcier et al. [33] have reported a case of pancreatic insulin-secreting islet cell tumor with metastases, that failed to respond to octreotide, diazoxide and continuous glucose infusion, but responded to oral dose of 2 mg/dl of rapamycin. The effect was due to inhibition of beta-cell growth and proliferation, as well as blockade of insulin production. This means that rapamycin can help the treatment of hypoglycemic states.

In conclusion, it has been forty years since one of us (REB) proposed that leucine-sensitive hypoglycemia of infancy might be related to the transformation of acinar and ductal elements into beta cells by the amino acid, leucine in hyperresponsive individuals [34]. Our study on two cases of diffuse variant of PHHI supports this concept by demonstrating that there is G0/G1 cell cycle arrest of both the mature exocrine cells undergoing transdifferentiation and in the islet cells in the exocrine pancreas and in the islets, and there is constitutive activation and overexpression of p-mTOR on the plasmalemmal aspect of the acinar cells, and activation on the plasmalemmal aspect of the ductal cells. We suggest that the particular distribution of expression of p-mTOR and PLD1 in PHHI, should allow rapamycin to act in an inhibitory fashion at the level of acini, where it will prevent the process of transdifferentiation, and at the level of already organized clusters and islets of beta-cells, where it will release the inhibitory effect of p-mTORC1 on autophagy, and will decrease insulin synthesis by decreasing the survival and potency of these cells. Calcium channel blockers would assist rapamycin in its action, by decreasing the intracellular level of calcium, which will lead to decrease activation of p-mTORC1, and inhibition of neoformation of insulin-secreting cells from acinar and ductal elements.

Despite all the recent progress in elucidating the intricacies of this entity there is still not a good management to stop the increase in number of insulin secreting cells. We believe that our findings might revolutionize the medical approach for infants with diffuse variant of persistent hyperinsulinemic hypoglycemia. However, the number of cases we studied is limited and further research is needed in this direction.
Aknowledgements

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