Original Article

Dipeptidyl peptidase-4 expression in pancreatic tissue from patients with congenital hyperinsulinism

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Abstract: Congenital hyperinsulinism (CHI) is caused by unregulated insulin release and leads to hyperinsulinaemic-hypoglycaemia (HH). Glucagon like peptide-1 (GLP-1), glucose-dependent insulinotropic peptide (GIP), peptide YY (PYY) and the enzyme; dipeptidyl peptidase-4 (DPP-4) all regulate appetite and glucose homeostasis. These proteins have been identified as possible contributors to HH but the mechanism remains poorly understood. We aimed to look at the expression pattern of pancreatic DPP-4 in children with focal and diffuse CHI (FCHI and DCHI, respectively). Using immunohistochemistry; we determined DPP-4 expression patterns in the pancreas of CHI patients. DPP-4 was found to be expressed in the pancreatic β, α and δ-cells in and around the focal area. However, it was predominantly co-localised with β-cells in the paediatric tissue samples. Additionally, proliferating β-cells expressed DPP-4 in DCHI, which was absent in the FCHI pancreas. Insulin was found to be present in the exocrine acini and duct cells of the DCHI pancreas suggestive of exocrine to endocrine transdifferentiation. Furthermore, 6 medically-unresponsive DCHI pancreatic samples showed an up-regulation of total pancreatic DPP-4 expression. In conclusion; the expression studies have shown DPP-4 to be altered in HH, however, further work is required to understand the underlying role for this enzyme.

Keywords: DPP-4, CHI, hypoglycaemia

Introduction

Congenital hyperinsulinism (CHI) is the most common cause of persistent and recurrent hypoglycaemia in neonates. Hence, this disorder has the potential to be life threatening causing neurological damage, requiring quick and effective treatment and management [1]. This disorder is rare and has an incidence of around 1:50,000 births in the general population [2]. Infants require constant feeding to stabilise the hypoglycaemia. Currently mutations in nine gene have been identified to be associated with CHI. These genes encode for glucokinase (GCK), glutamate dehydrogenase (GLUD1), 3-hydroxyacyl-CoA dehydrogenase (HADH), hepatocyte nuclear factor-1 and 4α (HNF1/4A), monocarboxylate transporter-1 (MCT1), uncoupling protein 2 (UCP2) and the two K⁺ ATP channel subunits: SUR-1 (ABCC8) and Kir6.2 (KCNJ11) [3].

Histologically, there are two types of CHI; focal (FCHI) and diffuse disease (DCHI). FCHI is most-sporadically inherited but DCHI can be autosomal recessive or dominantly inherited. Due to these factors, management of these two histological types of CHI is very different. FCHI only requires the removal of the lesion (potentially curing the patient), whereas, DCHI require medical therapy often with diazoxide; a K⁺ ATP channel activator. However, some patients are diazoxide-unresponsive; and therefore are treated with alternative drugs including glucagon as well as somatostatin analogues (octreotide and Lanreotide) to counteract the unregulated hyperinsulinaemic-hypoglycaemia (HH). If all these avenues fail, a near-total pancreatectomy is performed. This risks diabetes mellitus and pancreatic exocrine insufficiency.

Lately, the role of gut hormones in HH has become of interest due to the implications seen in bariatric surgery. Incretin hormones (glucagon like peptide-1: GLP-1 and glucose-dependent insulinotropic peptide: GIP) are gut hormones released in response to a meal [4]. The peptides promote insulin release and lowering
of postprandial blood glucose levels. It has been demonstrated that the GLP-1 receptor antagonist; exendin (9-39) causes a decrease in plasma insulin levels coupled with a rise in blood glucose concentration [5, 6]. More recently, two cases of unknown genetic cause of CHI have also been reported to have impaired incretin responses [7]. Hence, altering the incretins signalling/function appears to be important in reducing HH. At present, it remains unknown as to the mechanism of such observations. Moreover, there are currently no reports on the effects of peptide YY (PYY), another gut hormone which is known to be co-localised with and mediate GLP-1 action [8]. Additionally, to date no studies have assessed the pancreatic expression pattern of DPP-4, the enzyme that regulates all these gut hormones action. Hence, we aimed to identify DPP-4 expression patterns in CHI.

Materials and methods

Patients

CHI patients were recruited from Great Ormond Street Hospital for Children. All studies were approved by the UCL Ethics Committee. Information sheets were provided and detailed discussions were held with the families prior to obtaining informed consent from all the families.

Immunohistochemistry

5 µm pancreatic tissue sections were taken from FCHI or medically-unresponsive DCHI who underwent pancreatectomy. Immunohistochemistry for insulin, glucagon, somatostatin and DPP-4 were performed using methods described previously [9]. Cell proliferation was assessed using Ki67 immunostaining (anti-Ki67, Bond, UK). All antibodies were used according to the manufacturers’ instructions. Formalin-fixed, paraffin-embedded tissue sections were deparaffinised and hydrated. Heat-induced epitope retrieval was performed using citrate buffer (pH=6; Dako, UK) and a pressure cooker (Tinto Retriever, UK). After antigen retrieval, tissue sections were blocked with 0.01% serum-free protein solution (Dako, UK) for 30 minutes. Sections were then incubated with anti-goat biotinylated DPP-4 (R&D systems, UK) and anti-insulin (Cell signaling, UK) or with anti-glucagon (Abcam, UK) or with anti-somatostain (Millipore, UK) overnight in a humidified chamber at 4°C. Slides were then incubated for 30 minutes with secondary Alexafluor antibodies (strepatavidin 488, anti-goat 594 (Invitrogen, UK) to visualize sections via light, fluorescent or confocal microscopy (Zeiss, UK).

Microarray

Gene expression microarray was performed using Affymetrix Human GeneChip 1.0 ST Array (Santa Clara, CA, USA) with 40 ng of total RNA. Total pancreatic RNA was extracted from 6 medically unresponsive DCHI patients undergoing pancreatectomy and compared to 2 non-CHI controls obtained from a tissue biobank (Cambridge Bioscience Limited, UK).

Genetics

Genomic DNA was isolated from peripheral leukocytes using standard procedures as described previously [10]. Coding exons and the intron/exon boundaries of the ABCC8, KCNJ11, GCK and HNF4A genes were all amplified by PCR. Analysis of the HNF4A gene included the coding exons 1d-10 as well as the P2 pancreatic promoter. PCR products were sequenced using standard methods on an ABI 3730 (Applied Biosystems, Warrington, UK), and were compared to the published sequence NM_000457.3 (exons 2-10) and AY680697 (exon 1 aaaa only) using Mutation Surveyor v3.2 (SoftGenetics, State College, PA, USA) [11].

Statistics

Data are presented as mean + standard error or mean (SEM), unless otherwise indicated. All statistics were presented using GraphPad Prism 6 software (GraphPad, CA, USA).

Results

DPP-4 is co-localized with insulin, glucagon and somatostatin in β-, α- & δ-cells, respectively, in histologically normal pancreatic tissue surrounding the FCHI

CHI is histologically characterised into two subtypes; FCHI & DCHI. In FCHI, the lesion contains hyperplastic β-cells. The surrounding endocrine tissue in the FCHI pancreas is morphologically and functionally normal [12]. Therefore using
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these pancreatic regions as control, we assessed the DPP-4 localisation in the FCHI lesion and surrounding area. DPP-4 was predominantly expressed around the lesion, with lower expression found in the focal area (Figure 1). DPP-4 expression appeared to be distributed predominantly with insulin positive β-cells (Figure 1A and 1D), and lower levels with glucagon in α-cells (Figure 1B and 1E). A small population of δ-cells was also positive for the expression of DPP-4 (Figure 1C and 1F).

β-cells expressing DPP-4 are actively proliferating in DCHI

As previously reported, DCHI had large hyperchromatic islet nuclei, whilst FCHI had histologically normal area around the pancreatic lesion [13, 14]. Using Ki67 as a marker for cell proliferation, we found an increase in Ki67 in the pancreas of FCHI and DCHI (Figure 2A). In the FCHI pancreas, DPP-4 cells co-expressing Ki67 were found in cells absent of insulin, glucagon and somatostatin staining (Figure 2B). Next, we attempted to identify if islet cells in DCHI tissues were proliferating, and if so, did they co-express DPP-4. DCHI pancreatic tissue appeared to show islet β-cell proliferation in the presence of DPP-4 staining (Figure 2C). On the other hand, α-cells were absent of Ki67 staining suggesting a lack of proliferation in these cell-subtypes (Figure 2D), whereas, islet δ-cells expressed Ki67 (Figure 2E). In DCHI tissues, it has been suggested that transdifferentiation of

Figure 1. DPP-4 is predominantly expressed in β-, α- & δ-cells, respectively, in histologically normal pancreatic tissue around the focal lesion. Representative immunostained FCHI pancreatic tissues are shown. A-C. Immunostained pancreatic sections around the focal lesion (histologically normal). N=5, 2 sections per individual. D-F. Immunostained pancreatic sections within the focal lesion. N=3, 2 sections per individual. Reference line: 20 or 50 µm. INS; insulin. GCG; glucagon. SST; somatostatin. DPP-4; dipeptidyl peptidase-4.
exocrine to endocrine cells may occur which may have the potential to contribute to the HH phenotype [15, 16]. Hence, we co-stained for amylase as a marker for exocrine acinar cells with insulin or DPP-4 and found only insulin to be expressed in these cell types (Figure 3A and 3B). We next assessed whether insulin or DPP-4 were potentially localised to the pancreatic ducts using cytokeratin-19 (CK-19). The pancreatic ducts also expressed insulin but to a lesser extent than the expression found in the acinar cells. However, in both instances, DPP-4 was found closely expressed in particular to the periphery of these cells but not co-localised with the exocrine cells expressing insulin (Figure 3C and 3D).

Pancreatic DPP-4 expression is increased in children with hyperinsulinaemic hypoglycaemia

To complement the immunohistochemical data, pancreatic gene expression was assessed. Pancreatic RNA was extracted from six medically-unresponsive DCHI patients (D1-6) and compared to two controls (non-HH pancreatic tissues).

D1-D6 were all unresponsive to medical therapy (diazoxide and octreotide) and thus underwent a pancreatectomy. All patients required high concentrations of glucose at the time of surgery to maintain normoglycaemia. All
Figure 3. DCHI has insulin expression in the exocrine pancreas. A-D. Insulin is localised to ductal cells (CK-19) and acinar cells (amylose) in the absence of DPP-4 co-staining. Representative immunostained sections of the DCHI pancreas. N=3, 2 sections per individual. Reference line: 50 µm. E. Pancreatic DPP-4 expression is up-regulated in DCHI. Gene expression of RNA isolated from DCHI pancreata and control donors. N=6 DCHI and 2 control. Data shown as mean + SEM and as % of control gene expression. INS; insulin. DPP-4; dipeptidyl peptidase-4, CK-19; cytokeratin-19. GLP-1R; glucagon like peptide-1 receptor. GIP; glucose dependent insulinotropic peptide. PYY; peptide YY. GCG; glucagon. SST; somatostatin.
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Table 1. Details of pancreatectomised tissue samples used in microarray

<table>
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<th>Patient Information: Microarray data</th>
<th>Control 1</th>
<th>Control 2</th>
<th>D1</th>
<th>D2</th>
<th>D3</th>
<th>D4</th>
<th>D5</th>
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<td>Birth</td>
<td>2 w</td>
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<td>Near total</td>
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</tr>
<tr>
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<td>33 y</td>
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<td>2 m</td>
<td>7 m</td>
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<td>ABCC8</td>
<td>ABCC8</td>
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<td>ABCC8, KCNJ11 &amp; GCK negative</td>
</tr>
<tr>
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W: weeks.
patients were born at term and did not have risk factors like low birth weight, birth asphyxia or maternal diabetes with the exception of D3. There was no significant family history. All patients presented with severe hypoglycaemia within the first few weeks of life. Post operatively, patients D1 and D2 were cured whilst D3 and D4 required octreotide to maintain normoglycaemia. D5 required a small dose of diazoxide and DCHI6 required another pancreatectomy. Four of the six DCHI patients were found to have mutations in the \textit{ABCC8} gene (D1-D4). Whilst D5 and D6 were absent of mutations in the \textit{ABCC8}, \textit{KCNJ11} and \textit{GCK} genes (Table 1).

Using microarray analysis we found an up-regulation of pancreatic \textit{DPP-4} expression in DCHI by 21.3\% (Figure 3B) (control: 5.04±0.39 AU versus DCHI: 6.13±0.11 AU). A down-regulation of DCHI \textit{PYY} expression by 18.7\% was also observed (control: 5.32±0.01 AU versus DCHI: 4.85±0.08 AU. However, no changes were observed in pancreatic \textit{GLP-1R}, \textit{GIP}, \textit{GCG} and \textit{SST} expression in DCHI tissue.

\textbf{Discussion}

The current challenge faced in the management of CHI patients is to identify new drug therapy that would improve the hypoglycaemia in medically-unresponsive DCHI patients. At present, the only available option for this patient group is a near-total pancreatectomy. This is performed with the hope of reducing the hyperplastic \(\beta\)-cells; despite this surgery can lead to either recurrent hypoglycaemia or diabetes mellitus and pancreatic exocrine insufficiency. Thus, a detailed understanding of pathways that can be targeted by drugs in the management of these patients is required.

Whilst \textit{DPP-4} and its gut hormone substrates have been extensively researched in the past decade, the physiological roles of these regulators in glucose homeostatic processes are not fully understood. To our knowledge, apart from the effects of \textit{GLP-1} in HH, the role of other \textit{DPP-4} gut hormone substrates have not yet been investigated or examined fully in CHI [5-7, 17]. Hence this study aimed to identify the expression pattern of \textit{DPP-4} in the pancreas of CHI patients.

Histologically, CHI is characterised into FCHI and DCHI depending on pancreatic morphology. DCHI has large hyperchromatic nuclei dispersed throughout the pancreas, whereas FCHI has areas of \(\beta\)-cell hyperplasia [13]. Since the pancreatic tissue surrounding the lesion(s) in the FCHI pancreas have been shown to function normally [12], a lesionectomy usually cures the patient of the HH. Hence using the normal tissue from around the focal lesion as control, we identified the expression of \textit{DPP-4} in pancreatic \(\beta\)-, \(\alpha\)- and \(\delta\)-cells in children. Unlike reports looking at the adult pancreas [18, 19], we found the enzyme to be predominantly expressed in \(\beta\)-cells.

Reports have identified exocrine cells (in particular acinar and ductal cells) to express insulin, which is suggestive of exocrine transdifferentiation and is thought to be contributing to the \(\beta\)-cell hyperplasia, thus, potentially contributing to/worsening the HH phenotype [20]. Interestingly, we found the expression of insulin in the exocrine cells with \textit{DPP-4} localised to the periphery of these cells. It appears that the neoformation of these \(\beta\)-cells from the acini and ducts may arise from \(mTOR\) activation and the use of \(mTOR\) inhibitors have been shown to have the potential as effective therapy in medically-unresponsive DCHI patients [16, 20]. It has also been reported that \textit{DPP-4} inhibitors (which are known to reduce the degradation of \textit{GLP-1}), enhance islet insulin-like growth factor receptor (IGFR) and its downstream signalling targets (p-Akt and p-\(mTOR\)) in diabetic monkeys [21]. This would theoretically suggest that \textit{DPP-4} may have the ability to inhibit \textit{GLP-1}-mediated islet proliferation and exocrine transdifferentiation and this may be the reason behind its close location to the insulin-expressing exocrine cells. Moreover, the \textit{DPP-4} expression may otherwise be due to secondary or as an adaptive response to the hyperinsulinism or continuous feeding in these patients. However, the sample size for this study was small and these observations although very interesting, warrant further investigation.

We also assessed \textit{DPP-4} expression in the lesion of the FCHI and DCHI tissues. Here we found \textit{DPP-4} expression in all three islet-cell subtypes but at a much lower level in FCHI. As already known, the CHI pancreas have active proliferating pancreatic \(\beta\)-cells [13, 20, 22]. Thus, we assessed if cells expressing \textit{DPP-4} were also actively proliferating. In particular,
FCHI tissue expressed DPP-4 and Ki67 suggesting an increase in DPP-4 cellular growth. However, since these cells were absent of insulin, glucagon and somatostatin staining it would appear that FCHI proliferating DPP-4 cells may be of non-endocrine origin and requires further investigation to confirm. Thereafter, we identified the proliferating pancreatic endocrine cells as β-cells expressing DPP-4 in DCHI. Given this result, it comes as no surprise that pancreatic DPP-4 mRNA was also increased in DCHI patients. DPP-4 is known to inhibit GLP-1 and its effects on insulin synthesis, secretion and β-cell growth [23]. So, DPP-4 in DCHI may be attempting to inhibit the GLP-1 action and thus the hyperplasia seen in this group of patients. This difference between the paediatric and adult tissue remains unexplained since little is known about DPP-4 expression in children. Furthermore, a limitation with the control tissue is the difficulty in obtaining control tissue that is age-matched. Ways around this may be the use of foetal tissue, however, it has been shown that changes in pancreatic and gut hormones occur pre- and post-natally, thus, these cannot serve as true controls for these patients [24-26].

DPP-4 has been shown to be a marker for insulin resistance in obese individuals who have particularly high levels of circulating DPP-4 [27]. Moreover, DPP-4 directly activates mitogen-activated protein kinases (MAPK) pathways and has been shown to promote cell proliferation [28]. We were unable to assess these; however, it would be interesting to see if circulating DPP-4 activity has altered in these patients.

Medically-unresponsive HH persons require a pancreatectomy to prevent recurrent hypoglycaemia. However, complications of such invasive treatment include: (1) insulin-dependence in CHI and (2) reoccurrence of HH as a result of re-routing GI tract by the gastric bypass procedure. Currently, there is no literature except case reports assessing how the weight loss surgery promotes recurrent HH and nesidioblastosis even after pancreatectomy [29]. Hence an understanding of how gut hormones regulate this glucose dysfunction needs evaluation.

In summary, we found pancreatic DPP-4 to be expressed in the β-, α-, and δ-cells in morphologically normal paediatric tissue. In FCHI, pancreatic DPP-4 cells were proliferating in the absence of insulin, glucagon and somatostatin staining. Whereas, in DCHI we found islet β-cells were co-localised with DPP-4 and the proliferation marker, Ki67. Further work identified insulin expression in the exocrine cells, with DPP-4 expression on the cell surface in DCHI. Subsequently, we found this patient group to also have an increase in pancreatic DPP-4. In conclusion, our studies show that DPP-4 has the potential to be an important regulator of glucose metabolism in CHI and may serve as a possible target for the development of pharmacotherapy. However, more work is needed to examine more closely if (1) we are to gain full therapeutic benefit and (2) avoid possible adverse effects of current drug treatments for HH.

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Disclosure of conflict of interest

None.

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