Localization of nucleic acid-sensing toll-like receptors in human and mouse pancreas

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Abstract

Nucleic acid-sensing TLRs (3, 7, 8, 9) have a role both in antiviral innate immunity and in autoimmune disorders. We assessed the expression of TLR3, 7, 8 and 9 in human and mouse pancreas focusing on the subpopulations of cells in the Langerhans islets. We studied eight human samples with normal pancreatic islets and two samples from patients with type 1 diabetes. Additionally, 10 CD-1 mouse pancreases were analysed. Immunohistochemical double-stainings for the TLRs and insulin, glucagon or somatostatin, respectively, were performed along with appropriate controls. In human pancreas, strong immunoreaction of TLR7 and TLR8 was observed in the insulin positive beta cells, whereas glucagon or somatostatin expressing cells of the islets were weakly stained or negative. In type 1 diabetes, the expression in islets was weak or lost (TLR7: $p=0.014$, TLR8: $p=0.053$), correlating with loss of beta cells. TLR3 and 9 were expressed only weakly with no correlation to specific cell types. In mouse pancreas, only TLR9 was detected. Intra-pancreatic nerve ganglia strongly expressed TLR7. The strong expression of TLR7 and TLR8 in the beta cells of normal human islets could be an important piece in the puzzle of type 1 diabetes pathogenesis, and be linked with destruction of this particular subpopulation of the islet cells. In normal mice, only TLR9 can be constantly detected in the islets, highlighting differences between the species.

Keywords: Toll-like receptor; nucleic acid; type 1 diabetes; enterovirus; autoimmunity

Introduction

Toll-like receptors (TLRs) are innate immune receptors each of which having unique antigen-recognition domains. TLR3 and members of the TLR9 subfamily (TLRs 7, 8 and 9) specifically recognize different types DNA and RNA (1) triggering inflammatory response for anti-viral defence (2). In addition to immune cells, TLRs are found in epithelial cells and fibroblasts. Epithelial cells can thus regulate the inflammatory response to luminal microbes and endogenous danger signals by TLR-mediated activation (3). Recently, we reported TLR4 expression in human gastric G and D cells, and found evidence that TLR4 signalling modifies endocrine responses such as gastrin secretion (4). This suggests that functional links occur between the innate immunity and the endocrine systems.

Type 1 diabetes is characterized by immune mediated destruction of the pancreatic beta cells resulting with complete loss of insulin secretion (5). Incidence is rising suggesting the role of environmental factors in pathogenesis (6). It is known that T cells are dominated in insulinitis lesion resulting with damage of the beta cells (7). Viral infections have been suggested to trigger the autoimmunity in type 1 diabetes, especially enteroviruses (8). TLR3, 7 and 8 have been shown to contribute to coxsackievirus B induced type 1 diabetes in non-obese diabetic (NOD) mouse (9, 10) and additionally TLR9 contributes to interferon production during viral infection possibly predisposing to type 1 diabetes (11).

The aim of this study was to assess the expression of nucleic acid-sensing TLRs 3, 7, 8 and 9 in normal human pancreas and in pancreas of patients with type 1 diabetes. We also studied mouse pancreas specimens to compare of TLR expression in the islets of a species commonly used in experimental diabetes studies, and that in humans.

68 **Materials and methods**

69 *Samples*

70 We analyzed nine surgically removed pancreases. In eight cases, operation was due to ductal
71 adenocarcinoma of the pancreas and in one case due to intraductal papillary mucinous
72 neoplasm. Of these patients, seven had no evidence of diabetes in their clinical history and
73 had histologically normal islets, and two had previously been diagnosed with type 1 diabetes,
74 for 8 and 51 years earlier, respectively. Mean daily need for insulin was 28 units. None of the
75 subjects had any additional diseases affecting pancreas. For current analyses, samples
76 representing pancreas far from the neoplasms were selected, and the normal histological
77 structure of the specimens was confirmed by an experienced gastrointestinal pathologist. The
78 samples were obtained from files of Department of Pathology, Oulu University Hospital.
79 Additionally, a healthy human pancreas was obtained from a single organ donor in Oulu
80 University Hospital. The donor had no history of diabetes or any chronic disease. The use of
81 patient samples and the data inquiry were approved by the Oulu University Hospital Ethics
82 Committee. The need to obtain a written or oral consent from the patients for using the
83 samples in research was waived by the Finnish National Authority for Medicolegal Affairs
84 (VALVIRA; permission number 10832/06.01.03.01/2014). The acquisition of the pancreas
85 from the organ donor was also performed under permission (VALVIRA, permission number
86 10832).

87

88 A total of 10 CD-1 mice (5 male and 5 female, age 8 weeks) collected from the Animal
89 Research Center, Oulu University were analyzed. CD-1 strain has been previously used in
90 Cocksackie B virus triggered diabetes model (12).

91

92 *Immunohistochemistry*

TLR immunostainings were performed with a commercial antibodies (TLR3: NBP2-24875, TLR7: IMG-450, TLR8: NBP2-24917, TLR9: NBP2-24729, Novus Biological LLC, Littleton, CO and Imgenex, San Diego, CA) at a dilution of 1:30 (TLR3), 1:750 (TLR7), 1:800 (TLR8) and 1:300 (TLR9). For detection of the bound antibodies, we used the Dako Envision kit (Dako, Copenhagen, Denmark) with high temperature antigen retrieval in Tris-EDTA buffer for 15 minutes. Diaminiobenzidine (Dako basic DAB-kit) was used as a chromogen. Staining was done with Dako Autostainer (Dako, Copenhagen, Denmark). Double stains of TLR3, 7, 8 and 9, insulin, glucagon and somatostatin were performed by EnVision G|2 Doublestain kit (Dako) with antibodies for insulin (NCL-insulin, mouse monoclonal, Clone 2D11-H5, Leica Biosystems, Wetzlar, Germany, dilution 1:200), glucagon (A0565, rabbit polyclonal, Dako, dilution 1:3200), somatostatin (rabbit polyclonal, A0566, Dako, dilution 1:600). As a detection kit for single stainings of insulin and somatostatin we used Ultravision TP-125-HL (Thermo Fisher Scientific, MA, USA). For glucagon Envision kit was used.

The validation of immunohistochemical analysis was performed with negative controls including buffer solution or irrelevant antibodies instead of TLRs or insulin, glucagon and somatostatin antibodies, separately for human and mouse samples. For positive controls we used dendritic cells of small intestine lamina propria, reported to constantly express TLR3, 7, 8, 9 (13).

Assessment of TLR expression and quantification of Langerhan's islets

The histological slides were digitized using Aperio AT2 Console, Leica Biosystems Imaging Inc, Nussloch, Germany. Langerhans islets were recognized by using Hematoxylin and Eosin

117 stained sections and their areal proportion quantitated with the image analysis program
118 Imagescope (Leica).

119
120 Location and intensity of TLR expression was assessed by two independent investigators
121 (OH and HH), blinded from clinical data, using a four-point scale from 0 (negative) to 1
122 (weak), 2 (moderate) and 3 (strong). The extent of the staining was expressed as a percentage
123 (0-100%) of the stained cells or the nuclei in the samples. Nuclear expression of multiple
124 TLRs has been previously reported (14-16). The whole pancreatic tissue from histological
125 samples was analyzed. For comparison, mean value of two estimates was used and in case of
126 significant disagreement (more than one in intensity and more than 30% in extent), scores
127 were determined after re-evaluation by expert gastrointestinal pathologist (TJK) (17). In the
128 current study, no re-evaluations were needed.

130 *Statistical analysis*

131 We used IBM SPSS Statistics 22.0 (IBM corp., Armonk, NY) for statistical analyses. To
132 compare quantitative aspects of TLR expression between the groups Kruskal-Wallis Test was
133 used and comparison of the number of Langerhans islets between the groups was performed
134 with Mann-Whitney U. Median and interquartile range (IQR) was used due to skewed
135 distributions.

137 **Results**

138 *Histology of human pancreatic samples*

139 Histological structure of exocrine pancreas was normal all human subjects (n=10). Mean
140 areal proportion of Langerhans islets in subjects without type 1 diabetes (n=8) was 0.81
141 islets/mm², and in those with type 1 diabetes (n=2) 0.19 islets/mm² (p=0.036). Islets in

subjects without diabetes showed normal structure in all cases. Besides decreased size, the islets in the diabetic subjects showed collection of some intercellular hyaline material, but no inflammatory cell reaction.

Expression of TLR3, 7, 8 and 9 in exocrine human pancreas

TLR3 was expressed in majority (91%) of nuclei of exocrine cells and no cytoplasmic staining was seen. Cytoplasmic expression of TLR7 was detectable in 4/10 pancreases with median intensity of 0.5 and median proportion of positive cells 15%. TLR8 expression was cytoplasmic and slightly more abundant with detectable staining in all subjects with a median intensity of 1.0 and percentage of positive cells 100%. TLR9 was also expressed in the cytoplasm in all cases with a median intensity of 2.0 and percentage of 100%. Membrane expression of TLR9 was seen in about 15% of cells. Occasional lymphocytes and histiocytes were present in the studied pancreases, and expression of all studied TLRs were observed in these cells independent of staining intensity in the epithelial cells of exocrine pancreas.

Expression of TLR3, 7, 8 and 9 in human Langerhans islets

In humans without type 1 diabetes, all TLRs were expressed in Langerhans islets. Similarly to exocrine pancreas, TLR3 was expressed only in the cell nuclei and TLR7, 8 and 9 were expressed in the cytoplasm (Table 1, Fig. 1). Expression for TLR3 was similar in islets and the exocrine pancreas being present in majority of cells. In contrast, expression of TLR7 and 8 were clearly stronger in the islets this pattern greatly enhancing spotting the islets (Fig. 1d,g). Finally, TLR9 was detectable in Langerhans islets, although weaker than in exocrine pancreas (Fig. 1j). No inflammatory cells were detected in Langerhans islets.

In the diabetic subjects the location of expression was similar, but as compared to nondiabetic subjects, expression was less abundant for TLR3 (nuclei, $p=0.048$), TLR7 (intensity, $p=0.018$), and TLR9 (percentage, $p=0.046$). Expression for TLR8 showed a similar trend (intensity, $p=0.053$, percentage, $p=0.053$); Table 1, Fig. 1.

Interestingly, the proportion of positive cells in both normal and abnormal Langerhans islets was always less than 100% for TLR3, TLR7 and TLR8 (Fig. 1a,d,g) suggesting cell type specific expression. To analyse this phenomenon we performed double-stainings with TLR antibodies and those for the main endocrine hormones secreted by the subtypes of islet cells. Double-stainings indicated that TLR7 and TLR8 were expressed in all insulin-positive beta cells and only occasionally in alpha- and delta cells in the normal pancreas (Fig. 2). TLR3 showed no clear overlap with specific cell types. In islets from patients with type 1 diabetes cytoplasmic expression of TLR7 and TLR8 (Fig. 1e,h) was weak and diffuse and no single or double positive cells were found with insulin immunostaining, suggesting destruction of beta cells. In agreement with previous findings, single insulin positive cells were found in the exocrine pancreas of patients with type 1 diabetes (18), with TLR7 and 8 occasionally expressed in these cells.

TLR expression in mouse pancreas

In mouse pancreases, there were 0.46 islets/mm². The exocrine pancreas in mice was negative for TLR3, 7 and 8 (Fig. 1c,f,i). In contrast, TLR9 was always expressed exocrine cells with a median intensity of 3.0 and percentage of positive cells 100% (Fig. 1l). Nuclear TLR3 was seen in endocrine pancreas in 8/10 samples, with median percentage of positive nuclei 65%. In the islets, TLR9 was expressed weakly throughout (median intensity 1.0, percentage 100%). Weak expression of TLR7 (4/10) and TLR8 (1/10) was also detected in

the islets. Double-staining with TLR3, insulin, glucagon and somatostatin were performed with no clear overlap with specific endocrine cells.

Comparison of TLR expression in normal human and mouse pancreas

Expression of all studied TLRs differed significantly between normal human and mouse in exocrine pancreas. Membranous staining of TLR9 was observed only in human samples. Interestingly, TLR expression in Langerhans islets was also significantly higher in humans for TLR3 (nuclei $p < 0.001$), TLR7 (intensity $p = 0.002$, percentage $p < 0.001$) and TLR8 (intensity $p < 0.001$, percentage $p = 0.002$), Table 1.

TLR7 in intrapancreatic neural ganglia

Neural ganglia in all samples, both human and mouse, showed very high expression of TLR7 with intensity of 3.0 and percentage of 100% (Fig. 3). No other TLRs were detected in neural ganglia of either human or mouse samples. Ganglia were recognized by using Hematoxylin and Eosin stained sections.

Discussion

We have characterized nucleic acid-sensing TLR3, 7, 8 and 9 expression in exocrine and endocrine cells of histologically normal human pancreas and in patients with type 1 diabetes. For the first time, we describe evidence of strong expression of TLR7 and TLR8 specifically in insulin-secreting beta cells. In type 1 diabetes accentuated expression of TLR7 and 8 was lost and replaced with weak diffuse staining in the remaining islets without beta cells. In CD-1 mice, islets were negative or showed only occasional and unspecific TLR3, 7 and 8 expression with no correlation with specific cell types. TLR9 was expressed similarly to human with strong expression in exocrine and weak expression in endocrine pancreas.

216 Presence of TLR7 and TLR8 in the beta cells of human pancreas and the ability of these
217 innate receptors to recognize single stranded viral RNA support the hypothesis that beta cell
218 specific innate immune response triggered by a viral agent might launch the cascade leading
219 to type 1 diabetes.

220
221 Physiological role of TLR7 and TLR8 in the beta cells of human pancreas remains
222 speculative. Although widely expressed in diverse types of cells of the immune system and
223 epithelial cells, expression of TLRs in endocrine cells has only rarely been reported. Recently
224 we observed TLR4 in gastrin and somatostatin secreting cells of human gastric antral mucosa
225 (4). Furthermore we found association between genetic polymorphism of TLR4 and serum
226 gastrin suggesting that TLR4-ligand interaction might regulate endocrine function of G-cells.
227 Since gastric acid secretion is a major factor in gastrointestinal microbial defence, such TLR-
228 mediated regulation might be physiologically relevant innate response. More studies are
229 clearly needed to assess potential links between TLRs and the endocrine system function.
230 However, when considering animal model, it should be noted, that as shown in the present
231 study, TLR expression pattern in mice islets may be different from that in humans with no
232 beta cell predominance. Furthermore, the observed differences between human and mouse
233 pancreases in TLR expression suggests that in terms of viral pathogenesis of diabetes the
234 results from rodent studies might not be generalizable to humans.

235
236 Supporting our hypothesis of role of TLR-mediated beta cell inflammation in the
237 development of type 1 diabetes, single-stranded RNA viruses, especially enterovirus, have
238 been connected to the development of type 1 diabetes. Antibodies to enteroviruses are found
239 more frequently in recently diagnosed patients with diabetes than in healthy controls (19, 20).
240 Furthermore, enteroviral RNA and/or proteins can be detected in blood samples and intestine

241 biopsies of patients with type 1 diabetes (21). Recently in tail-resections of pancreases from
242 living patients with type 1 diabetes presence of viral capsid protein and viral RNA was
243 detected (22), suggesting possibility of persistent low-grade infection. Enteroviruses has been
244 suggested to associate with IFN- α mediated inflammation and initiation of autoimmunity (23,
245 24), which is also part of TLR7, TLR8 and MyD88 induced inflammation cascade (25).

246

247 Previously, it has been shown that the activation of TLR7 and 8 following viral exposure in
248 dendritic cells triggers a signal cascade which leads to intense secretion of pro-inflammatory
249 cytokines and type 1 interferons (2). TLR7 and 8 positive dendritic cells have been observed
250 in Langerhans islets in newly onset type 1 diabetes patients, in insulitis lesions and in non-
251 obese diabetic (NOD) mice, supporting importance of TLR7 mediated mechanisms (26, 27).
252 Furthermore, in NOD mice, TLR7 and 8 agonist (CL097) has been shown to shorten time to
253 the onset of type 1 diabetes, whereas the antagonist (IRS661) reduces the effect (10). Our
254 finding that TLR7 and 8 are constantly present even in normal beta cells supports the role of
255 innate immunity to viral antigens in pathogenesis of type 1 diabetes and indicates that
256 previous accumulation of dendritic cells to induce innate immunity response is not necessary
257 as the islet cells proper may be capable for the recognition of viral RNA.

258

259 TLR3 and 9 were also expressed in human Langerhans islets in our study, however showing
260 similar of less expression as compared with exocrine pancreas. Mouse Langerhans islets were
261 also at least occasionally positive for TLR3. Previously it has been shown that TLR3
262 knockout mice are protected from coxsackievirus induced type 1 diabetes and insulitis was
263 also less severe (9). TLR9 also contributes to resolving of viral infection with production of
264 cytokines, possibly contributing to dysfunctional inflammation leading to type 1 diabetes
265 (11).

266

267 Interestingly, the nerve ganglia within pancreatic tissue showed strong TLR7 expression.
268 Although the physiological role of this finding remains speculative, neural tropism and
269 dissemination through neural pathways (24, 28) of some viruses, suggests a function related
270 with innate immunity against such viral infections even for TLR7 in ganglion cells. Since
271 autonomic nervous system is known to regulate local inflammatory responses, our finding
272 provide an additional potential mechanisms linking viral infections and inflammation in the
273 pancreas. Additionally, possible TLR7 route might provide a specific targets for the
274 prevention of type 1 diabetes and other modes of viral related pancreatic inflammation as
275 new ways to modify responses are introduced, such as the recently recognized Thiostrepton
276 (29) or modified TLR7 ligands (30).

277

278 Our study was based on small number of patients and has to be considered as a preliminary
279 report needing confirmation with a larger series of pancreatic samples, especially of patients
280 with diabetes. It is noteworthy that all but one patient with histologically normal pancreas
281 were diagnosed with pancreatic cancer. However, this bias was minimized with careful
282 selection of specimens with normal basic histology for analysis. Also according to previous
283 studies adjacent carcinoma has only limited or no effect on normal epithelium suggesting
284 absence of any field impact in TLR expression (31). Our observations on TLR expression in
285 murine pancreas was based on only one strain, CD-1, which however, could be a relevant
286 model for human diabetes in case the etiological role of enteroviruses will be confirmed (12).
287 However, it would be interesting to explore the expression of nucleic acid-sensing TLRs also
288 in other mouse models for type 1 diabetes, such as the NOD mouse spontaneously developing
289 the disease by immune mechanisms.

290

291 We conclude that nucleic acid-sensing TLRs are present in human Langerhans islets. TLR7
292 and 8 are strongly expressed in human pancreatic beta cells. The expression is lost along with
293 disappearance of beta cells in patients with long-standing type 1 diabetes. In normal mice,
294 only TLR9 can be constantly detected in the islets, this finding highlighting differences
295 between the species. Based on their abundant expression on human beta cells, TLR7 and 8
296 may have important role in the development of type 1 diabetes.

297

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303 **Contribution statement** O.H. had full access to all of the data in the study and take
304 responsibility for the integrity of the data and the accuracy of the data analysis. H.H. and
305 O.H. had equal contribution as first authors.

306 *Study concept and design:* O.H., H.H., J.H.K., P.P.L., J.S., T.J.K.

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Fig. 1. Expression of nucleic acid-sensing TLRs in Langerhans islets of normal human pancreas, patients with type 1 diabetes and CD-1 mouse. TLR3 was expressed in cell nuclei with slightly higher expression in normal islet (a) compared to type 1 diabetes (b) and CD-1 mouse (c). TLR7 and TLR8 were strongly expressed in normal islets (d, g) and lost along with beta cells in long-standing type 1 diabetes (e, h). TLR7 and TLR8 immunostaining was negative in CD-1 mouse (f, i). TLR9 was detected in all islets (j, k, l), although weaker than in exocrine pancreas, without overlap with specific endocrine cells. Circles (b, e, h, k) indicate pseudoatrophic islets in patient with type 1 diabetes after loss of beta cells. Magnification 20x, except in e 10x.

Fig. 2. Double-stainings of TLR7 and TLR8 (brown) with endocrine hormones of the Langerhans islets, insulin, glucagon and somatostatin (gray). TLR7 and TLR8 co-localized with insulin (a, d) suggesting high expression in pancreatic beta cells. Glucagon (b, e) and somatostatin (c, f) positive cells were mostly negative for both TLR7 and TLR8. Magnification 20x.

Fig. 3. Strong TLR7 expression in human neural ganglia within pancreatic tissue (circle). Magnification 20x.

Table 1. Expression of TLR3, 7, 8 and 9 in human Langerhans islets with histologically normal pancreas (n=8), with type 1 diabetes (n=2) and in CD-1 mouse (n=10). Intensity was assessed with a four point scale from negative (0) to strong intensity (3). The extent of the staining was expressed as percentage of positive cells and positive cell nuclei (0-100%). Values are presented as median and interquartile range (IQR). Statistical testing was performed with Kruskal-Wallis test.

	intensity median	intensity IQR	statistical significance	percentage median	percentage IQR	statistical significance	nuclei median	nuclei IQR	statistical significance
TLR3									
Normal islets							100	18	
Type 1 diabetes							40	-	a
Mouse islets							65	48	a
TLR7									
Normal islets	3.0	0.8		90	18				
Type 1 diabetes	1.0	-	a	85	-				
Mouse islets	0.0	2.0	a	0	10	ab			
TLR8									
Normal islets	2.5	1.0		85	10				
Type 1 diabetes	1.0	-		100	-				
Mouse islets	0.0	0.0	ab	0	0	ab			
TLR9									
Normal islets	1.0	0.0		100	0				
Type 1 diabetes	1.0	-		95	-	a			
Mouse islets	1.0	0.0		100	0	b			

a compared to normal Langerhans islets, p<0.05

b compared to islets of patient with type 1 diabetes, p<0.05

Normal

Type 1 diabetes

Mouse

TLR3

a

b

c

TLR7

d

e

f

TLR8

g

h

i

TLR9

j

k

l

Insulin

Glucagon

Somatostatin