Expression of Toll-like Receptors in the Pancreas of Recent-onset Fulminant Type 1 Diabetes

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Abstract. Fulminant type 1 diabetes, established in 2000, is defined as a novel subtype of diabetes mellitus that results from remarkably acute and almost complete destruction of pancreatic beta cells at the disease onset. In this study, we aimed to clarify the pathogenesis of fulminant type 1 diabetes with special reference to insulitis and viral infection. We examined pancreatic autopsy samples from three patients who had died soon after the onset of disease and analyzed these by immunohistochemistry and in situ hybridization. The results were that both beta and alpha cell areas were significantly decreased in comparison with those of normal controls. Mean beta cell area of the patients just after the onset was only 0.00256 % while that of normal control was 1.745 %. Macrophages and T cells—but no natural killer cells—had infiltrated the islets and the exocrine pancreas. Although both of them had massively infiltrated, macrophages dominated islet infiltration and were detected in 92.6 % of the patients’ islets. Toll-like receptor (TLR) 3, a sensor of viral components, was detected in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (mean±SD) in all three patients. TLR7 and TLR9 were also detected in the pancreas of all three patients. Enterovirus RNA was detected in beta-cell positive islets in one of the three patients by in situ hybridization. In conclusion, our results suggest that macrophage-dominated insulitis rather than T cell autoimmunity contributes to beta cell destruction in fulminant type 1 diabetes.

Key words: Insulitis, Toll-like receptor, Enterovirus

TYPE 1 DIABETES, one of the two major forms of diabetes, results from nearly complete destruction of pancreatic beta cells [1]. We previously reported a novel subtype of type 1 diabetes that we called fulminant type 1 diabetes [2]. This subtype of diabetes is characterized by its clinical features, namely remarkably acute onset and absence of islet-related autoantibodies [2-4]. Fulminant type 1 diabetes has also shown high plasma glucose levels accompanied by ketosis or ketoacidosis. However, it also exhibits nearly normal glycosylated hemoglobin levels, a high serum pancreatic enzyme concentration and virtually no C-peptide secretion at the onset of disease.

A nationwide survey identified that this variant accounts for approximately 20 % of acute-onset type 1 diabetes cases in Japan [3]. Recently, 30.4 % of adult-onset type 1 diabetes was classified as fulminant type 1 diabetes in Korea according to the Japanese crite-
Our inclusion criteria for fulminant type 1 diabetes matched those previously published by our group [4]. Namely, 1) presence of ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms, 2) urinary c-peptide excretion <10 µg/day or fasting serum C-peptide level < 0.3 ng/mL (0.10 nmol/L) and peak serum C-peptide level < 0.5 ng/mL (0.17 nmol/L) after glucagon (1 mg) or a meal load soon after disease onset; and 3) plasma glucose level ≥ 288 mg/dL (16.0 mmol/L) and HbA1c level < 8.5 % at first visit.

In all three patients, postmortem dissections were performed, and we examined the pancreatic tissues. Patient 1 was diagnosed with diabetic ketoacidosis and died after 3 days of treatment. Patients 2 and 3 were diagnosed with diabetes after death. The direct causes of death of patient 2 and 3 were unknown at the time of autopsies. They ranged in age from 29 to 47 years, and the duration of disease was 3 to 6 days. Their blood glucose, Hba1c and serum c-peptide levels were 660 to 1585 mg/dL, 5.8 to 7.5 % and less than 0.39 ng/mL, respectively. GAD65 antibody, Ia-2 antibody and islet cell antibodies (ICA) were negative in all three patients. Patient 2 and 3 possessed HLA-DRB4-DQ8 haplotype that is susceptible to fulminant type 1 diabetes (Table 1) [4]. The pancreatic tissues were fixed in 10 % formalin and embedded in paraffin. The normal pancreatic tissues of six individuals were examined as non-diabetic control samples. These individuals were free from pancreatic diseases.

**Materials and Methods**

**Patients and samples**

We examined three patients with fulminant type 1 diabetes who had died just after the onset of overt diabetes. Our inclusion criteria for fulminant type 1 diabetes matched those previously published by our group [4]. Namely, 1) presence of ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms, 2) urinary C-peptide excretion <10 µg/day or fasting serum C-peptide level < 0.3 ng/mL (0.10 nmol/L) and peak serum C-peptide level < 0.5 ng/mL (0.17 nmol/L) after glucagon (1 mg) or a meal load soon after disease onset; and 3) plasma glucose level ≥ 288 mg/dL (16.0 mmol/L) and HbA1c level < 8.5 % at first visit.

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**Immunohistochemistry and morphometry**

Formalin-fixed paraffin embedded pancreatic sections cut at a thickness of 4 µm were deparaffinized and rehydrated using xylene and graded descending series of alcohol. After washing in distilled water once for 5 min, the slides only for natural killer (NK) cells were exposed to microwave pretreatment in a target retrieval solution (Dako Japan, Kyoto, Japan) at 100 °C for 15 min to enhance antigenicity. Endogenous
peroxidase activity was blocked for all sections using ice-cold 3 \% H_{2}O_{2}/methanol for 30 min. All slides were incubated for 30 min in 10 \% normal serum. The slides were then incubated at room temperature for 1 hour with guinea pig anti-insulin antibody (1:1000; Dako Japan), rabbit anti-glucagon antibody (1:1000; Linco Research, Ellisville, MO, USA), rabbit anti-human CD3 antibody (1:100, Dako Japan), monoclonal mouse anti-human CD68 antibody (1:100, KP1, Dako Japan), monoclonal mouse anti-human CD56 antibody (1:100, SNCL-CD56-1B6, Novocastra, Newcastle, UK), goat anti-human Toll-like receptor (TLR) 3 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human TLR7 antibody (1:100, Santa Cruz) and monoclonal mouse anti-human TLR9 antibody (1:200, 26C593, Imgenex, San Diego, CA, USA). As a negative control for TLR9 antibody, the same concentration of mouse IgG1 (Dako, Japan) was applied as a primary antibody. As negative controls for TLR3 and TLR7, those antibodies incubated with 10-fold higher concentration of TLR3 and TLR7 blocking peptides (Santa Cruz) were applied. Then slides were incubated with secondary antibodies; mouse or rabbit Envision kit/HRP (Dako Japan), Elite ABC mouse or goat IgG kit (Vector Laboratories, Burlingame, CA, USA), following the manufacturers’ instructions. Finally, antibody binding was detected using 3',5'-diaminobenzidine (DAB) (Dako Japan). All the washes were performed in PBS (pH 7.4). Sections were counterstained with methyl green or hematoxylin and were mounted in oil mounting medium (Mount-quick, Tokyo, Japan) before microscopy (BH-2, Olympus, Tokyo, Japan).

The total areas of all sections, for both insulin- and glucagon-positive cells, were measured using Image-J and a digital light microscope (BZ-8000, Keyence, Osaka, Japan). For each subject, three sections separated by more than 250 \( \mu \)m were assessed to eliminate the risk of measurement dispersion. Insulin- and glucagon-positive cell areas were measured by two different observers and expressed as a percentage of the total area of each section.

We used a double-immunofluorescence method to detect insulitis. The sections were incubated at room temperature for 1 hour with rabbit anti-human CD3 antibody (1:25, Dako Japan) or monoclonal mouse anti-human CD68 antibody (1:25, KP1, Dako Japan). The sections were then incubated at room temperature for 30 min with biotinylated anti-rabbit or anti-mouse immunoglobulins (Vector Laboratories), and then for an additional 15 min with fluorescein avidin D (Vector Laboratories). These procedures were followed by incubation with guinea-pig anti-glucagon antibody (1:200) or guinea-pig anti-insulin antibody (1:200), and incubated with the secondary antibody, namely, Alexa Fluor goat anti-guinea-pig immunoglobulins (Molecular Probes, Carlsbad, CA, USA). Each section was washed in PBS and mounted in aqueous mounting medium (Perma Fluor, Immunon, Pittsburgh, PA, USA) prior to fluorescence microscopy (BX 50, Olympus). We examined more than 125 islets for each subject to detect insulitis. When we observed two or more mononuclear cells infiltrating an islet, we determined that the subject was insulitis-positive, as we had previously shown [17]. This criterion guarded against false negatives in evaluating human insulitis because massive infiltration of mononuclear cells is rare in comparison with non-obese diabetic (NoD) mice. The criterion was also robust to false positives because single mononuclear cells sometimes become lodged in an islet even in subjects who do not have diabetes. Infiltration rates are defined as the percentage of insulitis-positive islets out of total islets examined. To clarify the TLR3 expression on mononuclear cells, the staining by goat anti-human TLR3 antibody (1:200) was followed by the second staining by rabbit anti-human cD3 antibody (1:25) or monoclonal mouse anti-human CD68 antibody (1:25).

**In situ hybridization**

The used method was modified from a previously published method [18, 19] and has been described in detail by Oikarinen et al. [20]. An enterovirus-specific oligonucleotide probe designed to hybridize with the conserved 5’ non-coding sequence was used (sequence from 5’ to 3’ GAA ACA CGG ACA CCC AAA GTA GTC GGT TCC GCT GCR GAG TTR CCC RTT ACG ACA) to detect all known enterovirus types. The probe was 3’end –labelled with digoxigenin using a kit (DIG oligonucleotide tailing kit; Roche Diagnostics, Welwyn Garden City, UK). A 10 pmol sample of the probe was used for one labelling reaction. Hybridization was performed using earlier published conditions [20]. The amount of probe in the hybridization cocktail was 250 ng, the hybridization time was 3 hour. Binding of the probes was revealed by anti-digoxigenin antibody, which was conjugated with alkaline phosphatase. This enzyme together with
its substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate yields an insoluble purple precipitate, which can be detected using a light microscope. Enterovirus-infected and mock-infected green monkey kidney cells were used as controls.

**Statistical analysis**

The significance of differences between the two groups was evaluated using Mann-Whitney’s U-test. \( P < 0.05 \) was considered statistically significant.

**Results**

**Insulin- and glucagon-positive cell regions**

Insulin- and glucagon-positive cells were markedly decreased in all patients with fulminant type 1 diabetes. The mean (±SD) insulin- and glucagon-positive cell areas were 0.00256±0.00158 % and 0.0278±0.0140 % in fulminant type 1 diabetes and 1.745±0.336 % and 0.266±0.049 % in normal control subjects, respectively (Table 2). In fulminant type 1 diabetes, both the beta and alpha cell regions were decreased significantly in comparison with those in normal control subjects (\( P<0.001 \)).

**Cellular infiltration of CD3+, CD68+ and CD56+ cells**

We detected the infiltration of CD3+ cells and CD68+ cells in and around the islets as well as in exocrine tissue by H&E stain. Decreased numbers of insulin+ cells and glucagon+ cells were seen in the islet. Original magnification: x300.
TLR9 in all patients with fulminant type 1 diabetes (Figure 3). Double staining method revealed that TLR3 was positive in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (Table 2). TLR3-positive macrophages and T cells were detected both in the islets and exocrine areas (Figure 4). No positive cells were observed in 92.6±2.7 % in fulminant type 1 diabetes but only in 1.3±1.2 % in control subjects (P<0.001) (Table 2).

Expression of TLRs
We detected the expression of TLR3, TLR7 and TLR9 in all patients with fulminant type 1 diabetes (Figure 3). Double staining method revealed that TLR3 was positive in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (Table 2). TLR3-positive macrophages and T cells were detected both in the islets and exocrine areas (Figure 4). No positive cells were observed in 92.6±2.7 % in fulminant type 1 diabetes but only in 1.3±1.2 % in control subjects (P<0.001) (Table 2).
and alpha cells as well as macrophage predominant insulitis was also observed.

The expression of TLRs is an important finding from the view of viral infection. TLRs are pattern recognition receptors (PRRs) that detect conserved structures found across a broad range of pathogens and protect the gateways to innate immune systems. Of these, TLR3, TLR7/8 and TLR9 are known to recognize viral components and induce type I interferon for anti-viral defense. Notably, TLR 3 is upregulated when coxsackie B5 virus itself or interferon alpha, a cytokine induced by viral infection, is incubated with the isolated human islets [21, 22]. These findings suggest that the expression of TLRs, especially TLR3, in the pancreases of fulminant type 1 diabetes soon after the disease onset. Destruction of beta

Expression of enterovirus RNA

We detected the expression of enterovirus RNA in 11 islets at case 1 by in situ hybridization. One islet contained insulin-positive cells (Figure 5). No enterovirus RNA was detected in the exocrine pancreas. We could not detect enterovirus RNA in case 2, case 3 and normal controls.

Discussion

In the present study, we have revealed the expression of multiple TLRs, especially TLR3, and enterovirus RNA in the pancreases of fulminant type 1 diabetes soon after the disease onset. Destruction of beta and alpha cells as well as macrophage predominant insulitis was also observed.

The expression of TLRs is an important finding from the view of viral infection. TLRs are pattern recognition receptors (PRRs) that detect conserved structures found across a broad range of pathogens and protect the gateways to innate immune systems. Of these, TLR3, TLR7/8 and TLR9 are known to recognize viral components and induce type I interferon for anti-viral defense. Notably, TLR 3 is upregulated when coxsackie B5 virus itself or interferon alpha, a cytokine induced by viral infection, is incubated with the isolated human islets [21, 22]. These findings suggest that the expression of TLRs, especially TLR3, in the pancreases of fulminant type 1 diabetes soon after the disease onset. Destruction of beta and alpha cells as well as macrophage predominant insulitis was also observed.

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the innate immune system by the virus. Moreover, the expression of TLR3 in T cells and macrophages infiltrating to the pancreas in those patients, is an important evidence of viral infection in fulminant type 1 diabetes. In addition, several TLRs and type I interferon are reported to express simultaneously in immune cells [23], suggesting different types of TLRs might be detected in a same immunocyte in our specimens. The finding that enterovirus RNA was detected in a beta-cell-positive islet in one of the three patients by in situ hybridization is a direct evidence of enterovirus infection in this patient, while Ylipaasto et al studied autopsy pancreases from 65 type 1 diabetic patients (not subclassified into type 1A or type 1B) for presence enterovirus RNA by in situ hybridization and they found positive results in just 4 out of 65 patients. [24].

Second, both beta and alpha cell regions were decreased significantly in fulminant type 1 diabetes, even very soon after the onset of overt diabetes. The beta cell region in fulminant type 1 diabetes patients was 0.1 % of that in normal controls in our study. We previously reported that the beta cell area was decreased to 0.4 % of that of normal controls in pancreatic biopsy specimens with fulminant type 1 diabetes obtained 1 to 5 months after the onset of overt diabetes. We also reported that the beta cell area in autoimmune (type 1A) diabetes was decreased to only 14.5 % of that in normal controls [8]. These data indicated that almost all the beta cells were destroyed within a short period in fulminant type 1 diabetes. The result contrasts strikingly with type 1A diabetes where the process of beta cell destruction usually progresses gradually [25]. In addition, the alpha cell area in fulminant diabetes was also markedly decreased to 9.6 % of that in normal controls, indicating that both beta and alpha cells are damaged at the onset of fulminant type 1 diabetes. This finding also contrasts to the mild decrease of alpha cells in type 1A diabetes.

Third, we detected the infiltration of CD3+ cells and CD68+ cells in and around the islets, as well as in the exocrine pancreas, in all patients with fulminant type 1 diabetes just after the onset of clinical diabetes. CD68+ macrophages infiltrates predominantly and are observed in 92.6 % of islets examined in this study. In our previous report, no insulitis was observed in the biopsy specimens of three patients obtained 1 to 5 months after onset [2]. Overall, it is reasonable to believe that mononuclear cell infiltration into the islets exist at the time of disease onset but disappear soon after both beta cell destruction and elimination of possible viral antigens in fulminant type 1 diabetes.

From these results, we suggest that not autoimmune but antiviral inflammation plays an etiopathological role in fulminant type 1 diabetes. In the classical type 1A diabetes, autoimmunity is believed to be an etiology and insulin is the most likely candidate as a primary antigen [25]. It is well known that T cells are dominated in insulitis lesion, alpha cells are not affected, and beta cells are specifically damaged because of selective recognition of beta cell autoantigens by T cells [26]. However, in fulminant type 1 diabetes, the infiltration of macrophages (but not T cells) is dominant. Macrophages are initially activated in viral infected lesion and generate inflammatory cytokines, and chemokines to kill the target cells [27], though it also observed in low-dose Streptozotocin-induced diabetes model mice [28]. They are less selective than T cell-oriented target cell death. This hypothesis of a less selective mechanism is in accord with the fact that both beta and alpha cell regions are decreased significantly in fulminant type 1 diabetes as shown in this study.

In conclusion, our study showed remarkably decreased numbers of pancreatic beta and alpha cells, macrophage-dominated insulitis and the expression of TLRs, a signature of viral infection, in fulminant type 1 diabetes soon after the disease onset. These results suggest a new mechanism of virus-induced macrophage-dominated inflammatory process, rather than autoimmune T cell response, plays a major role in beta cell destruction in this novel subtype of diabetes.

Acknowledgements

We would like to thank Shinobu Mitsui and Fumie Katsube for their excellent technical assistance and Prof. Tadatsugu Taniguchi (University of Tokyo) for the helpful discussion and Dr. Kensuke Miyake (University of Tokyo) for the generous gifts of the antibodies. This study was supported in part by a Grant-in-Aid from the Japanese Society for the Promotion of Science (KAKENHI 19790641, 19591087, 19591069, 21591184), a grant from The Naito Foundation (2007), a grant from Takeda Science Foundation (2007), a Grant-in-Aid from the Japanese Ministry of Health, Labor and Welfare and a grant from Juvenile Diabetes Research Foundation (HH).
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219


