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HSP 10 is a new autoantigen in both autoimmune pancreatitis and fulminant type 1 diabetes

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ABSTRACT

To search autoantigens in autoimmune pancreatitis (AIP), we have screened the human pancreas cDNA library with a patient's serum and obtained 10 positive clones. Seven out of 10 clones were amylase α-2A, the autoantibody to which was specifically detected in sera from patients with AIP and fulminant type 1 diabetes (FT1DM) [T. Endo, S. Takizawa, S. Tanaka, M. Takahashi, H. Fujii, T. Kamisawa, T. Kobayashi, Amylase α-2A autoantibodies: novel marker of autoimmune pancreatitis and fulminant type 1 diabetes mellitus, Diabetes 58 (2009) 732–737]. Sequencing of 1 out of remaining 3 positive clones revealed that it was identical to heat shock protein 10 (HSP 10) cDNA. Using a recombinant HSP 10, we have developed enzyme-linked immunosorbent assay (ELISA) system for detecting autoantibodies against HSP 10. We found that autoantibody against HSP 10 was also produced with high frequency in sera from patients with AIP (92%) and FT1DM (81%), but not in chronic alcoholic pancreatitis (8%) or healthy volunteers (1.4%). These results suggest that an autoantibody against HSP 10 is also a new diagnostic marker for both AIP and FT1DM.

Autoimmune pancreatitis (AIP), a distinct form of chronic pancreatitis [1], is characterized by (1) irregular narrowing of the main pancreatic duct and swelling of the pancreas, both of which are due to abundant lymphoplasmacytic inflammation [2], (2) increased levels of serum IgG and IgG4, with positive autoantibodies such as anti-lactoferrin antibody (LFAb) or anti-carbonic anhydrase II antibody (CAIIAb) [3,4], and (3) diabetes, which is a frequent complication and can be resolved by corticosteroid treatment [5].

We previously reported that pancreatic islets, as well as exocrine pancreatic cells, were associated with the inflammatory process involving $CD8^+$ and $CD4^+$ T cells, which might induce diabetes mellitus in AIP [6]. These data support the concept that autoimmune mechanisms play pivotal roles in the destruction of endocrine and exocrine pancreatic functions in AIP with diabetes.

Clinically, initial symptoms of AIP include obstructive jaundice and mild abdominal pain, but some patients are asymptomatic, making it difficult to distinguish AIP from idiopathic chronic pancreatitis or cancer of the pancreas. In such cases, detection of autoantibodies is an important means for diagnosing AIP; however, some proportion of patients with AIP are negative for LFAb and CAIIAb [3,4].

We encountered an AIP patient whose serum IgG and IgG4 levels were 3498 mg/dl and 2430 mg/dl, respectively. High concentrations of IgG in this case prompted us to search for new autoantigens primarily associated with AIP. We have screened λTriplEx2 human pancreas cDNA library with the patient's serum and obtained 10 positive clones. Seven out of 10 clones were identical to amylase α-2A (AMY) [7].

In this report, we further analyzed remaining positive clones other than AMY. Sequencing of 1 out of 3 clones revealed that it was identical to heat shock protein 10 (HSP 10) cDNA. We determined frequency of autoantibody against HSP 10 in AIP and other pancreatic diseases.

Materials and methods

Subjects. Serum used for screening the human pancreas cDNA library was obtained from a 67-year-old male patient with AIP (A.O.), whose detail laboratory data were described previously [7]. Additional 19 AIP sera, 24 sera from patients with chronic alcoholic pancreatitis, 24 serum from patients with pancreas tumor [cancer ($n = 10$) and intraductal papillary mucinous tumor (IPMT, $n = 14$)] were recruited. Sera from FT1DM ($n = 16$, 11 cases at the onset and 5 cases after onset) was diagnosed by criteria (fasting C-peptide $\leqslant 0.033$ nmol/L and HbA_{1c} is $\leqslant 8.0\%$ or $\sum$C-peptide $\leqslant 0.540$ nmol/L and HbA_{1c} is $\leqslant 8.0\%$), type 2 diabetes (T2DM) ($n = 50$), Hashimoto's thyroiditis ($n = 54$) and control sera (healthy

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Table 1
Clinical characteristics of the subjects.

Type of diabetes	*N*	Age (Y)	Sex (M/F)	Duration[a] of diabetes month (M)/day (D)	Treatment by insulin (*N*)
Autoimmune pancreatitis	20	67.1 (9.8)	18 / 2	–	12
At the onset[a] and before corticosteroid treatment	12				
After corticosteroid treatment	8				
Chronic alcoholic pancreatitis	24	62.8 (11.7)	18 / 6	–	–
Pancreatic tumor	24	69.0 (10.1)	10 / 14	–	–
Cancer	10				
IPMT	14				
Fulminant type 1 diabetes	16	41.6 (14.6)	11 / 5	4.7 M (8.4)	16
At the onset[b]	11			23 M (27)	
After the onset	5			406 D (343)	
Acute onset type 1 diabetes	40	24.9 (16.2)	13 / 27	28.8 M (45.0)	40
At the onset[b]	18			0.7 M (0.9)	
After the onset	22			51.8 M (50.3)	
Type 2 diabetes	50	62.2 (12.7)	35 / 15	138.5 M (100.3)	27
Normal	71	40.7 (21.5)	39 / 32	–	–
Hashimoto's thyroiditis	54	57 (12.1)	6/48	–	–

Mean (SD), IPMT; intraductal papillary mucinous tumor.
[a] Duration; from onset of diabetes to time of sample collection.
[b] At onset; within 3 months after onset.

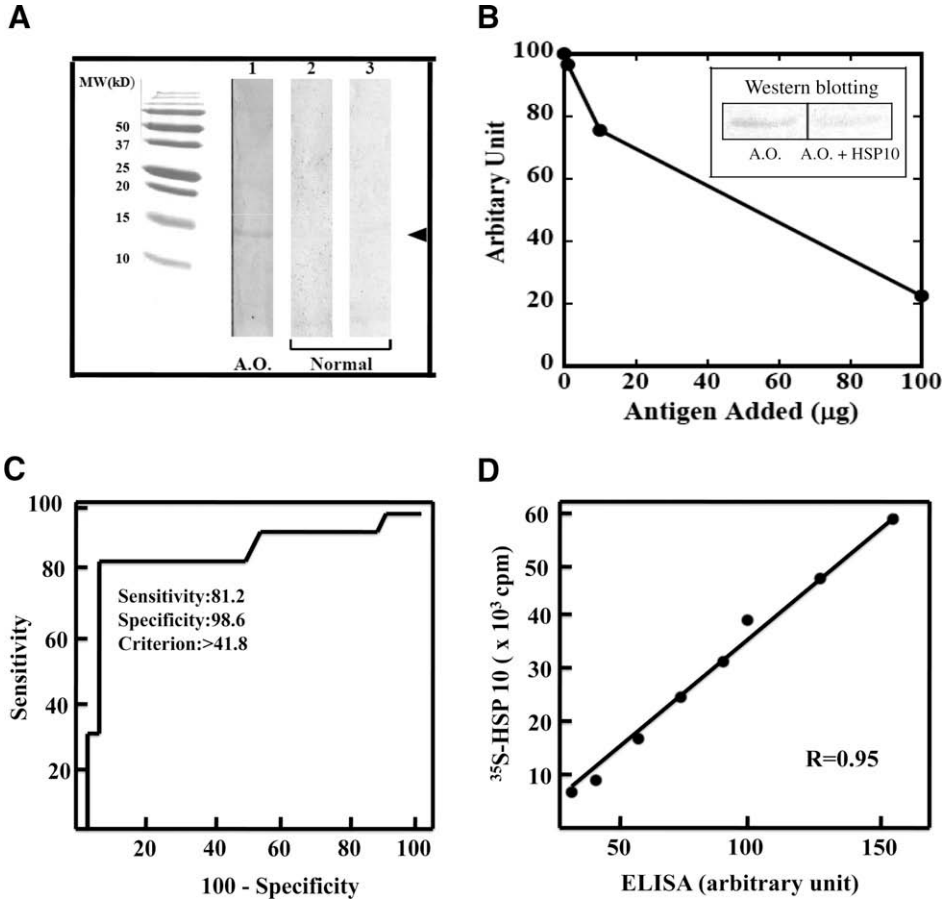

Fig. 1. Western blot analysis and development of an ELISA system for detecting HSP 10 autoantibody. (A) Western blot analysis. Recombinant human HSP 10 was electrophoresed in a 0.1% SDS–15% polyacrylamide gel and transferred onto a PVDF membrane. The membrane was reacted with serum (1000×) from a patient with AIP (lane 1) and normal control sera (lanes 2 and 3). (B) By coating the plate with the recombinant protein, we developed an ELISA system for detecting anti-HSP 10. One milliliters of the patient's serum (1:1000) was preincubated with 1, 10, and 100 mg/ml of the recombinant protein overnight at 4 °C and then added to the plate and incubated for 1 h at 37 °C. The data were the mean of triplicate assays. The inset shows that the positive reaction in Western blotting was reduced when serum from a patient with AIP (A.O.) was preincubated with 100 mg of the recombinant protein. (C) ROC analysis. We carried out ROC analysis of the healthy normal volunteers (n = 71) and fulminant type 1 diabetes patients (n = 16) with MedCalc. (D) Correlation between the result of ELISA and that of immunoprecipitation. Sera from 11 patients with AIP (●) were assayed by ELISA and IP for detecting the autoantibody against HSP 10.

volunteer, $n = 71$ (39 male and 32 female), were also recruited from our cohort (Table 1). These patients' sera were almost overlapped with our previous report [7], in which detail diagnostic criteria for each disease and patients' profiles were described.

Immunoscreening. λTriplEx2 human pancreas large insert cDNA library (HL5517u) was screened with a serum from AIP patient (A.O.) as described previously [7].

Preparation of the recombinant human heat shock protein 10 (HSP 10). A cDNA fragment of the positive clone was amplified by polymerase chain reaction (PCR) with the sense primer 5′-ATGGGGA TCCGCAGGACAAGCGTTTAGA-3′ and anti-sense primer 5′-CTTCG AATTCTCAGTCTACGTACTTTCC-3′. The PCR product was digested with BamHI and EcoRI, and then ligated into pTrcHisB (Invitrogen Co., Carlsbad, CA). After sequencing, the plasmid was transfected into *Escherichia coli* BL21 (Novagen, Darmstadt, Germany). The production of the recombinant protein was induced with 1 mM IPTG, and purified by HisBond® column chromatography (Invitrogen Co., Carlsbad, CA).

Western blot analysis. The 0.1% SDS–15% polyacrylamide gel electrophoresis and transference of the proteins onto the polyvinylidene difluoride (PVDF) membrane were carried out as described previously [8]. The membrane was reacted with goat horseradish peroxidase-conjugated anti-human IgG (1:2000) for 30 min at room temperature. Positive reaction was detected by the same way as described in the section on immunoscreening.

Enzyme-linked immunosorbent assay (ELISA) for detecting autoantibody against human HSP 10. Autoantibody against human HSP 10 was measured by ELISA using the methods as described previously [5,7]. The bound antibody was specifically reacted with goat horseradish peroxidase-conjugated anti-human IgG (1:2000) in 1% BSA for 30 min at room temperature. After washing, the plate was incubated with 100 ml of 1-Step Slow TMB-ELISA (PIERCE, Rockford, IL) for 30 min. The reaction was terminated by adding 100 ml of 1 M H_2SO_4. Intra-assay CV was 1.6% and inter-assay CV was 8.8%.

In vitro translation and immunoprecipitation assay. cDNA fragment of HSP 10 was amplified by PCR, and then ligated into pcDNA3.1. ^{35}S labeled human HSP 10 was prepared with PROTEIN script II (Ambion, Austin, TX) and [^{35}S] methionine (GE Healthcare, Piscataway, NJ). ^{35}S labeled HSP 10 was incubated with patients' sera (100×) in 100 μl of phosphate buffer saline (PBS) containing 1% bovine serum albumin at 4 °C overnight. Bound antigens were catched, washed and released from the column using Catch and Release v2 immunoprecipitation system (MILLIPOR, Temecula, CA).

Ethics. An ethics committee approved all study protocols, and patients and first-degree relatives of patients with FT1DM gave informed consent.

Statistical analysis. Statistical analysis was carried out using Fisher's exact test (JMP, Cary, NC), in which we considered statistically significant if P values were <0.05. ROC analysis was carried out with MedCalc (MedCalc Software, Mariakerk, Belgium).

Results

Cloning of HSP 10 cDNA from human pancreas cDNA library

We screened λTriplEx2 human pancreas cDNA library with the serum from the patient with AIP (A.O.) and obtained 10 positive clones. Of 10 clones, 7 were AMY cDN [7], but remaining 3 clones did not cross-hybridized with ^{32}P-AMY cDNA. Insert size of 1 (clone 34) out of 3 clones is 800 bp, and sequencing of it revealed that the clone was identical to human HSP 10. When compared to the nucleotide sequence of the human HSP 10 cloned by Monzini et al. [8], the clone contained the full coding sequence, the 5′ end of which started from −75 bp (A in ATG is designated as +1) and the 3′ end was +724 bp.

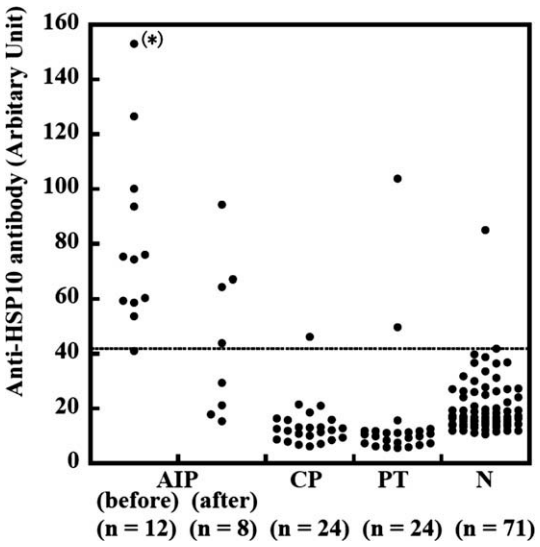

Fig. 2. Prevalence of autoantibody against human HSP 10 in patients with various pancreatic diseases. The prevalence of autoantibody against HSP 10 in patients with autoimmune pancreatitis (AIP; before treatment with corticosteroid, $n = 12$; after treatment with corticosteroid, $n = 8$), chronic alcoholic pancreatitis (CP, $n = 24$), pancreatic tumor (PT, $n = 24$), and normal controls (healthy volunteers N; $n = 71$) was studied by ELISA as described in Materials and methods. Cut-off value is shown by dotted line. The data were the mean of triplicate assays. (∗): $p < 0.001$ by Fisher's exact test.

Western blot analysis, ELISA system and immunoprecipitation assay for detecting HSP 10 autoantibody

We produced recombinant human HSP 10 in *E. coli* BL21 and carried out Western blot analysis. The patient's serum (A.O.) clearly recognized the 14 kDa recombinant protein (lane 1), but sera from healthy volunteers (lanes 2 and 3) did not (Fig. 1A). When the patient's serum was preincubated with the recombinant protein, positive staining was abolished (Fig. 1B, inset), suggesting that the autoantibody reacted with the recombinant protein.

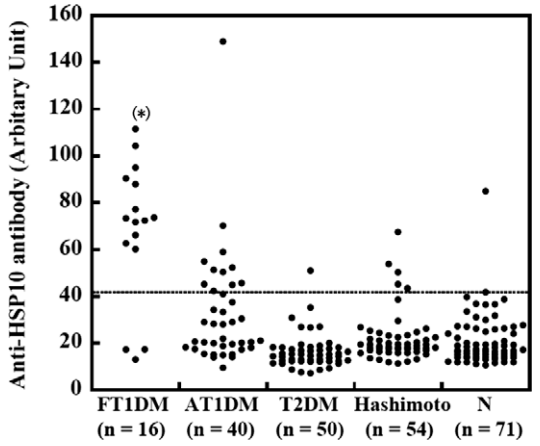

Fig. 3. Prevalence of autoantibody against HSP 10 in patients with various types of diabetes. The prevalence of autoantibody against HSP 10 in patients with fulminant type 1 diabetes (FT1DM, $n = 16$), acute onset type 1 diabetes (AT1DM, $n = 40$), type 2 diabetes (T2DM, $n = 50$), normal controls (N, $n = 71$) and patients with Hashimoto's thyroiditis (Hashimoto, $n = 54$) was studied by ELISA as described in Materials and methods. The data were the mean of triplicate assays. The dotted line is the cut-off value. (∗): $p < 0.001$ by Fisher's exact test.

Next, by coating the protein onto the plate, we developed an ELISA system for detecting anti-HSP 10 antibodies in the serum. When compared to the normal serum, patient serum showed a strong signal. This reaction was absorbed when the patient's serum was preincubated with recombinant HSP 10 protein (Fig. 1B). To obtain a cut-off value for positivity, we carried out ROC analysis of the control (healthy volunteers ($n = 71$)) and FT1DM patients ($n = 16$) with MedCalc software. Analysis of the criterion values and coordinates of the curve indicated that, at value 41.8, sensitivity, specificity, positive predictive value and negative predictive value were 81.25%, 98.59%, 92.85% and 99.87%, respectively (Fig. 2C) (area under the ROC curve: 0.88, significance level P: 0.0001). So, we set 41.8 as cut-off value for positivity.

We further prepared ^{35}S labeled HSP 10 by in vitro transcription and translation, and then immunoprecipitation assay was carried out using IgGs from the patients with AIP. The amounts of precipitated ^{35}S-HSP 10 were well correlated with the ELISA signals (Fig. 1D).

Prevalence of autoantibody against human HSP 10 in patients with AIP

Using the ELISA system, we determined the prevalence of autoantibody against HSP 10. Of the patients with AIP ($n = 12$) who were newly diagnosed but not yet treated with corticosteroid, 92% were positive for HSP 10 autoantibodies ($p < 0.0001$, Fisher's exact test). When 8 out of these 12 patients with AIP were treated with corticosteroid, 4 patients (63%) became to be negative for the autoantibody. Only 2 (8%) were positive in sera from 24 patients with chronic alcoholic pancreatitis, and 2 (8%) were positive in sera from 24 patients with a pancreas tumor (pancreatic cancer, $n = 10$; IPMT, $n = 14$) (Fig. 2).

Prevalence of autoantibody against human HSP 10 in the patients with fulminant type 1 diabetes, acute onset type 1 diabetes and type 2 diabetes

Interestingly, of the 16 patients in whom FT1DM was newly diagnosed, 13 (81%) were positive ($p < 0.0001$, Fisher's exact test) for the HSP 10 autoantibody, with titers nearly comparable to those of patients with AIP (Fig. 3). The autoantibody was detected with low frequency in patients with AT1DM (29%), and only 1 in the patients with T2DM (2%). Antibodies were detected in 9% of patients with Hashimoto's thyroiditis, a representative organ-specific autoimmune disease.

Longitudinal changes of HSP 10 autoantibodies in patients with AIP or fulminant type 1 diabetes

The levels of HSP 10 autoantibodies were measured by ELISA in the patients with AIP ($n = 2$), who were followed up to 12–14 months, and in the patients with FT1DM ($n = 2$), who were followed up to 7–10 weeks immediately after the clinical onset. HSP 10 autoantibodies from 2 patients with AIP were positive at onset and were sustained until the initiation of corticosteroid treatment, while the titer of the autoantibodies decreased or disappeared after

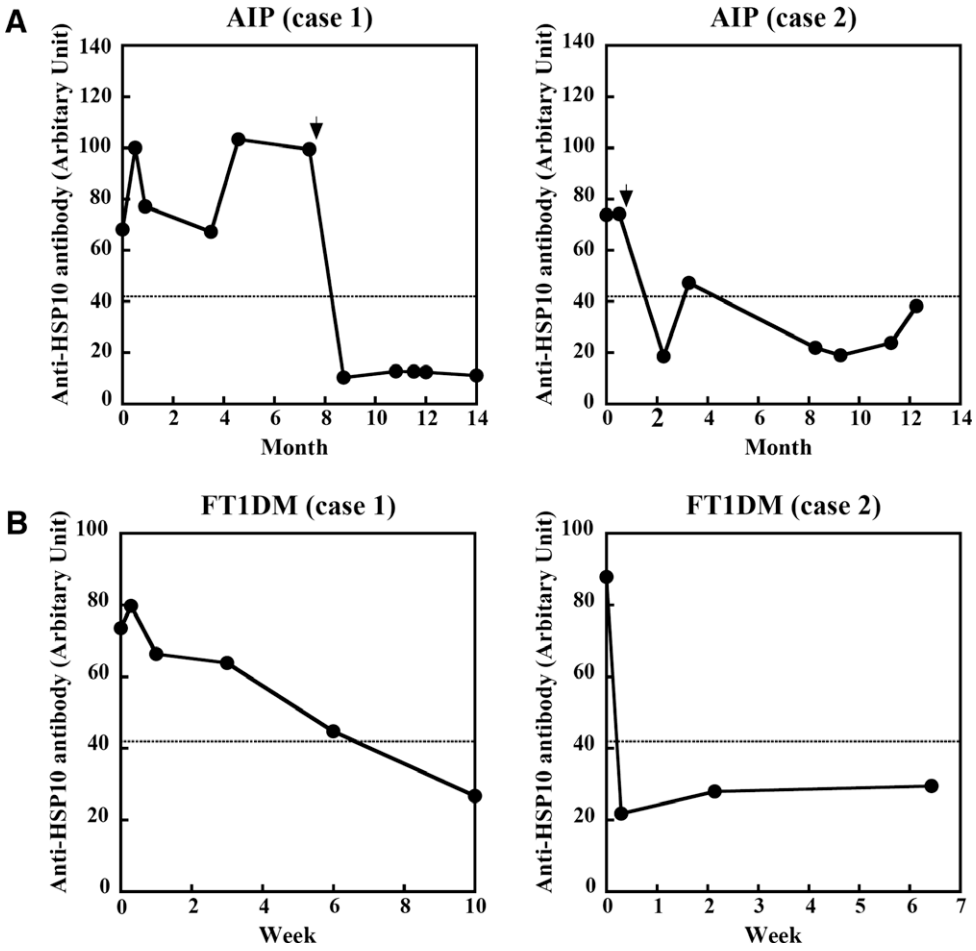

Fig. 4. Longitudinal changes of HSP 10 autoantibodies in patients with AIP or fulminant type 1 diabetes. (A) Time course of HSP 10 autoantibodies in two patients with AIP. The titers of HSP 10 autoantibodies from Case 1 and Case 2 are shown. Each value is the mean of triplicate assays. Arrows indicate the initiation point of corticosteroid treatment in each case. The dotted line shows the cut-off value. (B) Time course of HSP 10 autoantibodies in two patients with FT1DM. The titers of HSP 10 autoantibodies from Case 1 and Case 2 are shown. Each value is the mean of triplicate assay.

initiation of corticosteroid treatment (Fig. 4A) in parallel with decline of serum IgG concentration (data not shown). HSP 10 autoantibodies from two patients with FT1DM were also positive at onset and the titer decreased with the duration of diabetes (Fig. 4B).

Discussion

In the present study, we detected a new autoantibody against HSP 10 in patients with active AIP, but not in patients with chronic alcoholic pancreatitis or pancreatic tumors. Titers of the autoantibody were high at onset, and rapidly decreased in response to corticosteroid treatment, suggesting that HSP 10 is a new diagnostic and clinical marker for AIP.

It is particularly interesting that HSP 10 autoantibodies are detected in a high proportion of the patients with newly diagnosed FT1DM and AT1DM. AMY autoantibodies were also detected with similar prevalence both in patients with AIP and FT1DM [7], suggesting that both diseases are closely related with each other.

At present, the pathogenesis of FT1DM is associated with autoimmunity is still unknown [9], but we have demonstrated $CD4^+$ and $CD8^+$ T cell infiltration into pancreatic exocrine cells as well as the islets, which decreased immediately after the onset of FT1DM [10]. These results, as well as the presence of an autoantibody against HSP 10, suggest that the disease might be autoimmune-related, involving the exocrine and the endocrine pancreas functions. Our data also suggest that the measurement of the autoantibodies against HSP 10 is useful for diagnosing FT1DM, which sometimes causes serious diabetic ketoacidosis and is life-threatening for affected individuals who may be pregnant [11].

HSP 10 and HSP 60 form mitochondrial chaperoning complexes and are believed to play a role in the maintenance of normal mitochondrial function. However, overexpression of these proteins during cellular stress makes them an important part of immune system recognition. Following their release from inflamed or necrotizing tissues, they may be recognized by the cell surface receptors of the host immune systems [12].

Recently, it has been proposed that HSP 60, a counterpart of HSP 10, is an early antigen in the triggering of insulin-dependent diabetes mellitus; as the presence of an antibody to HSP 60 precedes the disease, HSP-reactive T cells can transfer the disease to prediabetic NOD mice, and vaccination with the protein blocks disease induction [13,14]. Furthermore, studies using HSP 60 transgenic mice indicated that it is involved in the islet-cell destruction that occurs in NOD mice [15].

With respect to HSP 10, immunohistochemical studies revealed the presence of HPS 10 in the pancreas, especially in acinal cells and also islet cells [16]. It is also reported that HSP 10 is detected outside the cells [17]. So, possibility exists that positive HSP 10 antibody in AIP and FT1DM may reflect only pancreatic destruction.

However, recent findings have revealed that extracellular HSP 10 interacts with Toll-like receptor 4 and inhibits the induction of nuclear factor-κB, followed by the reduction of serum tumor necrosis factor and RANTES (regulated upon activation, normal T cell expressed and secreted) levels and the elevation of serum interleukin-10 levels [18]. Therefore, HSP 10 itself has anti-inflammatory properties or has a role in the modulation of innate immune response. Indeed, administration of HSP 10 to the patients with rheumatoid arthritis reduces the signs and symptoms of the disease [19]. So, we speculate that HSP 10, like HSP 60, might be involved in the pathogenesis of both diseases.

Taking into account these observations, further studies are needed to clarify the roles of HSP 10 or its autoantibody in the development of both AIP and FT1DM.

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