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Intravascular and Intra-atrial Extension of Follicular Thyroid Carcinoma

Keiji Tanimoto,[1] Tetsuya Hiraiwa,[1] Ranko Ibata,[1] Jungo Terasaki,[1] Akiko Soyama,[2] Takahiro Katsumata,[3] and Toshiaki Hanafusa[1]

Dear Editor:

Follicular thyroid carcinoma (FTC) accounts for 10%–27% of thyroid cancers in published series. Microscopic vascular invasion is well recognized in FTC, but massive invasion of tumor into the great veins is rare (1). Only 10 cases of thyroid cancer with invasion into the right atrium have been documented. We report a patient with FTC that invaded not only the great veins but also the right atrium.

An 80-year-old woman presented with dyspnea, cyanosis, orthopnea, and pitting edema. Medical history was significant for a right hemithyroidectomy at age 66 for a 6-cm atypical adenoma. She had been treated with diuretics for chronic heart failure since age 73. Four months before admission, she presented with dyspnea. Diuretics were increased but her symptoms did not improve. She was referred and admitted to our hospital.

Physical examination revealed edema of the face and neck and dilatation of the right jugular vein, suggesting superior vena cava syndrome. There was a surgical scar on the right side of the neck but no palpable tumor there. The electrocardiogram showed atrial fibrillation. Thyroid function tests were normal but serum thyroglobulin was elevated to 16,300 ng/mL (normal, <30 ng/mL). Serum antithyroglobulin antibody levels were 0.8 U/mL (normal range, <0.3 U/mL). Cardiac ultrasound revealed a tumor occupying the right atrium and extending beyond the tricuspid valve (see Supplementary Data, available online at www.liebertonline.com/thy). Enhanced computed tomography (CT) showed that a >17-cm tumor with a cystic component extended from the right internal jugular vein into the superior vena cava and right atrium (see Supplementary Data). There were no masses in the left lobe of the thyroid. Thallium 201 (Tl-201) scintigram revealed concentrations of radioactivity in the same area as the tumor (see Supplementary Data). She was treated with diuretics, cardiac stimulants, and alpha-human atrial natriuretic peptide but there was no symptomatic improvement. She underwent surgical removal of the tumor in the brachiocephalic vein, superior vena cava, and right atrium but not the right internal jugular vein. Pneumonia developed after surgery and she died 2 months later. Histopathological examination of the tumor revealed FTC.

Intra-atrial extension of thyroid cancer is rare as only 10 cases have been reported since 1930 (2–4). Six were FTC and the others were papillary thyroid carcinoma, atypical thyroid cancer, adenocarcinoma, and undifferentiated cancer. Of the six cases with FTC, two were untreated and died within 2 months and four were treated with tumor thrombectomy. A patient with FTC who had extension of tumor to the heart was reported in 1978 (3). That patient received aggressive surgical therapy with removal of the tumor thrombus from the neck to the right atrium. Of the four cases in the literature treated with tumor thrombectomy, two survived for more than 2 years, suggesting that surgical treatment might prolong survival in some patients. In the present case, the tumor was removed surgically because of uncontrolled heart failure and obstruction of the tricuspid valve but she died 2 months after surgery.

Two types of extension of FTC to the heart have been described (3). One is direct extension through the great veins of the chest, as in the present case. The other is metastatic seeding to other organs. Most patients with direct extension died shortly after their diagnosis as a result of vascular or cardiac involvement. There are only two reports in which FTC with direct vascular invasion appeared to respond to therapy (3,4).

In conclusion, enhanced CT and Tl-201 thyroid scintigraphy is useful for evaluating intra-atrial extension of FTC through the veins. Surgical treatment may ameliorate cardiac failure in a few patients but intra-atrial extension of FTC has a poor short-term prognosis and high mortality rate.

Disclosure Statement

The authors declare that no conflicts of interest exist.

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1. Hyer SL, Dandekar P, Newbold K, Haq M, Wechalakar K, Harmer C 2008 Thyroid cancer causing obstruction of the great veins in the neck. World J Surg Oncol **6**:36.
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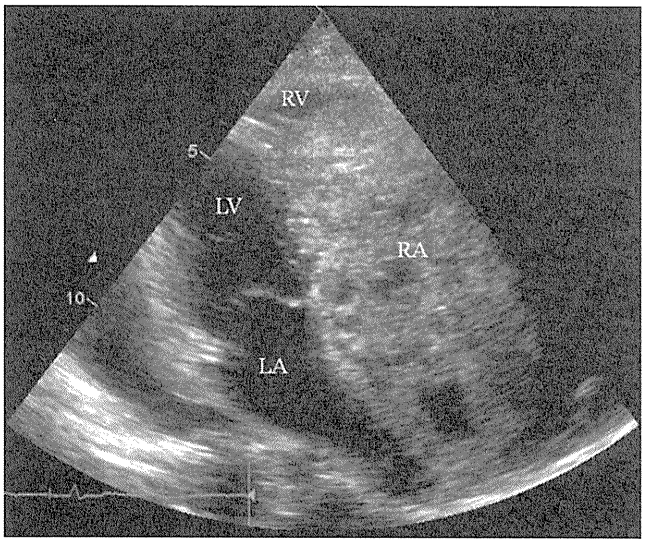
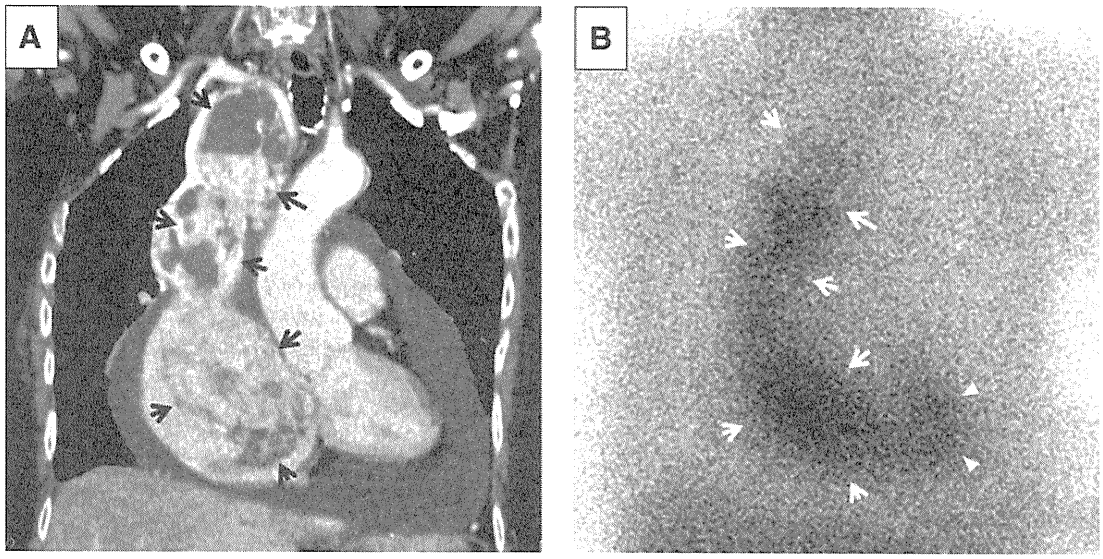
Supplementary Data



SUPPLEMENTARY FIG. S1. Cardiac ultrasound showing a tumor occupying the right atrium (RA). The tumor extended into the right ventricle (RV) beyond the tricuspid valve in diastolic phase. LA, left atrium; LV, left ventricle.



SUPPLEMENTARY FIG. S2. **(A)** Contrast-enhanced computed tomography image showing a bulky tumor extending into the right internal jugular vein, the brachiocephalic vein, superior vena cava, and right atrium. A large amount of pericardial fluid is apparent. **(B)** Thallium 201 thyroid scintigram showing abnormal accumulation in the thorax. Concentrations of radioactivity are in the same area as the tumor (arrow). Accumulation of cardiac muscle is also apparent (triangle).

RIG-I– and MDA5-Initiated Innate Immunity Linked With Adaptive Immunity Accelerates β-Cell Death in Fulminant Type 1 Diabetes

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OBJECTIVE—The contribution of innate immunity responsible for aggressive β-cell destruction in human fulminant type 1 diabetes is unclear.

RESEARCH DESIGN AND METHODS—Islet cell expression of Toll-like receptors (TLRs), cytoplasmic retinoic acid–inducible gene I (RIG-I)-like receptors, downstream innate immune markers, adaptive immune mediators, and apoptotic markers was studied in three autopsied pancreata obtained 2 to 5 days after onset of fulminant type 1 diabetes.

RESULTS—RIG-I was strongly expressed in β-cells in all three pancreata infected with enterovirus. Melanoma differentiation–associated gene-5 was hyperexpressed in islet cells, including β- and α-cells. TLR3 and TLR4 were expressed in mononuclear cells that infiltrated islets. Interferon (IFN)-α and IFN-β were strongly expressed in islet cells. Major histocompatibility complex (MHC)-class I, IFN-γ, interleukin-18, and CXC motif ligand 10 were expressed and colocalized in affected islets. CD11c+ MHC-class II+ dendritic cells and macrophage subsets infiltrated most islets and showed remarkable features of phagocytosis of islet cell debris. CD4+ forkhead box P3+ regulatory T cells were not observed in and around the affected islets. Mononuclear cells expressed the Fas ligand and infiltrated most Fas-expressing islets. Retinoic acid–receptor responder 3 and activated caspases 8, 9, and 3 were preferentially expressed in β-cells. Serum levels of IFN-γ were markedly increased in patients with fulminant type 1 diabetes.

CONCLUSIONS—These findings demonstrate the presence of specific innate immune responses to enterovirus infection connected with enhanced adoptive immune pathways responsible for aggressive β-cell toxicity in fulminant type 1 diabetes. ***Diabetes*** **60:884–889, 2011**

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Fulminant type 1 diabetes is a unique subtype of type 1 diabetes and is characterized by an abrupt onset of severe hyperglycemia/ketoacidosis and severe β-cell damage that is preceded by flu-like symptoms (1–3). Recently, we have reported unique enterovirus-induced mechanisms for β-cell destruction involving CXC chemokine ligand 10 (CXCL10) and chemokine receptor CXCR3 in fulminant type 1 diabetes (4).

In this study, we examined the in situ status of innate and adaptive immunity of enterovirus-induced fulminant type 1 diabetes. This includes expression of Toll-like receptors (TLRs) TLR3 and TLR4 and cytoplasmic retinoic acid–inducible gene I (RIG-I)-like receptors (RLRs) RIG-I and melanoma differentiation–associated gene-5 (MDA5). TLRs and RLRs are major receptor systems for detecting RNA viruses like enterovirus (5). As interferon (IFN)-α and -β potentially inhibit viral replication and enhance cytotoxic β-cell immunity (6), their expression, cytokine/chemokine expression, and activity of dendritic cells (DCs)/macrophages, CD4+ forkhead box P3 (Foxp3)+ regulatory T cells (Tregs) in affected islet cells were examined.

RESEARCH DESIGN AND METHODS

Patients. Clinical profiles of three autopsied patients with fulminant type 1 diabetes have been reported (4). Briefly, case 1 was a 14-year-old boy who died from diabetic ketoacidosis, following flu-like symptoms 5 days earlier. Case 2 was a 25-year-old man who died from diabetic ketoacidosis, following sudden symptoms of nausea and epigastralgia 2 days earlier. Case 3 was a 29-year-old man who died from diabetic ketoacidosis, following slight fever, nausea, and vomiting 2 days earlier.

Control subjects. Pancreatic tissues from 10 nondiabetic men (mean age ± SD, 62 ± 10 years) with gastric carcinoma who had undergone partial pancreatectomy and from five autopsied nondiabetic men (65 ± 11 years) were used as nondiabetic control subjects for immunohistochemical analysis. Pancreatic tissues from four autopsied type 1 diabetic patients (44 ± 9 years) who had histopathological insulitis and glutamic acid decarboxylase autoantibodies (titer: 3.0 ± 1.5 U/mL, cutoff <1.5) were examined as type 1 diabetic control subjects.

Immunostaining. Methods for immunohistochemical analyses have been reported previously (4). Primary antibodies used in this study are listed in Supplementary Table 1. The definition of insulitis and frequencies of insulitis and mononuclear cell (MNC) phenotypes in islets of cases 1–3 have been documented previously (4). The number of pancreatic acinar cells surrounded by CD8+ T cells was counted in the randomly selected 60 photos of pancreatic section in each case. A confocal laser-scanning microscope, Fluoview FV1000 (Olympus, Tokyo, Japan), was also used.

Measurement of serum IFN-γ. We obtained sera from 18 patients with fulminant type 1 diabetes (age [range]: 32.3 ± 13.5 [17–58] years, sex [man/woman]: 12/6, duration: 31.0 ± 64.1 [0–240] days), 27 patients with typical type 1 diabetes (age: 31.4 ± 14.7 [6–55] years, sex: 12/15, duration: 12.5 ± 25.4

[0.1–108] months), and 30 nondiabetic control subjects (age: 33.3 ± 13.3 [20–60] years, sex: 17/13). Serum level of IFN-γ was measured by ELISA (PBL Biomedical Laboratories, R&D Systems, Piscataway, NJ).

Ethics. The Ethics Committee of the University of Yamanashi approved all of the procedures performed in this study. All patients gave informed consent for measuring serum IFN-γ.

Statistical analysis. Differences in variables between groups were compared using Student *t* test and ANOVA. Fisher exact test was used to compare frequencies of islets. Values are expressed as means ± SD unless otherwise mentioned.

RESULTS

MDA5, RIG-I, and enterovirus-capsid protein expression. MDA5 was strongly expressed in β-cells, α-cells, and other types of islet cells of fulminant type 1 diabetic pancreata (Fig. 1*A–D*). In nondiabetic control and type 1 diabetic control subjects, weak MDA5 expression was observed in a few α-cells (Supplementary Fig. 1). Significant expression of RIG-I was observed preferentially in β-cells in all three patients with fulminant type 1 diabetes (Fig. 1*E–H*), yet it was not expressed in nondiabetic and type 1 diabetic control subjects (Supplementary Fig. 1). Enterovirus-capsid protein (VP1) was detected in β- and non–β-cells of fulminant type 1 diabetic pancreata confirming our previous report (Supplementary Fig. 2) (4) but not type 1 diabetic control and nondiabetic control subjects.

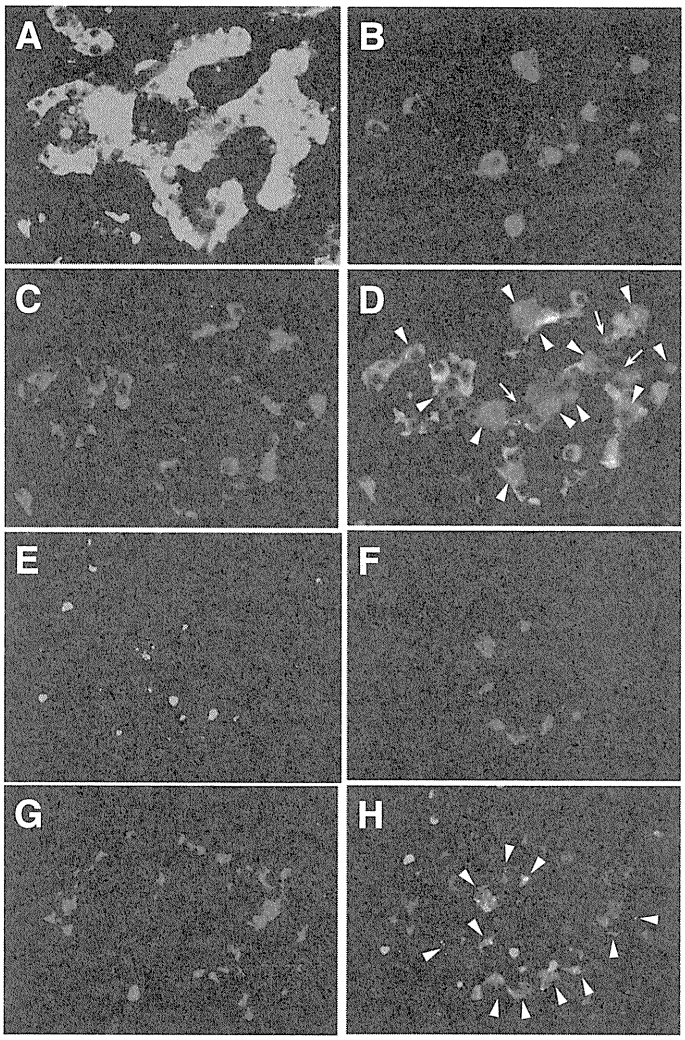

FIG. 1. Intracytoplasmic double-stranded virus RNA receptor expression in enterovirus-associated human fulminant type 1 diabetes. *A–C*: Triple-immunostaining of MDA5 (*A*), insulin (*B*), and glucagon (*C*). The merged image (*D*) demonstrates hyperexpression of MDA5 in β-cells (light blue, arrowheads), α-cells (orange), and other types of islet cells (green, arrows) (×400, case 2). Triple-immunostaining of RIG-I (*E*), insulin (*F*), and glucagon (*G*) is also shown. The merged image (*H*) demonstrates specific expression of RIG-I in β-cells (light blue, arrowheads) (×400, case 2). (A high-quality digital representation of this figure is available in the online issue.)

TLR3 and TLR4 expression. Both TLR3 and TLR4 were expressed in MNCs that had infiltrated islets of fulminant type 1 diabetic pancreata but not nondiabetic and type 1 diabetic control subjects (Table 1).

IFN-α, IFN-β, interferon regulatory factor-7, and major histocompatibility complex class I expression. In all three pancreata of the patients with fulminant type 1 diabetes, IFN-α and -β_1 were strongly expressed (Fig. 2*A–F*). Some MNCs that had infiltrated around or in islets and pancreatic acinar and ductal cells also expressed theses cytokines in fulminant type 1 diabetes (Fig. 2*A* and *B*) but not in either control subject. The numbers of pancreatic acinar cells surrounded by CD8+ T cells were 11/mm^2, 24/mm^2, and 22/mm^2, respectively, in the pancreatic sections of cases 1, 2, and 3. Most IFN-α–expressing cells were β-cells, α-cells, and islet non–β- and non–α-cells (Fig. 2*C–F*). Interferon regulatory factor (IRF)-7 (7) was strongly expressed in β- and α-cells (Fig. 2*G–I*) and mostly stained around and in the nucleus of the islet cells (Fig. 2*G*). Major histocompatibility complex class I (MHC-I) was hyperexpressed in all islet cell subsets of fulminant type 1 diabetic pancreata (Fig. 2*J*). Nondiabetic control and type 1 diabetic control subjects did not show expression of IFN-α, IFN-β_1, or IRF-7 and hyperexpression of MHC-I in their islets.

CD11c+ cells in islets. Remarkable CD11c+ cells migration to the islets was observed in most islets of fulminant type 1 diabetic pancreata (Table 1). Intraislet CD11c+ cells expressed MHC class-II molecules (Fig. 2*K–N*). Confocal microscopy showed that some CD11c+ cells contained β-cell debris positive for insulin (Fig. 2*O*). Such findings were not observed in islets of nondiabetic or type 1 diabetic control subjects. Most CD11c+ cells were also positive for CD1a and some for CD68 (Fig. 2*P*), likely representing DCs and macrophage subsets. CD56+ or CD57+ NK cells and Tregs (CD4+ Foxp3+ cells) were not detected in or around islets of fulminant type 1 diabetic pancreata and either control. Tregs, CD4+ Foxp3+ cells, were not detected in or around the islets or in exocrine regions of the pancreas in fulminant type 1 diabetic, nondiabetic control, or type 1 diabetic control subjects (Table 1).

IL-18, IFN-γ, and CXCL10 expression. IL-18 was expressed in islet cells in all three fulminant type 1 diabetes (Fig. 3*A* and *E*). Most residual β-cells expressed both IFN-γ and IL-18 (Fig. 3*G* and *H*). IL-18, IFN-γ, and CXCL10 colocalized in most β- and islet non–β-cells (Fig. 3*A–H*). IL-12 was not expressed in any cells in affected pancreata. A few islets of type 1 diabetic control subjects (mean [range]: 2.8% [0–5.2]) expressed IL-18 and IFN-γ but not CXCL10. Nondiabetic control subjects did not express IL-18, IFN-γ, and CXCL10.

Serum IFN-γ levels in patients with fulminant type 1 diabetes. Serum levels of IFN-γ in patients with fulminant type 1 diabetes were approximately three times higher than those in nondiabetic and type 1 diabetic control subjects (Fig. 3*I*).

TABLE 1
Frequency of islets with MNCs that express TLR3, TLR4, CD11c, CD4+Foxp3, and FasL in three fulminant type 1 diabetic cases, and nondiabetic and type 1 diabetic control subjects

	Frequency of TLR3+ MNCs (%)	Frequency of TLR4+ MNCs (%)	Frequency of CD11c+ cells (%)	Frequency of CD4+ Foxp3+ cells (%)	Frequency of FasL+ MNCs (%)
Case					
1	11.3 (7/62)	1.6 (1/62)	95.9 (70/73)	0 (0/75)	82.6 (38/46)
2	5.0 (2/40)	1.8 (1/56)	100 (64/64)	0 (0/63)	90.0 (36/40)
3	18.8 (6/32)	9.4 (5/53)	91.2 (52/57)	0 (0/70)	95.2 (40/42)
Mean					
FT1D (n = 3)	11.7 ± 6.9 (15/134)	4.3 ± 4.4 (7/171)	95.7 ± 4.4 (186/194)	0 (0/208)	89.3 ± 6.3 (114/128)
Nondiabetic control subjects (n = 15)	0 (0/652)	0.3 ± 0.6 (0–1.7) (3/742)	0.2 ± 0.5 (0–1.4) (2/863)	0 (0/692)	0.3 ± 0.7 (0–1.9) (3/763)
Type 1 diabetic control subjects (n = 4)	0 (0/228)	0 (0/284)	2.4 ± 4.2 (0–9.8) (10/392)	0 (0/351)	18.2 ± 16.9 (3.8–46.6) (81/417)
P value					
FT1D vs. nondiabetes	<0.0001	<0.003	<0.0001	NS	<0.0001
FT1D vs. type 1 diabetes	<0.002	NS	<0.0001	NS	=0.0005
Nondiabetes vs. type 1 diabetes	NS	NS	NS	NS	=0.001

Values are means ± SD unless otherwise indicated. FT1D, fulminant type 1 diabetes.

Fas expression in islet cells and infiltration of islet Fas-ligand–bearing MNCs. Elevated expression of Fas in islet cells coincided with marked MNC infiltration in fulminant type 1 diabetic pancreata (Fig. 3*J*). The subsets of islet cells with Fas expression were mostly β-cells. Fas-ligand (FasL)-bearing MNCs infiltrated most islets of fulminant type 1 diabetic pancreata (Fig. 3*K–M*) (Table 1). In islet cells of nondiabetic control and type 1 diabetic control subjects, Fas was not expressed (Supplementary Fig. 3). FasL-bearing MNC infiltration of islets was observed in type 1 diabetic but not nondiabetic control subjects (Table 1) (Supplementary Fig. 3).

Expression of retinoic acid–receptor responder 3 and activated caspases 8, 9, and 3 in islet β-cells. Retinoic acid–receptor responder 3 (RARRES3) (8,9) was expressed in β-cells of fulminant type 1 diabetic pancreata (Supplementary Fig. 4). Cleaved caspase 8, a marker of the Fas-mediated extrinsic apoptotic pathway, cleaved caspase 9, a marker of the activated non–Fas-mediated apoptotic pathway, and activated caspases 3, a marker of the end stage of β-cell apoptosis, were expressed specifically in islet β-cells (Supplementary Fig. 4). In islets of autopsied nondiabetic control subjects, RARRES3, cleaved caspases 8, -9, and -3 were not expressed (Supplementary Fig. 5). In type 1 diabetic control subjects, RARRES3, cleaved caspases 8, -9, and -3 were expressed weakly in some islet β-cells (Supplementary Fig. 5).

DISCUSSION

Both RIG-I and MDA5 were strongly expressed in β-cells of fulminant type 1 diabetic pancreata. MDA5 was also hyperexpressed in α-cells and non−β-/non–α-cells in affected islets. Hyperexpression of RIG-I and MDA5 with expression of IFN-α and -β_1 in β-cells suggests a crucial role of RIG-I and MDA5 for sensing and responding to enterovirus infection in the pancreas of patients with fulminant type 1 diabetes. Mutations of MDA5 genes have been implicated in reducing the risk of type 1 diabetes (10). Reports also noted RIG-I mRNA expression in human islets infected with Coxsackievirus B3 and B5 (11,12). We showed that IRF-7, a master transcription factor of IFN-α and -β (7), translocated to the nucleus and that IFN-α and -β, essential factors that protect β-cells against viral infection (6), were strongly expressed in both β- and α-cells. These results indicate that all islet cells are in an activated state of innate immunity in response to enterovirus in patients with fulminant type 1 diabetes.

Increased TLR3+ MNCs that infiltrate affected islets should participate in sensing viral RNA and subsequently destroy β-cells with RIG-I– and MDA5-initiated proinflammatory signal axes in the innate immune response against Coxsackievirus B3 (13). Intra- and peri-islet DCs and macrophage subsets drastically increased in number and showed active phagocytosis of enterovirus-infected β-cells, whereas MHC-I was hyperexpressed in all islet subsets. Some DCs and macrophage subsets also expressed MHC-II molecules. Activated innate immune responses including virus sensing by RIG-I and MDA5 with subsequent IFN-α and -β production and DC and macrophage activation will not only protect for enteroviral infection by upregulating RIG-I and MDA5 (12,14) but will also enhance the adaptive immunity cascades for islet cell destruction (6,15,16). Indeed, patients with fulminant type 1 diabetes showed elevated serum levels of IFN-γ.

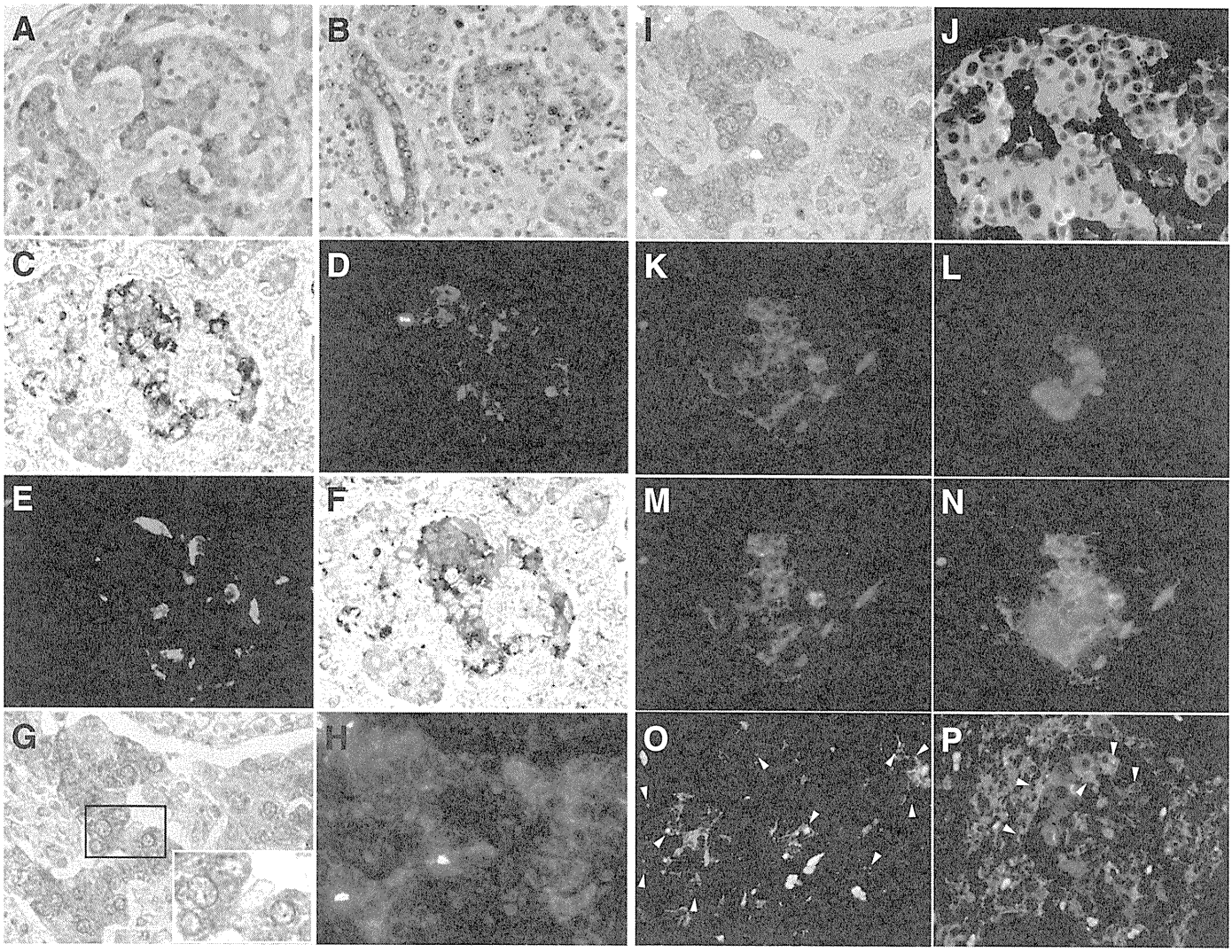

FIG. 2. Immunohistochemical staining of IFN-α, IFN-β_1, IRF-7, and MHC-class I in a pancreas with fulminant type 1 diabetes (×200, case 2). *A* and *B*: Immunostaining of IFN-α (*A*) and IFN-β_1 (*B*). *C–F*: Triple-immunohistochemical staining of IFN-α (*C*), insulin (*D*), and glucagon (*E*). A merged image (*F*) demonstrates a high proportion of β-cells and α-cells expressing IFN-α. Color balance of *F* has been adjusted. *G–I*: Double-immunohistochemical staining of IRF-7 (*G*) and insulin (*H*). Insert in *G* demonstrates strong peri- and intranuclear staining of IRF-7, indicating translocation of IRF-7 from the cytoplasm to the nuclease, thus acting as an activated transcription factor. The merged image (*I*) shows strong expression of IRF-7 in both islet β-cells and islet non–β-cells. Color balance of *I* has been adjusted. *J*: Triple-immunostaining shows MHC-class I molecules are hyperexpressed at the cell surface (green) in β-cells (blue), α-cells (orange), and non–β-/non–α- (nonstained for cytoplasm) islet cells. *K–N*: Triple-immunostaining of CD11c (*K*), insulin (*L*), and MHC-II (*M*). Merged image (*N*) demonstrates that CD11c+ cells expressing MHC-II migrate around and into the islets (×200, case 1). *O*: Confocal microscopic demonstration of intraislet CD11c+ cells (green), showing phagocytosis of the unprocessed β-cell antigen, insulin (red; arrowheads) (×400, case 1). *P*: Merged image of triple-immunostaining of CD11c (red), CD68 (green), and insulin (blue). Arrowheads indicate positive cells (yellow) both for CD11c and CD68 (×200, case 1). (A high-quality digital representation of this figure is available in the online issue.)

CD4+ Foxp3+ cells, which represent a pivotal subset of Tregs, were not observed in or around the islets of fulminant type 1 diabetic pancreata, suggesting that the extremely polarized local condition to predominance for Th1 in response to enteroviral infection suppresses Treg differentiation from naive T CD4+ precursors (17). In turn, the Treg-depleted islet condition enhances Th1 cytokine (i.e., IFN-γ) generation.

Notably, IL-18, an IFN-γ–inducing factor, was extensively expressed in islet cells of infected fulminant type 1 diabetic pancreata. In response to viral infection, IL-18 is promptly secreted from virus-activated macrophages, DCs, and T cells (18), stimulating production of IFN-γ synergistically with IFN-α and -β through a unique pathway that sometimes occurs independently of IL-12 or NK cells (19). Conversely, IL-18 can be induced by IFN-γ alone or in combination with other cytokines in islet β-cells (20). Thus, for fulminant type 1 diabetes, enterovirus itself or enterovirus-activated T cells and macrophages most likely infiltrate islets to induce IL-18 production in these cells. In addition, IFN-α and -β, produced in islet cells and islet-infiltrating MNCs, can enhance IL-18–mediated signaling (21). Subsequently, islet-secreted IL-18 may induce IFN-γ production via receptors on the islet cells or islet stromal cells in an autocrine/paracrine manner. Once this positive autocrine/paracrine circuit for production of IL-18, IFN-γ, and CXCL10 is established in islet cells, destructive mechanisms involving CXCR3+ T cells and macrophages might persist until complete destruction of the β-cells (4).

We found that Fas was highly expressed in affected islet β-cells and islet-infiltrating FasL+ cells. Taken together with the finding that MHC-I and IFN-α, -β, and -γ were

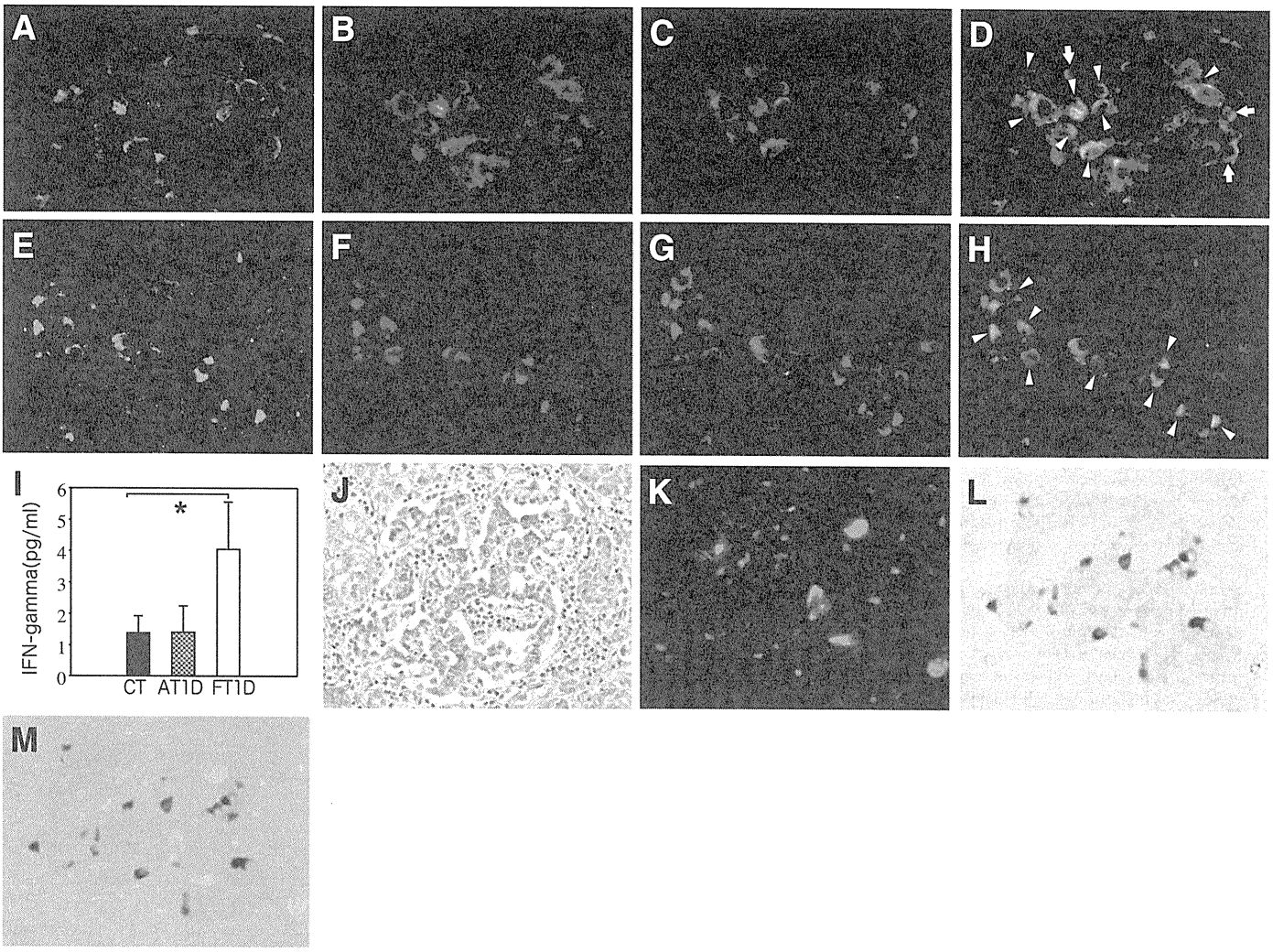

FIG. 3. Immunohistochemical staining of IL-18, insulin, CXCL10, IFN-γ, and FasL. *A–D*: Triple-immunostaining of IL-18 (*A*), insulin (*B*), and CXCL10 (*C*). The merged image (*D*) shows that most β-cells express both IL-18 and CXCL10 (arrowheads). Some islet non–β-cells also express IL-18 and CXCL10 (orange, arrows) (×400, case 2). *E–H*: Triple-immunostaining of IL-18 (*E*), insulin (*F*), and IFN-γ (*G*). The merged image (*H*) shows that most β-cells express both IL-18 and IFN-γ (arrowheads) and that some islet non–β-cells also express IFN-γ (red) (×400, case 2). *I*: Serum levels of IFN-γ in patients with fulminant type 1 diabetes (FT1D) or with typical acute-onset type 1 diabetes (AT1D) and nondiabetic control (CT) subjects. Values are expressed as means ± SE; *$P < 0.05$. *J*: Immunostaining of Fas in islets affected by fulminant type 1 diabetes (brown) demonstrates strong expression of Fas in islet cells (×200, case 2). *K–M*: Double-immunofluorescent staining of insulin (*K*) and FasL (*L*). The merged image (*M*) shows that FasL-positive cells infiltrate the islets (×400, case 3). Color balance of *M* has been adjusted. (A high-quality digital representation of this figure is available in the online issue.)

strongly expressed in affected islet cells, effecter mechanisms for β-cell apoptosis in fulminant type 1 diabetes are likely mediated in part by MHC-I and by the Fas-FasL pathway (22). Inflammation-induced Fas-FasL expression in β-cells was reported to lead to rapid and massive β-cell destruction (23). Other apoptotic mechanisms through the IFN-γ–dependent JAK/STAT pathway (24) and innate immune pathway (25) will also exert β-cell destruction.

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K.A. and Y.N. conducted immunohistochemical staining, RT-PCR data analysis, and discussed, reviewed, and edited the article. S.T. contributed to planning and discussion and edited the article. T.M. and A.S. sampled autopsied pancreas and participated in discussion. T.A., M.S., and H.S. contributed to discussion and reviewed and edited the article. S.T., T.M., M.I., D.A., and T.A. recruited serum samples, measured serum levels of IFN-γ, and participated in discussion. F.F., A.K., and M.K. contributed to analysis of immunostained sample data. J.I., H.F., and T.E. contributed to analysis of nondiabetic pancreas and Treg+ lymph nodes. T.K. analyzed data and wrote and edited the article.

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RESEARCH ARTICLE

Pathological changes in the pancreas of fulminant type 1 diabetes and slowly progressive insulin-dependent diabetes mellitus (SPIDDM): innate immunity in fulminant type 1 diabetes and SPIDDM

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Abstract

Objective The contribution of innate immunity responsible for beta-cell destruction in fulminant type 1 diabetes (FT1D) and slowly progressive insulin-dependent diabetes mellitus (SPIDDM) is unclear.

Research Design and Methods Islet-cell expression of Toll-like receptors (TLRs) including TLR3 and TLR4, the cytoplasmic retinoic acid-inducible protein I (RIG-I)-like helicases, RIG-I, melanoma differentiation-associated gene-5 and laboratory of genetics and physiology 2 in the affected islets were studied immuno-histochemically on three pancreases obtained 2–5 days after the onset of FT1D and a pancreas from a patient with SPIDDM.

Results Laboratory of genetics and physiology 2 and RIG-I strongly expressed in beta cells in all three FT1D pancreases infected with enterovirus (VP1 antigen). Melanoma differentiation-associated gene-5 was hyper-expressed in all subsets of islet cells including beta cells and alpha cells. TLR3 and TLR4 were expressed in mononuclear cells that infiltrated to islets. IFN-alpha/beta was strongly expressed in islet cells. In contrast, pancreas of a patient with SPIDDM, enterovirus and expression of innate immune receptors including RIG-I, melanoma differentiation-associated gene-5, hyperexpression of laboratory of genetics and physiology 2 and mononuclear cells, which were positive for TLR3 and TLR4, and infiltration to the islets were not detected.

Conclusions These findings demonstrate that retinoic acid-inducible protein I (RIG-I)-like helicases and TLRs play a crucial role on beta-cell destruction in enterovirus-induced FT1D. The presence of distinct mechanism(s) of slowly progressive beta-cell failure in SPIDDM was suggested.

Keywords LGP2; RIG-I; MDA5; innate immunity; CXCL10; fulminant type 1; diabetes; slowly progressive insulin-dependent diabetes mellitus

Introduction

Fulminant type 1 diabetes is a unique subtype of type 1 diabetes characterized by an abrupt onset of severe hyperglycaemia and ketoacidosis that is preceded

by flu-like symptoms including fever, abdominal pain and headache [1–3]. Recently, we have reported enterovirus-induced unique mechanisms for beta-cell destruction in human fulminant type 1 diabetes [4]. Enterovirus infection initiates the expression of CXC chemokine ligand 10 (CXCL10) in beta cells. T cells bearing CXCR3, the receptors for CXCL10, macrophages are activated by CXCL10 and are attracted to virus-infected islets. These infiltrating autoreactive T cells and macrophages release inflammatory cytokines including interferon (IFN)-gamma in the islet, which not only damages beta cells but also accelerates the generation of CXCL10 in residual beta cells. This further activates cell-mediated immunity until complete beta-cell destruction occurs [4].

In this study, we describe the *in situ* status of viral infection and innate immunity in the islets of human fulminant type 1 diabetes. This includes expression of enterovirus RNA and enterovirus capside protein (VP1), Toll-like receptors (TLRs): TLR3 and TLR4, the cytoplasmic retinoic acid-inducible protein I (RIG-I)-like helicases (RLHs): RIG-I, melanoma differentiation-associated gene-5 (MDA5) and laboratory of genetics and physiology 2 (LGP2) in the affected islets. TLRs and RLHs are two major receptor systems for detecting RNA viruses including enterovirus [5,6]. In addition, we investigated possible superimposed infection of influenza virus because the influenza virus is accessible to RIG-I [5,6], and influenza infection-induced fulminant type 1 diabetes has been reported [7]. The expression status of type I IFN including IFN-alpha and IFN-beta, which not only potentially inhibits viral replication but also enhances cytotoxic beta cell immunity [8], was examined on affected islet cells.

SPIDDM is characterized by islet cell antibodies-positive non-insulin-dependent diabetes and subsequent gradual loss of beta-cell function lapsing into insulin dependency during several years [9–12]. Involvement of enterovirus infection followed by specific innate immunity for beta-cell destruction is potentially considered. In the present study, expression of VP1, TLRs and RLHs was examined in a patient with SPIDDM, which was reported previously [4].

Our unique findings on innate immunity which is closely linked to adaptive immunity of the affected pancreas by fulminant type 1 diabetes and SPIDDM will provide new insights into the understanding of the specific mechanisms for beta-cell failure and possible preventions of two subtypes of type 1 diabetes.

Methods

Patients

The clinical profiles of three autopsied patients with newly developed fulminant type 1 diabetes are reported [4]. In brief:

Case 1: A 14-year-old boy died from diabetic ketoacidosis preceded by flu-like symptoms 5 days earlier. *Case 2*: A 25-year-old man died from diabetic ketoacidosis. He had sudden symptoms of nausea and epigastralgia for 2 days before. *Case 3*: A 29-year-old man died from diabetic ketoacidosis. This was preceded by slight fever, nausea and vomiting 2 days before. All subjects had the susceptible HLA haplotype [13] for fulminant type 1 diabetes.

In addition, pancreatic tissue from an autopsied patient (a 56-year-old woman who died due to cerebral infarction) with slowly progressive insulin-dependent (type 1) diabetes [14] was also examined for the presence of enterovirus and innate immune receptors. She had been treated with insulin and had shown diminished urinary C-peptide secretion (1.1 nmol/day) and high serum glutamic acid decarboxylase autoantibodies titre [12.5 U/mL (221.4 WHO U/mL)].

Controls

Pancreatic tissues from five autopsied non-diabetic male patients (65 ± 11 years) were used as non-diabetic controls for immunohistochemical analysis.

Real-time PCR for enterovirus, RIG-I and MDA5

RNA was extracted from two 5-μm paraffin sections of pancreatic tissue using a Recover All total nucleic acid isolation kit (Ambion, Austin, TX, USA), according to the manufacturer's instructions. Nested reverse transcription polymerase chain reaction (PCR) targeting the 5′ nontranslated region and VP1 region was performed using primers described previously [14–17]. We then performed real-time PCR to detect enterovirus sequences with specific primers and probes [18,19]. Real-time PCR for RIG-I and MDA5 was performed using TaqMan probes (Applied Biosystems, Foster City, CA, USA). However, we were unable to detect the enterovirus, RIG-I or MDA5 genome sequences. Also, we could not amplify 18S rRNA and/or glyceraldehyde phosphate dehydrogenase cDNAs from the pancreatic sections of diabetic patients, although we could detect 18S rRNA and/or glyceraldehyde phosphate dehydrogenase sequences from control pancreata. We therefore assumed that most RNA in the autopsied pancreata had already degraded.

Immunostaining and immunofluorescent staining

Methods and sera used for immuno-stainings except for rabbit anti-LGP2 (DHX58, Sigma, MO, USA) and goat anti-influenza A H1N1 antibody (AbD Serotec, Oxford, UK), mouse anti-influenza A monoclonal antibody blend (Millipore, Temecula, CA, USA) were reported [20].

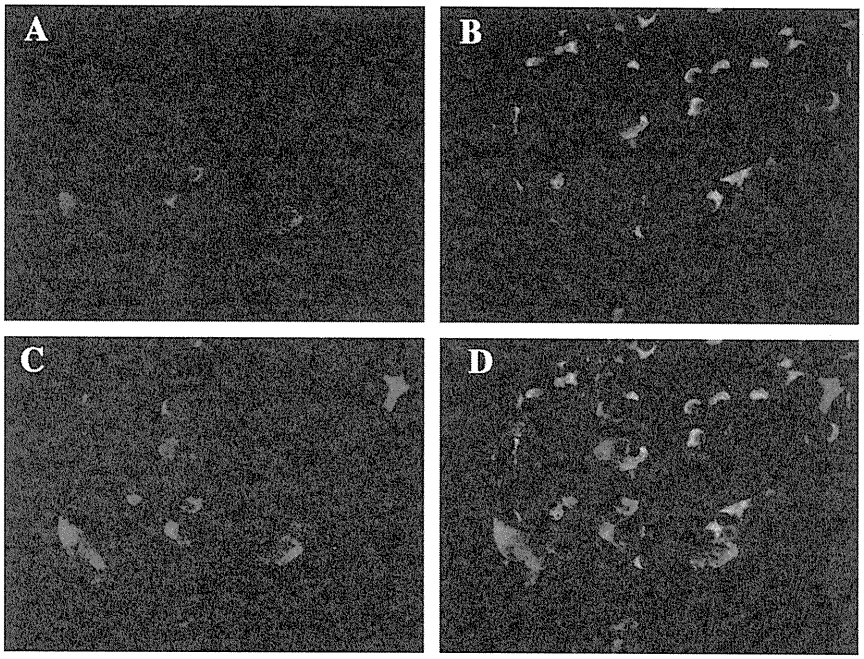

Figure 1. Intra-cytoplasmic laboratory of genetics and physiology 2 expression in enterovirus-associated human fulminant type 1 diabetes. Triple-immunostaining for: laboratory of genetics and physiology 2 (A), glucagon (B) and insulin (C). The merged image (D) demonstrates specific hyper-expression of laboratory of genetics and physiology 2 in beta cells (×400)

Ethics

The Ethics Committee of the University of Yamanashi approved all of the procedures performed in this study.

Statistics

Differences in variables between groups were compared using Student's *t*-test and analysis of variance.

Results

LGP2, MDA5 and RIG-I expression, enterovirus capsid protein (VP1) and influenza A virus protein in the pancreas

LGP2 was preferentially hyper-expressed in beta cells of three patients with fulminant type1 diabetes (Figures 1A–D). In non-diabetic control pancreas, basal expression of LGP2 was observed in beta cells (Figures 2A–D).

MDA5 was strongly expressed in beta cells, alpha cells and other types of islet cells in affected islets of three patients with fulminant type1 diabetes in accordance with our previous report [20]. In non-diabetic control pancreas, weak MDA5 expression was observed in a few alpha cells.

Significant expression of RIG-I was observed preferentially in beta cells in all three patients with fulminant type 1 diabetes, yet it was not expressed in non-diabetic control pancreas. Enterovirus capsid protein (VP1) was detected in beta cells and non-beta cells in the islets of fulminant type 1 diabetes, in accordance with our previous study [4]. In addition, we investigated possible superimposed infection of influenza virus. However, we did not detect any influenza A virus protein in the affected pancreas of fulminant type 1 diabetes patients.

In the pancreas of a patient with SPIDDM, we could not detect VP1 antigen and expression of innate immune receptors including RIG-I, MDA5 and hyperexpression of LGP2.

TLR3 and TLR4 expression in the pancreas

TLR3 was expressed in mononuclear cells (MNCs) that had infiltrated to the islets as well as around the islets of the patients with fulminant type 1 diabetes. TLR4-bearing MNCs were observed in the patients with fulminant type 1 diabetes. Numbers of MNCs positive for TLR3 or TLR4 were not increased in the affected pancreas of SPIDDM in composed with non-diabetic controls. TLR3- or TLR4-expressing MNCs were not observed in non-diabetic controls.

IFN-alpha, IFN-beta expression in the islets

In all three pancreases of the patients with fulminant type 1 diabetes, IFN-alpha and IFN-beta1 were strongly

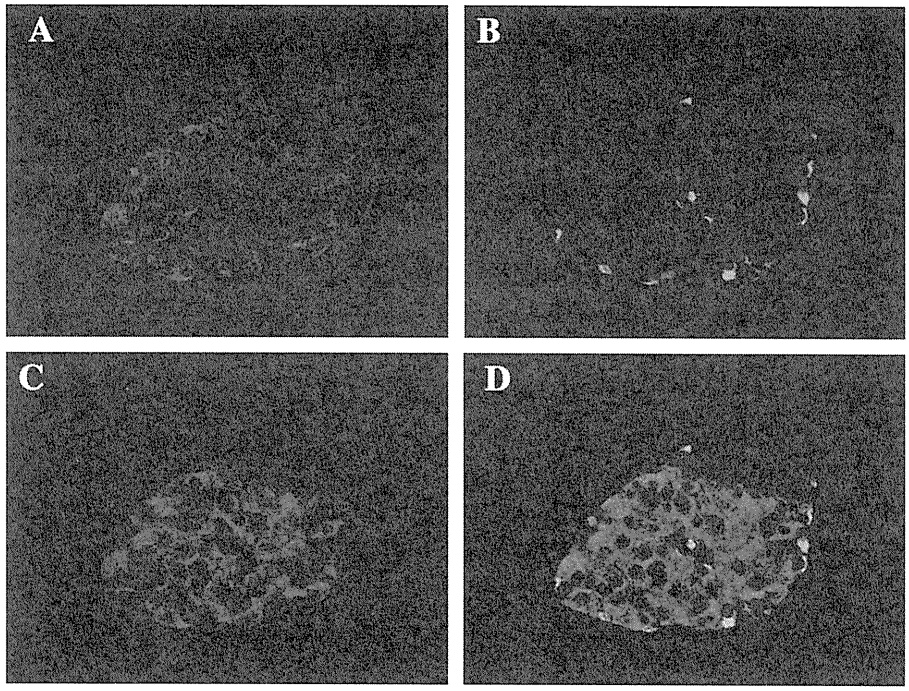

Figure 2. Intra-cytoplasmic laboratory of genetics and physiology 2 expression in non-diabetic controls. Triple-immunostaining for: laboratory of genetics and physiology 2 (A), glucagon (B) and insulin (C). The merged image (D) demonstrates specific basal expression of laboratory of genetics and physiology 2 in beta cells (×400)

expressed in most islet cells. In the pancreas with SPIDDM neither IFN-alpha nor IFN-beta1 was expressed.

Discussion

We found that LGP2 was hyper-expressed specifically in beta cells of the patients with fulminant type 1 diabetes. LPG2 is particularly important for the recognition of picornavirus including encephalomyocarditis virus, mengovirus and probably enterovirus [21]. LPG2 acts as a positive regulator of MDA5- and RIG-I-mediated viral recognition at the upstream site [21]. It, therefore, is highly probable that LPG2 enhance RIG-I- and MDA5-mediated enterovirus sensing pathway and subsequently accelerate specific beta cell destruction. MDA5 was also hyper-expressed in alpha cells and non-beta/non-alpha cells in the affected islets. Mutations of the MDA5 genes have been implicated in reducing the risk of type 1 diabetes [22], suggesting important engagements for virus infection and the occurrence of type 1 diabetes. These results, together with the finding that the enterovirus had infected all subsets of islet cells [4], indicate that all of the subsets of islet cells are in an activated state of innate immunity in response to enterovirus in the patients with fulminant type 1 diabetes.

Some viruses in the picornavirus family including enterovirus and rhinovirus have essentially MDA5-mediated virus sensing mechanisms in immune-mediated cells, such as dendritic cells [23,24]. Our data of hyper-expressed RIG-I as well as MDA5 with the expression of

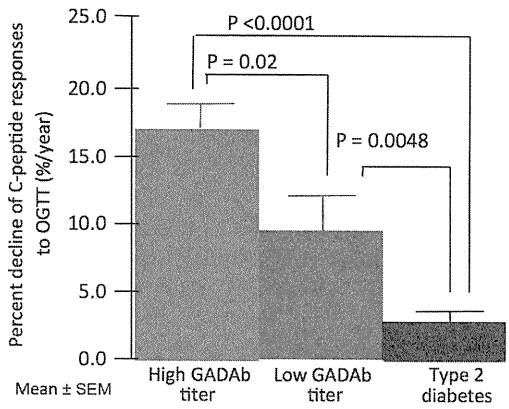

Figure 3. Annual decline rate of C-peptide responses to 75-g oral glucose tolerance tests in the SPIDDM patients. Left column: high titre of glutamic acid decarboxylase autoantibodies (≥180 WHO U/mL), Middle column: low titre of glutamic acid decarboxylase autoantibodies (<180 WHO U/mL), Right column: glutamic acid decarboxylase autoantibodies-negative type 2 diabetes. The values among the three groups are significant ($p < 0.0001$)

IFN-alpha/beta1 in beta cells suggest a crucial role of RIG-I as well as MDA5 for sensing and responding to enterovirus infection in the pancreas of patients with fulminant type 1 diabetes. Increased TLR3-positive MNCs that infiltrated to the affected islets will participate in sensing viral RNA and subsequently destruct the beta cell in corporation with RIG-I- and MDA5-initiated axis, thus producing pro-inflammatory cytokines and chemokines [25,26]. TLR3 has a critical link with the

pro-inflammatory signal axis in the innate immune response against Coxsackievirus B3 [26].

Our studies, in which we have reported that enterovirus (VP1 antigen) was immunostained in the pancreas with fulminant type 1 diabetes, do not exclude the possibility that virus other than enterovirus is involved in destructive mechanism(s) of beta cells in fulminant type 1 diabetes [8]. In addition double infection of two kinds of different viruses cannot be excluded. However, present study failed to demonstrate superimposed infection of influenza virus to enterovirus infection pancreas.

In this study we could not find any evidence of involvement of enterovirus (VP1 antigen) in the affected pancreas by SPIDDM. In addition, expression of RLHs and TLRs to the affected pancreas from a patient with SPIDDM was not observed. Already we could find mild insulitis and peri-insulitis composed of CD8+ T cells and CD68+ macrophages in this case [4]. Considering together with the present findings and our previous results [27] that the rate of decline of beta-cell loss in SPIDDM is faster than that in type 2 diabetes (Figure 3), we can suggest that different immunological mechanism(s) from that of fulminant type 1 diabetes and type 2 diabetes may be related with beta-cell dysfunction in SPIDDM. *In situ* studies using larger number of pancreases of SPIDDM patients will be needed.

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Conflict of interest

The authors have declared that no conflict of interest exists.

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ARTICLE

Association of genetic variation in *FTO* with risk of obesity and type 2 diabetes with data from 96,551 East and South Asians

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Abstract

Aims/hypothesis *FTO* harbours the strongest known obesity-susceptibility locus in Europeans. While there is growing evidence for a role for *FTO* in obesity risk in Asians, its association with type 2 diabetes, independently of BMI, remains inconsistent. To test whether there is an association of the *FTO* locus with obesity and type 2 diabetes, we conducted a meta-analysis of 32 populations including 96,551 East and South Asians.

Methods All studies published on the association between *FTO*-rs9939609 (or proxy [r^2>0.98]) and BMI, obesity or type 2 diabetes in East or South Asians were invited. Each study group analysed their data according to a standardised analysis plan. Association with type 2 diabetes was also adjusted for BMI. Random-effects meta-analyses were performed to pool all effect sizes.

Results The *FTO*-rs9939609 minor allele increased risk of obesity by 1.25-fold/allele (p=9.0×10^{-19}), overweight by

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1.13-fold/allele ($p=1.0\times10^{-11}$) and type 2 diabetes by 1.15-fold/allele ($p=5.5\times10^{-8}$). The association with type 2 diabetes was attenuated after adjustment for BMI (OR 1.10-fold/allele, $p=6.6\times10^{-5}$). The *FTO*-rs9939609 minor allele increased BMI by 0.26 kg/m^2 per allele ($p=2.8\times10^{-17}$), WHR by 0.003/allele ($p=1.2\times10^{-6}$), and body fat percentage by 0.31%/allele ($p=0.0005$). Associations were similar using dominant models. While the minor allele is less common in East Asians (12–20%) than South Asians (30–33%), the effect of *FTO* variation on obesity-related traits and type 2 diabetes was similar in the two populations.

Conclusions/interpretation *FTO* is associated with increased risk of obesity and type 2 diabetes, with effect sizes similar in East and South Asians and similar to those observed in Europeans. Furthermore, *FTO* is also associated with type 2 diabetes independently of BMI.

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Keywords Asians · *FTO* · Meta-analysis · Obesity · Type 2 diabetes

Abbreviations

GWAS	Genome-wide association study
MAF	Minor allele frequency
PAR	Population-attributable risk
SNP	Single-nucleotide polymorphism

Introduction

Large-scale genome-wide association studies (GWAS) in mainly white Europeans have identified at least 50 genetic loci to be robustly associated with obesity-related traits [1–

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12]. A cluster of common variants in the first intron of the fat mass and obesity-associated gene (*FTO*) was the first obesity-susceptibility locus to be identified by two independent GWAS in 2007 [1, 2] and has since been consistently replicated by many others and for a variety of obesity-related traits [7, 9, 13–15]. Of all currently identified obesity-susceptibility loci, the *FTO* locus has the most pronounced effect on BMI and obesity risk, at least in individuals of European descent. Each minor allele of any commonly investigated variant in *FTO* increases BMI by 0.30–0.40 kg/m^2 (equivalent to 870–1,150 g for a person 1.7 m tall) and risk of obesity by ~20% [7, 15]. The minor allele of the *FTO* variant is common (minor allele frequency (MAF)=~42%) in white Europeans, such that 66% of Europeans carry at least one risk allele and 18% carry two risk alleles. Because of the high prevalence of the risk allele and its relatively strong effect on BMI, the *FTO* locus explains most (0.34%), yet little, of the variation in BMI in Europeans [7].

FTO has also been examined as an obesity-susceptibility locus in populations of non-white European origin. While the initial replication efforts in East Asian populations were inconsistent [16, 17], a growing number of studies have provided evidence that genetic variation in *FTO* influences

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