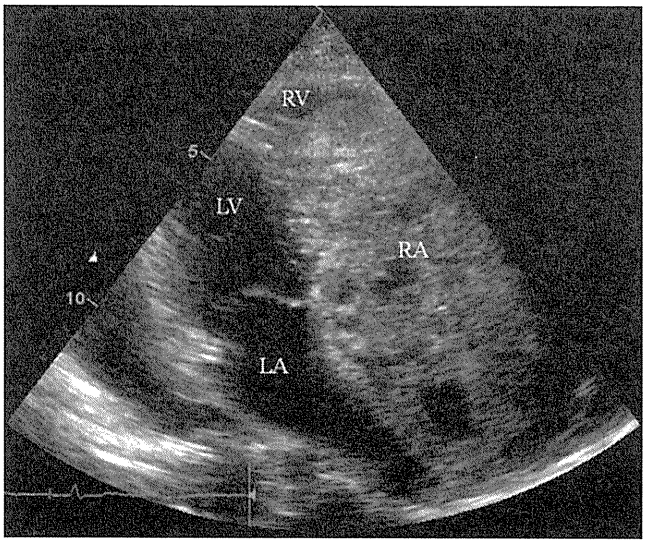
3. Thompson NW, Brown J, Orringer M, Sisson J, Nishiyama R 1978 Follicular carcinoma of the thyroid with massive angioinvasion: extension of tumor thrombus to the heart. Surgery **83:**451–457.
4. Niederle B, Hausmaninger C, Kretschmer G, Polterauer P, Neuhold N, Mirza DF, Roka R 1990 Intraatrial extension of thyroid cancer: technique and results of a radical surgical approach. Surgery **108:**951–956; discussion 956–957.

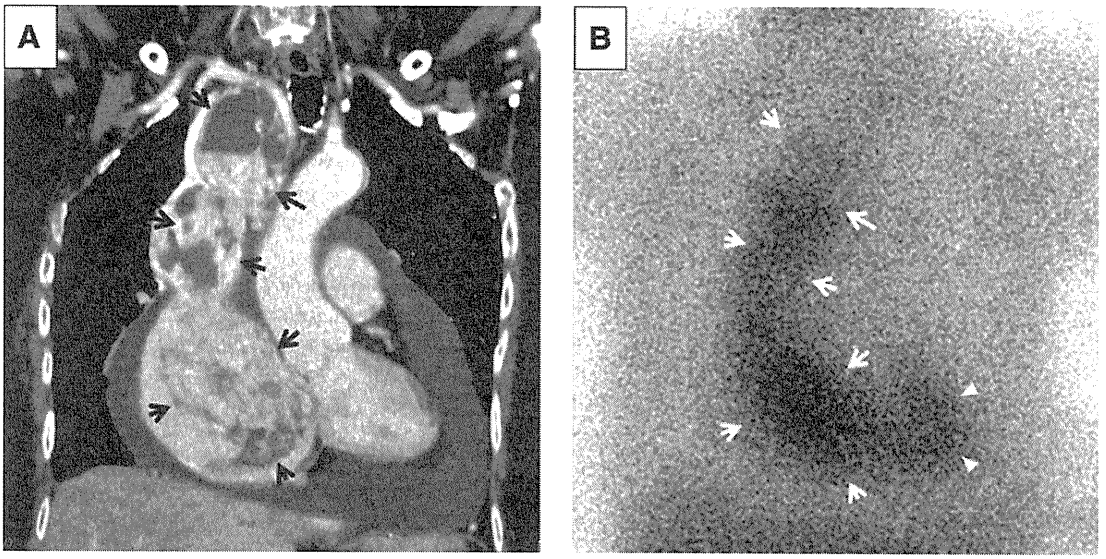
Address correspondence to:
Keiji Tanimoto, M.D., Ph.D.
Department of Internal Medicine (I)
Osaka Medical College
2-7 Daigaku-machi
Takatsuki City 569-8686
Japan

E-mail: in1120@poh.osaka-med.ac.jp

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).

Endocrine Journal 2011, **58** (3), 193-198

ORIGINAL

Hypopituitarism in a patient with transsphenoidal cephalocele: longitudinal changes in endocrinological abnormalities

Keiji Tanimoto, Saori Onda, Hideaki Sawaki, Tetsuya Hiraiwa, Hiroyuki Sano, Mineki Ohnishi, Jungo Terasaki and Toshiaki Hanafusa

Department of Internal Medicine (I), Osaka Medical College, Takatsuki 569-8686, Japan

Abstract. We report a 21-year-old man with severe fatigue due to hypopituitarism. At the age of 6 years, he was diagnosed with short stature due to a GH deficiency accompanied by a sphenoid cystic lesion. Laboratory findings and provocative tests for pituitary hormone function revealed ACTH, LH, FSH, TSH, and GH deficiency. Computed tomography and magnetic resonance imaging revealed transsphenoidal cephalocele due to a defect in the floor of the sella turcica. At 6 years, he only had severe GH deficiency and poor response of LH to LHRH. Hypothalamic-pituitary dysfunction and pituitary herniation have progressed subsequently; we observed a longitudinal progression of hypothalamic-pituitary dysfunction caused by transsphenoidal cephalocele. This dysfunction requires the selection of a treatment that will not aggravate the condition further.

Key words: Hypothalamic-pituitary dysfunction, Panhypopituitarysm, Pituitary herniation, Follow-up, MRI

CONGENITAL basal encephaloceles are rare, with an estimated incidence of 1 in every 35,000 live births [1]. Transsphenoidal cephalocele is the least frequent anomaly, representing only 5% of basal encephaloceles (estimated incidence: 1 in 700,000 live births). In transsphenoidal cephalocele, transsphenoidal lesions collapse into the epipharynx or nasopharynx [2]. The diagnosis of transsphenoidal cephalocele is usually made in infancy or early childhood [3].

Because the floor of the sella turcica is lacking in this anomaly, hypothalamic-pituitary function may deteriorate. The natural course of hypothalamic-pituitary dysfunction due to transsphenoidal cephalocele remains unclear. There are only a few reports describing endocrinological follow-up due of transsphenoidal cephalocele for a period of 10 years or more. We report the longitudinal progression of hypothalamic-pituitary dysfunction due to transsphenoidal cephalocele in a patient. Surgical treatment for transsphenoidal cephalocele was not performed in the follow-up period; the natural course of hypothalamic-pituitary dysfunction was able to be observed.

Received Dec. 24, 2010; Accepted Jan. 19, 2011 as K10E-405
Released online in J-STAGE as advance publication Feb. 17, 2011

Correspondence to: Keiji Tanimoto, Department of Internal Medicine (I), Osaka Medical College, 2-7 Daigaku-machi, Takatsuki city, Osaka 569-8686, Japan
E-mail: in1120@poh.osaka-med.ac.jp

Case Report

A 21-year-old man was referred to our hospital with severe fatigue. At 6 years of age, he had consulted the department of pediatrics in our hospital because of his short height (97.4 cm, −3.56 SD). He was diagnosed with short stature due to GH deficiency accompanied by a sphenoid cystic lesion. Until 10 years old, he did not visit his doctor. GH replacement therapy was administered from the age of 10 to 13 years. However, the patient could not continue the GH replacement therapy after the age of 13 years. After this, he visited the doctor about only once a year, and he did not receive a treatment. At 21 years of age, he was hospitalized in a local hospital due to infectious enteritis; he was also diagnosed with secondary hypothyroidism. Following this, he was admitted to our hospital for further evaluation of hypopituitarism.

His height was 144 cm and body weight was 38 kg. He had no external malformations, but had peripapillary staphyloma in left eye. No axillary or pubic hair was present. His Tanner stages were G1 and P1, and each of his testes was 3 mL in volume. The labora-

Table 1 Laboratory findings

White blood cell	5620 /μL
Neutrophil	62.9 %
Lymphocyte	28.6 %
Eosinophil	3.2 %
Red blood cell	359×10^4 /μL
Hemoglobin	10.2 g/dL
Hematocrit	30.3 %
Platelet	25.4×10^4 /μL
Albumin	4.2 g/dL
Aspartate aminotransferase	24 IU/L
Alanine aminotransferase	13 IU/L
Lactate dehydrogenase	204 IU/L
Creatinine kinase	83 IU/L
Alkaline phosphatase	254 IU/L
Blood urea nitrogen	6 mg/dL
Creatinine	0.56 mg/dL
Uric acid	4.2 mg/dL
Total-Cholesterol	200 mg/dL
Triglyceride	105 mg/dL
Sodium	141 mEq/L
Potassium	3.9 mEq/L
Chloride	108 mEq/L
Calcium	8.9 mg/dL
Fasting serum glucose	84 mg/dL

Table 2 Endocrinological laboratory findings

		Reference value
TSH	2.790 μU/mL	(0.500~5.000)
Free T3	1.62 pg/mL	(2.30~4.30)
Free T4	0.77 ng/dL	(0.90~1.70)
PRL	11.26 ng/mL	(3.58~12.78)
GH	0.08 ng/mL	(<0.17)
IGF-I	12 ng/mL	(133~368)
ACTH	13.0 pg/mL	(7.2~63.3)
Cortisol	4.7 μg/dL	(4.0~18.3)
DHEA-S	8 μg/dL	(85~690)
UFC	8.1 μg/day	(11.2~80.3)
LH	<0.1 mIU/mL	(0.79~5.72)
FSH	0.3 mIU/mL	(2.00~8.30)
Testosterone	<0.05 ng/mL	(2.07~7.61)
Plasma Osm	279 mOsm/kg	(276~292)
Urine Osm	350 mOsm/kg	(50~1200)
ADH	1.0 pg/mL	(0.3~3.5)

Free T3: free triiodothyronine, Free T4: free thyroxine, IGF-I: insulin-like growth factor-I, ADH: antidiuretic hormone, DHEA-S: dehydroepiandrosterone sulfate, UFC: urinary free cortisol

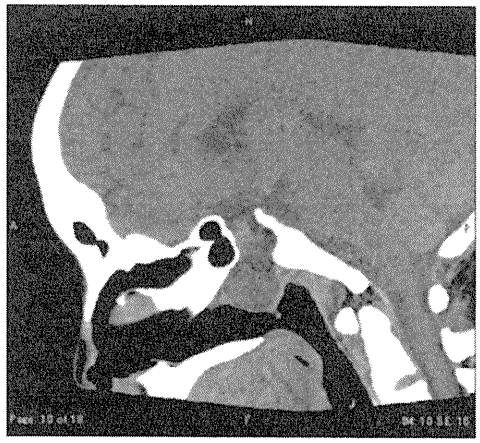


Fig. 1 Sagittal head CT demonstrated a bone defect in the floor of the sella turcica by a transsphenoidal soft tissue mass extending into the epipharynx.

tory examination is displayed in Table 1. Red blood cell, hemoglobin, and hematocrit decreased slightly. Endocrinological laboratory findings are shown in Table 2. Although the serum levels of both free triiodothyronine and free thyroxine were low, TSH was normal. IGF-I (insulin-like growth factor-I) level was low for his age and sex; IGF-I SD score was −5.7 SD. Levels of plasma ACTH, serum cortisol and dehydroepiandrosterone sulfate (DHEA-S), and urinary free cortisol were all low. LH and testosterone levels were lower than the measurable limit. Provocative tests for pituitary hormone function are shown in Table 3. The response of cortisol to 250 μg of synthetic ACTH was impaired; the peak value of cortisol was 12.9 μg/dL. Both LH and FSH levels were consistently low in response to LHRH injection. After TRH administration, TSH rose to high values, but total T3 levels did not rise enough to 1.3 times or more. These findings suggested that bioactivity of TSH is decreased in the patient. The patient had almost no response of GH after GHRP-2 (growth hormone releasing peptide-2) injection. According to hand X-rays, his bone age was equivalent to a 12.6-year-old Japanese boy. Computed tomography (CT) of the head showed a defect in the floor of the sella turcica (Fig. 1.). Magnetic resonance imaging (MRI) revealed a cystic mass extending into the epipharynx through the bone defect (Fig. 2a and 2b).

On the basis of the above mentioned findings, he was diagnosed with ACTH, LH, FSH, TSH, and GH

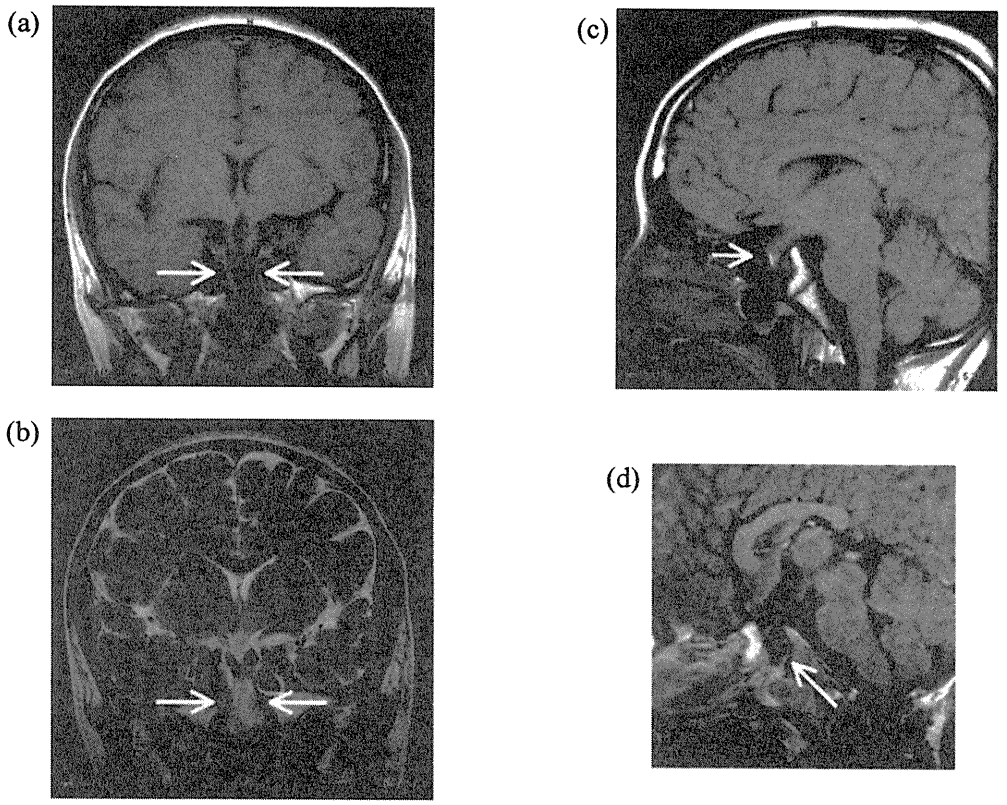


Fig. 2 T1-weighted (a) and T2-weighted (b) coronal and T1-weighted sagittal (c) MRI revealed a cystic mass with a surrounding structure extending through the bone defect into the epipharynx. The longitudinal diameter of the cystic mass was 3.5 centimeters. T1-weighted sagittal MRI at the age of 6 years (d) demonstrated a sphenoid cystic lesion 1.5 centimeters in diameter.

deficiency due to transsphenoidal cephalocele. Nasal obstruction or nasal discharge, meningitis, and cerebrospinal fluid rhinorrhea were not present; therefore, he did not undergo surgery. He received replacement therapy of adrenocortical hormone (hydrocortisone 20 mg), thyroid hormone (levothyroxine sodium hydrate 75 μg), after which his complaints were resolved. His weight was increased to 44kg, though his height did not change. Ten months after, he accepted the replacement therapy of sex hormone (testosterone enanthate 125mg, once a month) and GH (somatropin 1.3mg). However, he did not come to our hospital three months later.

Discussion

Transsphenoidal cephalocele often leads to hypothalamic-pituitary dysfunction. The incidence of hypothalamic-pituitary insufficiency was estimated at about 50–60% in previous reports [1, 4, 5]. Hypothyroidism, GH deficiency, hypogonadotrophic hypogonadism, and diabetes insipidus were frequent disorders. Central hypoadrenalism was rare in previous reports [6]. In our case, hypothyroidism, GH deficiency, hypogonadotrophic hypogonadism, and hypoadrenalism were found, but diabetes insipidus was not detected from laboratory findings and MRI (Table 2 and Fig. 2c).

We obtained the clinical examination data from the hospital where this patient had consulted a doctor in his childhood. Results of his endocrinological examination at the age of 6 years are shown in Table 4. Severe GH deficiency and poor response of LH to LHRH were observed. No evidence of ACTH, FSH, TSH, or ADH deficiency was present at that point. He was diagnosed with short stature due to GH deficiency. His subsequent clinical course is shown in Fig. 3. Free T4 level decreased gradually; nevertheless, TSH did not rise sufficiently. Testosterone levels were lower than the measurable limit at all time points. By the time he was admitted to our hospital at the age of 21, he

Table 3 Provocative tests for pituitary hormone function

	Before	15 min	30 min	45 min	60 min	90 min	120 min
ACTH (250 μg) administration test							
Cortisol (μg/dL)	2.8		11.6		12.9		
CRH (100 μg), LHRH (100 μg), and TRH (200 μg) administration test							
ACTH (pg/mL)	40.2		124		88.1	86.8	
Cortisol (μg/dL)	10.3		17.5		17.8	21.3	
LH (mIU/mL)	0.2		0.4		0.4	0.4	
FSH (mIU/mL)	0.7		1.2		1.4	1.6	
TSH (μU/mL)	8.65		27.7		25.7	21.2	
PRL (ng/mL)	20.7		28.3		22.0	17.7	
Total T3 (ng/mL)	0.75						0.97
GHRP-2 (growth hormone releasing peptide-2) (100 μg) administration test							
GH (ng/mL)	<0.03	0.12	0.08	0.04			

Table 4 Endocrinological findings when the patient was 6 years old

IGF-I	38 ng/mL	Total T4	12.9 μg/dL	Plasma Osm	279 mOsm/kg
Overnight GH	3.85 ng/mL (Mean)	Testosterone	<0.05 ng/mL	Urine Osm	799 mOsm/kg
				ADH	2.0 pg/mL

	Before	15 min	30 min	60 min	90 min	120min	150min
Insulin tolerance test (1.8U, 0.1U/kg)							
Glucose (mg/dL)	82	22	50	80	92		
GH (ng/mL)	0.41		1.4	1.2	0.3		
Cortisol (μg/dL)	11.4			26.0			
Propranolol-Glucagon administration test							
Glucose (mg/dL)	84		122	92	81	66	70
GH (ng/mL)	0.66		0.74	0.64	1.2	2.8	3.4
CRH (27 μg), LHRH (36 μg), and TRH (180 μg) administration test							
Cortisol (μg/dL)	12.6			21.5			
LH (mIU/mL)	<0.5		1.8	1.3	1.4		
FSH (mIU/mL)	0.8		4.0	4.2	5.9		
TSH (μU/mL)	2.7		13.0	8.7			
PRL (ng/mL)	4.3		13.0	7.6			

had not only GH deficiency, but also ACTH, LH, FSH, and TSH deficiency. MRI when he was 6 years old revealed a sphenoid cystic lesion 1.5 cm in diameter (Fig. 2d). The size of his transsphenoidal cephalocele had increased to 3.5 cm by the time of his admission to our hospital (Fig. 2a, 2b, and 2c). To summarize, both hypothalamic-pituitary dysfunction and pituitary herniation had progressed significantly in 15 years.

There are only a few case reports showing endocrinological follow-up for a time period of ten years or more in patients with transsphenoidal cephaloceles and pituitary herniation [2, 4, 7]. The natural course of hypothalamic-pituitary dysfunction is still unclear. Although hypothalamic-pituitary function was not necessarily examined at the first visit or during the follow-up time in all previous case reports, it has been reported that most patients (7 out of 9 patients: 77.8%) with transsphenoidal cephaloceles exhibited a progression of hormonal disturbances [8]. On the other hand, neurosurgical intervention could not effectively prevent pituitary dysfunction [4, 9]. In hypothalamic-pituitary dysfunction, surgical treatment might be required to prevent a progression of hormonal insufficiency. Since a valid surgical procedure for transsphe-

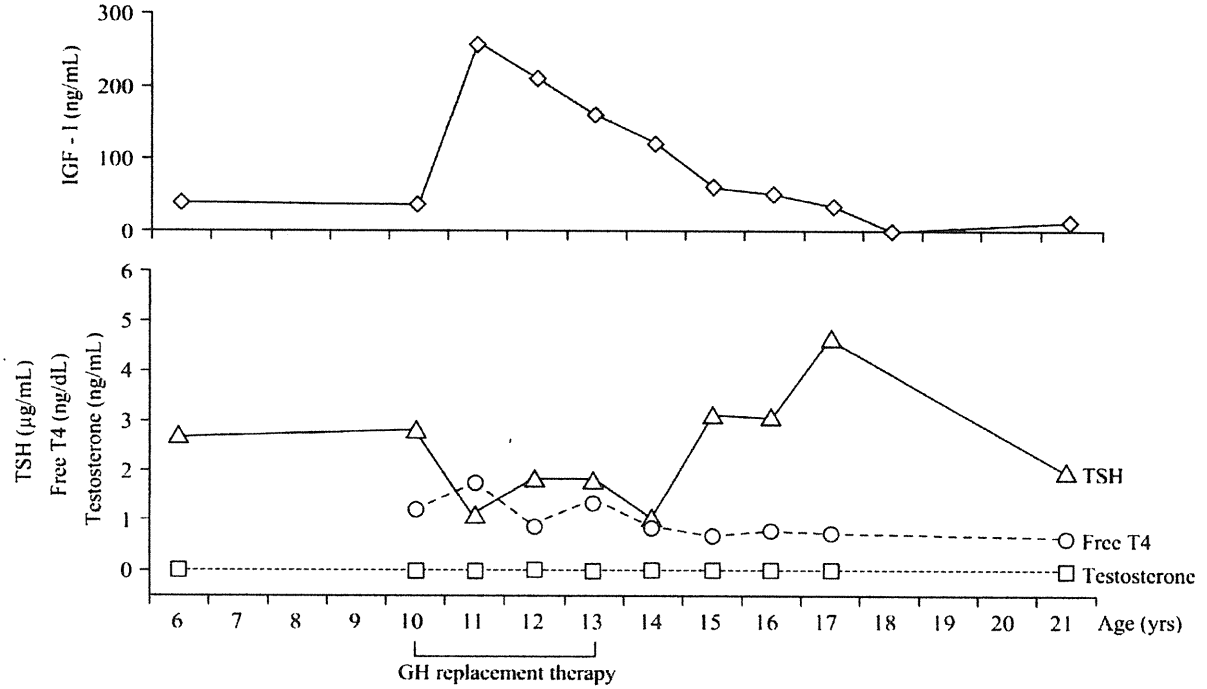


Fig. 3 Clinical course of endocrinological abnormality from 6 years to 21 years is shown. IGF-I (◇) rose temporarily in response to the GH replacement therapy. Free T4 (○) decreased gradually; nevertheless, TSH (△) did not rise appropriately. Testosterone (□) levels were lower than the measurable limit at all time points.

noidal encephalocele was recently reported, the number of treatable cases will probably increase in the near future [10-12].

The surgical treatment for transsphenoidal encephalocele has not always been beneficial [3, 13]. There is a risk of damaging the functioning tissue within the wall of encephalocele [10]. In our case, MRI revealed that a cystic mass contained a surrounding structure, and the pituitary hormones were not completely lack. In addition, surgical treatment could not effectively prevent pituitary dysfunction [4, 9]. If there was an evidence of respiratory obstruction, meningitis, cerebrospinal fluid rhinorrhea, and progressive visual defects, we will conduct a surgical repair to transsphenoidal encephalocele.

The mechanism of this hormonal abnormality is still unknown. In a child with diabetes insipidus complicated by cephalocele, the degeneration of the hypothalamus and agenesis of the supraoptic nuclei may be detected, but the description of pituitary gland anomaly had not been reported [2]. One report described a male patient with GH, TSH, and LH deficiency, and pituitary fibrosis, but a normal hypothalamus [4]. Therefore, the abnormalities of the hypothalamus and pituitary gland are variable. In the present case, MRI revealed that the periphery of the cephalocele was enhanced by gadolinium, and the posterior pituitary bright spot was observed in the sella turcica (Fig. 2). These findings suggest that both the anterior pituitary gland and the neurohypophysis existed. Accordingly, damage to the pituitary stalk might have caused hypothalamic-pituitary dysfunction in this case.

In conclusion, we have reported a patient who had ACTH, LH, FSH, TSH, and GH deficiency due to a transsphenoidal cephalocele. Both hypothalamic-pituitary dysfunction and pituitary herniation have gradually progressed in the past 15 years. In the future, surgical treatment might be a promising choice to prevent further progression of hormonal insufficiency in similar patients.

Acknowledgments

We would like to thank Dr. Kazutaka Konishi, Abuyama Pediatrics Clinic, and Dr. Okasora Keisuke, Department of Pediatrics, Hirakata Municipal Hospital, for providing the childhood clinical data of this patient.

References

1. Smith DE, Murphy MJ, Hitchon PW, Babin RW, Abu-Yousef MM (1983) Transsphenoidal encephaloceles. *Surg Neurol* 20: 471-480.
2. Pollock JA, Newton TH, Hoyt WF (1968) Transsphenoidal and transethmoidal encephaloceles. A review of clinical and roentgen features in 8 cases. *Radiology* 90: 442-453.
3. Yokota A, Matsukado Y, Fuwa I, Moroki K, Nagahiro S (1986) Anterior basal encephalocele of the neonatal and infantile period. *Neurosurgery* 19: 468-478.
4. Ellyin FM, Potchynok A, Song C (1991) Long-term follow-up of hypothalamic-pituitary function in patients with transsphenoidal pituitary herniation and midfacial anomalies. *Clin Endocrinol (Oxf)* 35: 263-266.
5. Koral K, Geffner ME, Curran JG (2000) Transsphenoidal and sphenoethmoidal encephalocele: report of two cases and review of the literature. *Australas Radiol* 44: 220-224.
6. Lieblich JM, Rosen SE, Guyda H, Reardan J, Schaaf M (1978) The syndrome of basal encephalocele and hypothalamic-pituitary dysfunction. *Ann Intern Med* 89: 910-916.
7. Durham LH, Mackenzie IJ, Miles JB (1988) Transphenoidal meningohydroencephalocoele. *Br J Neurosurg* 2: 407-409.
8. Morioka M, Marubayashi T, Masumitsu T, Miura M, Ushio Y (1995) Basal encephaloceles with morning glory syndrome, and progressive hormonal and visual disturbances: case report and review of the literature. *Brain Dev* 17: 196-201.
9. Sailer HF, Landolt AM (1987) A new method for the correction of hypertelorism with preservation of the olfactory nerve filaments. *J Craniomaxillofac Surg* 15: 122-124.
10. Tsutsumi K, Asano T, Shigeno T, Matsui T, Ito S, Kaizu H (1999) Transcranial approach for transsphenoidal encephalocele: report of two cases. *Surg Neurol* 51: 252-257.
11. Abe T, Ludecke DK, Wada A, Matsumoto K (2000) Transsphenoidal cephaloceles in adults. A report of two cases and review of the literature. *Acta Neurochir (Wien)* 142: 397-400.
12. Faggin R, Pentimalli L, Grazzini M, Saetti R, Drigo P, d'Avella D (2009) Combined endoscopic-microsurgical approach for transsphenoidal (sphenopalatine) encephalocele with an intralesional pituitary gland. Case report. *J Neurosurg Pediatr* 4: 262-265.
13. David DJ (1993) Cephaloceles: classification, pathology, and management--a review. *J Craniofac Surg* 4: 192-202.

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

Kaoru Aida,[1] Yoriko Nishida,[1] Shoichiro Tanaka,[1] Taro Maruyama,[2] Akira Shimada,[3] Takuya Awata,[4] Masako Suzuki,[5] Hiroki Shimura,[1] Soichi Takizawa,[1] Masashi Ichijo,[1] Daiichiro Akiyama,[1] Fumihiko Furuya,[1] Akio Kawaguchi,[1] Masahiro Kaneshige,[1] Jun Itakura,[6] Hideki Fujii,[6] Toyoshi Endo,[1] and Tetsuro Kobayashi[1]

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**

From the [1]Third Department of Internal Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan; the [2]Department of Internal Medicine, Saitama Social Insurance Hospital, Saitama, Japan; the [3]Department of Internal Medicine, Keio University, Tokyo, Japan; the [4]Division of Endocrinology and Diabetes, Department of Medicine, Saitama Medical School, Saitama, Japan; the [5]Department of Pathology, Sayama Hospital, Saitama, Japan; and the [6]First Department of Surgery, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan.

Corresponding author: Tetsuro Kobayashi, tetsurou@yamanashi.ac.jp.

Received 4 June 2010 and accepted 15 December 2010.

DOI: 10.2337/db10-0795

This article contains Supplementary Data online at http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db10-0795/-/DC1.

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.

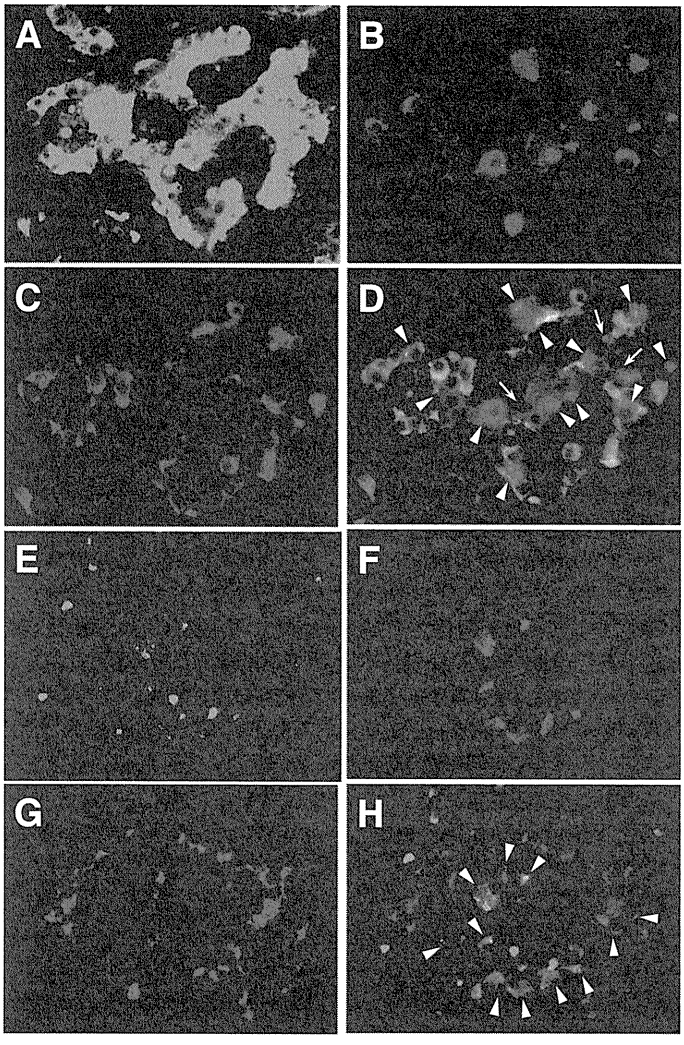


FIG. 1. Intracytoplasmic double-stranded virus RNA receptor expression in enterovirus-associated human fulminant type 1 diabetes. *A–C*: Triple-immunostaining of MDA5 (*A*), insulin (*B*), and glucagon (*C*). The merged image (*D*) demonstrates hyperexpression of MDA5 in β-cells (light blue, arrowheads), 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.

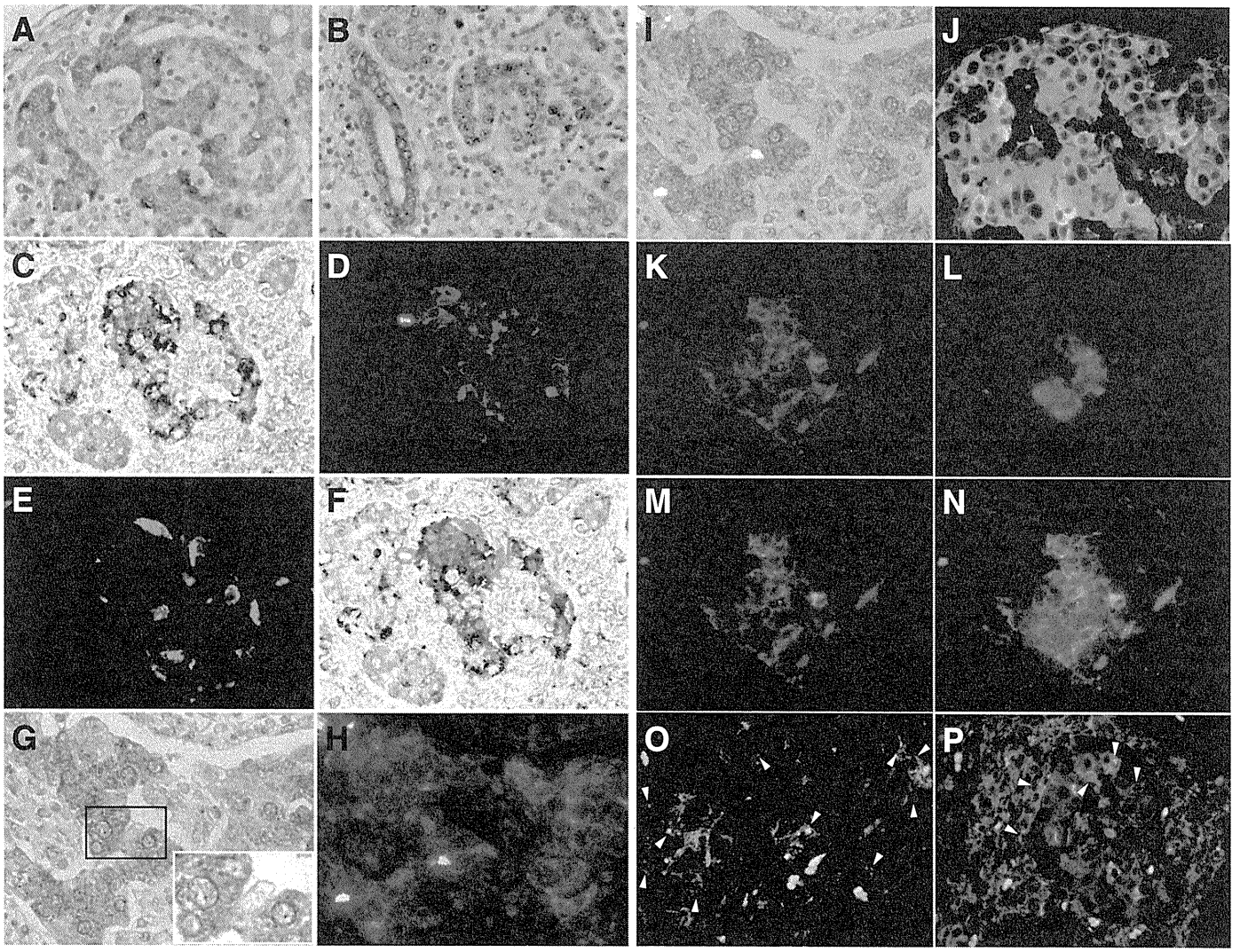


FIG. 2. Immunohistochemical staining of IFN-α, IFN-β_1, IRF-7, and MHC-class I in a pancreas with fulminant type 1 diabetes (×200, case 2). *A* and *B*: Immunostaining of IFN-α (*A*) and IFN-β_1 (*B*). *C–F*: Triple-immunohistochemical staining of IFN-α (*C*), insulin (*D*), and glucagon (*E*). A merged 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

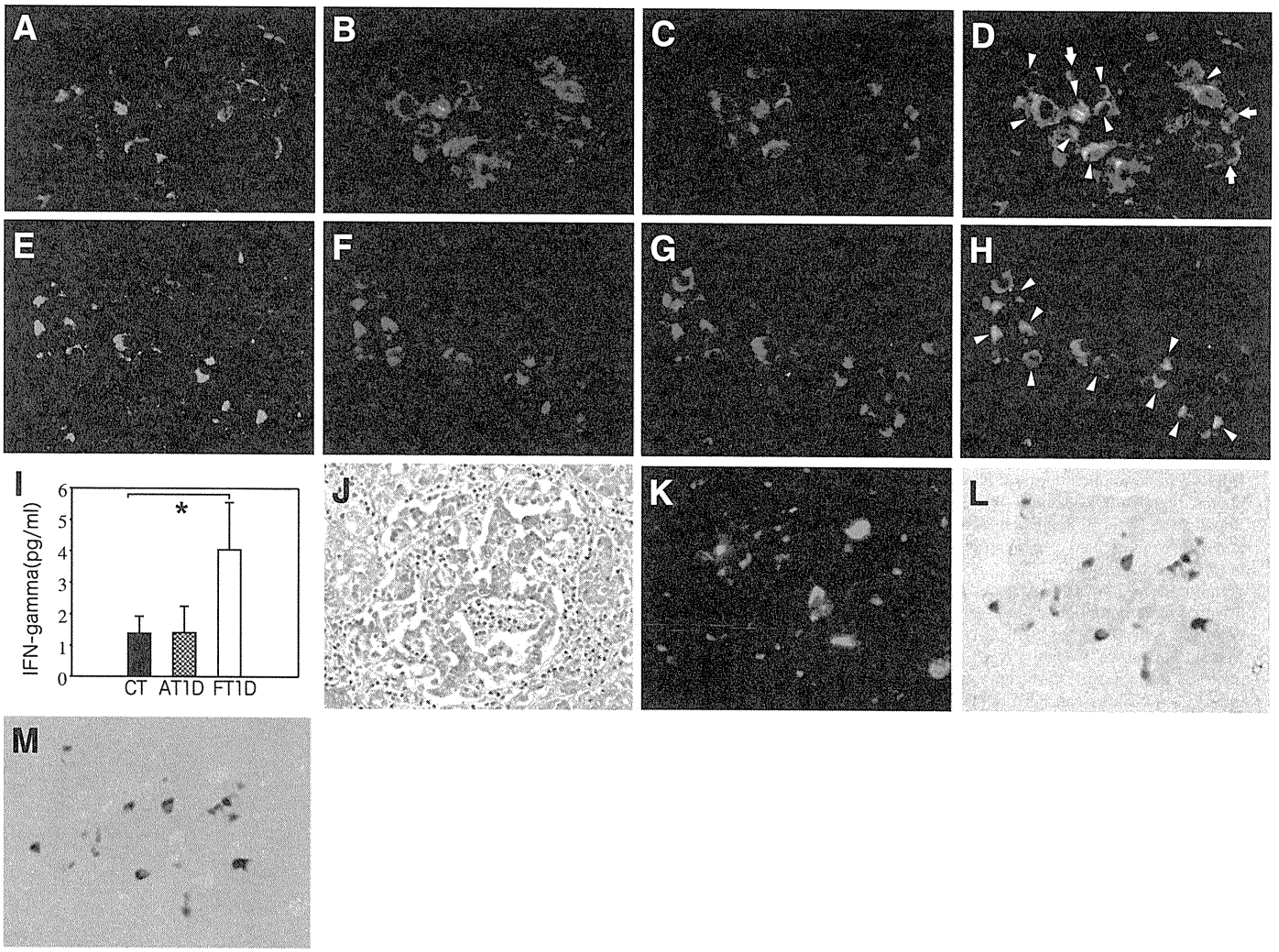


FIG. 3. Immunohistochemical staining of IL-18, insulin, CXCL10, IFN-γ, and FasL. *A–D*: Triple-immunostaining of IL-18 (*A*), insulin (*B*), and CXCL10 (*C*). The merged image (*D*) shows that most β-cells express both IL-18 and CXCL10 (arrowheads). Some islet non–β-cells also express IL-18 and CXCL10 (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.

ACKNOWLEDGMENTS

This study was partly supported by grants from the Ministry of Education, Science, Sports and Culture and Ministry of Health, Labor and Welfare (Japan).

No potential conflicts of interest relevant to this article were reported.

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.

The authors thank K. Hosaka, C. Imai, and S. Takei for excellent secretarial work; Drs. T. Momotsu and E. Okazaki (Niigata City General Hospital) for generous assistance; and Professors A. Nakao (Department of Immunology) and R. Kato (Department of Pathology) at the University of Yamanashi for critical assessment and assistance of the article.

REFERENCES

1. Kobayashi T. Immunology and immunogenetics of type 1 diabetes in Japan. IDF Bull 1990;35:34–37

2. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y; Osaka IDDM Study Group. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. N Engl J Med 2000; 342:301–307
3. Tanaka S, Kobayashi T, Momotsu T. A novel subtype of type 1 diabetes mellitus. N Engl J Med 2000;342:1835–1837
4. Tanaka S, Nishida Y, Aida K, et al. Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes. Diabetes 2009;58:2285–2291
5. Takeuchi O, Akira S. MDA5/RIG-I and virus recognition. Curr Opin Immunol 2008;20:17–22
6. von Herrath M. Diabetes: A virus-gene collaboration. Nature 2009;459: 518–519
7. Honda K, Yanai H, Negishi H, et al. IRF-7 is the master regulator of type-I interferon-dependent immune responses. Nature 2005;434:772–777
8. Sturniolo MT, Dashti SR, Deucher A, et al. A novel tumor suppressor protein promotes keratinocyte terminal differentiation via activation of type I transglutaminase. J Biol Chem 2003;278:48066–48073
9. Sarkar SA, Wong R, Hackl SI, et al. Induction of indoleamine 2,3-dioxygenase by interferon-gamma in human islets. Diabetes 2007;56:72–79
10. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. Science 2009;324:387–389
11. Ylipaasto P, Kutlu B, Rasilainen S, et al. Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets. Diabetologia 2005;48:1510–1522
12. Schulte BM, Kramer M, Ansems M, et al. Phagocytosis of enterovirus-infected pancreatic beta-cells triggers innate immune responses in human dendritic cells. Diabetes 2010;59:1182–1191
13. Negishi H, Osawa T, Ogami K, et al. A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. Proc Natl Acad Sci USA 2008;105:20446–20451
14. Hultcrantz M, Hühn MH, Wolf M, et al. Interferons induce an antiviral state in human pancreatic islet cells. Virology 2007;367:92–101
15. Le Bon A, Durand V, Kamphuis E, et al. Direct stimulation of T cells by type I IFN enhances the CD8+ T cell response during cross-priming. J Immunol 2006;176:4682–4689
16. Blanco P, Palucka AK, Pascual V, Banchereau J. Dendritic cells and cytokines in human inflammatory and autoimmune diseases. Cytokine Growth Factor Rev 2008;19:41–52
17. Wei J, Duramad O, Perng OA, Reiner SL, Liu YJ, Qin FX. Antagonistic nature of T helper 1/2 developmental programs in opposing peripheral induction of Foxp3+ regulatory T cells. Proc Natl Acad Sci USA 2007;104: 18169–18174
18. Okamura H, Tsutsi H, Komatsu T, et al. Cloning of a new cytokine that induces IFN-gamma production by T cells. Nature 1995;378:88–91
19. Pien GC, Nguyen KB, Malmgaard L, Satoskar AR, Biron CA. A unique mechanism for innate cytokine promotion of T cell responses to viral infections. J Immunol 2002;169:5827–5837
20. Frigerio S, Holländer GA, Zumsteg U. Functional IL-18 Is produced by primary pancreatic mouse islets and NIT-1 beta cells and participates in the progression towards destructive insulitis. Horm Res 2002;57:94–104
21. Sareneva T, Julkunen I, Matikainen S. IFN-alpha and IL-12 induce IL-18 receptor gene expression in human NK and T cells. J Immunol 2000;165: 1933–1938
22. Nagata S. Apoptosis by death factor. Cell 1997;88:355–365
23. Christen U, Darwiche R, Thomas HE, et al. Virally induced inflammation triggers fratricide of Fas-ligand-expressing beta-cells. Diabetes 2004;53: 591–596
24. Eizirik DL, Mandrup-Poulsen T. A choice of death—the signal-transduction of immune-mediated beta-cell apoptosis. Diabetologia 2001;44:2115–2133
25. Besch R, Poeck H, Hohenauer T, et al. Proapoptotic signaling induced by RIG-I and MDA-5 results in type I interferon-independent apoptosis in human melanoma cells. J Clin Invest 2009;119:2399–2411

DIABETES/METABOLISM RESEARCH AND REVIEWS
Diabetes Metab Res Rev 2011; **27**: 965–970.
Published online in Wiley Online Library (wileyonlinelibrary.com) **DOI:** 10.1002/dmrr.1237

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

Tetsuro Kobayashi*
Yoriko Nishida
Shoichiro Tanaka
Kaoru Aida

Third Department of Internal Medicine, Interdisciplinary Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan

*Correspondence to:
Tetsuro Kobayashi,
Third Department of Internal Medicine,
Interdisciplinary Graduate School of Medicine and Engineering,
University of Yamanashi,
1110 Shimokato, Chuo,
Yamanashi 409-3898,
Japan
E-mail: tetsurou@yamanashi.ac.jp

Received: 24 March 2011
Revised: 11 May 2011
Accepted: 31 May 2011

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].

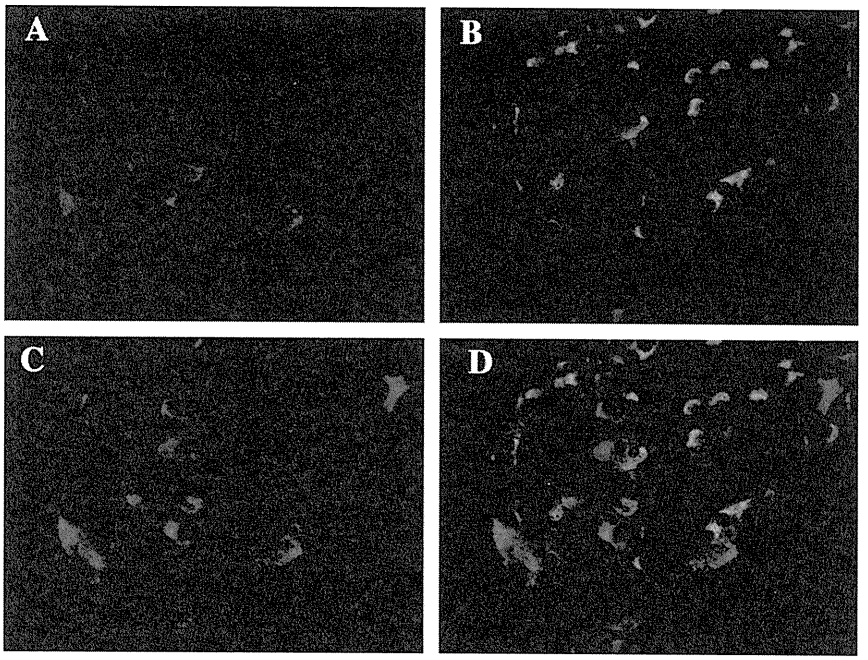


Figure 1. Intra-cytoplasmic laboratory of genetics and physiology 2 expression in enterovirus-associated human fulminant type 1 diabetes. Triple-immunostaining for: laboratory of genetics and physiology 2 (A), glucagon (B) and insulin (C). The merged image (D) demonstrates specific hyper-expression of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

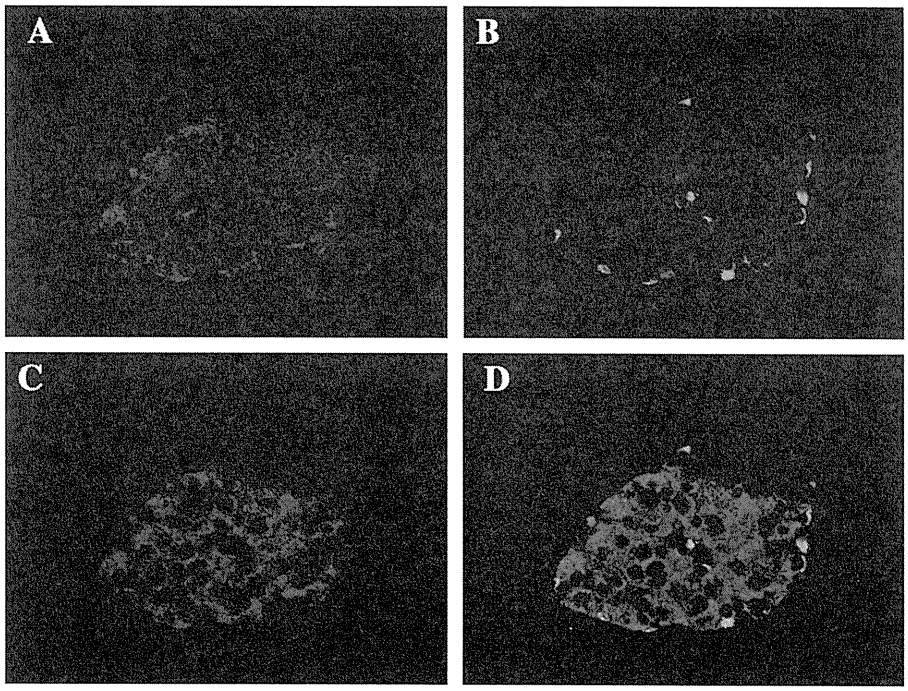


Figure 2. Intra-cytoplasmic laboratory of genetics and physiology 2 expression in non-diabetic controls. Triple-immunostaining for: laboratory of genetics and physiology 2 (A), glucagon (B) and insulin (C). The merged image (D) demonstrates specific basal expression of laboratory of genetics and physiology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

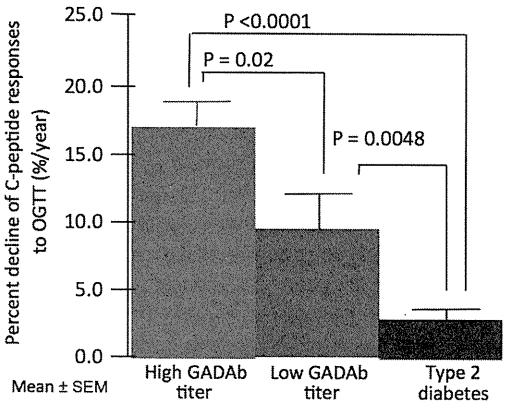


Figure 3. Annual decline rate of C-peptide responses to 75-g oral glucose tolerance tests in the SPIDDM patients. Left column: high titre of glutamic acid decarboxylase autoantibodies (≥180 WHO U/mL), Middle column: low titre of glutamic acid decarboxylase autoantibodies (<180 WHO U/mL), Right column: 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.

Acknowledgements

We wish to thank K. Hosaka, C. Imai and S. Takei for their excellent secretarial work, and to Drs T. Momotsu and E. Okazaki (Niigata City General Hospital) for their generous assistance and to Profs A. Nakao (Department of Immunology) and R. Kato (Department of Pathology) at the University of Yamanashi for their critical assessment and generous assistance of the manuscript. This study was partly supported by grants from the Ministry of Education, Culture, Sports, Science and Technology and grants from the Ministry of Health, Labor and Welfare in Japan.

Conflict of interest

The authors have declared that no conflict of interest exists.

References

1. Kobayashi T. Immunology and immunogenetics of type 1 diabetes in Japan. *IDF Bulletin* 1990; **35:** 34–37.
2. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y. A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. Osaka IDDM Study Group. *N Engl J Med* 2000; **342:** 301–307.
3. Tanaka S, Kobayashi T, Momotsu T. A novel subtype of type 1 diabetes mellitus. *N Engl J Med* 2000; **342:** 1835–1837.
4. Tanaka S, Nishida Y, Aida K, *et al.* Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes. *Diabetes* 2009; **58:** 2285–2291.
5. Takeuchi O, Akira S. MDA5/RIG-I and virus recognition. *Curr Opin Immunol* 2008; **20:** 17–22.
6. Kawai T, Akira S. Toll-like receptor and RIG-I-like receptor signaling. *Ann N Y Acad Sci* 2008; **1143:** 1–20.
7. von Herrath M. Diabetes: A virus-gene collaboration. *Nature* 2009; **459:** 518–519.
8. Sano H, Terasaki J, Tsutsumi C, Imagawa A, Hanafusa T. A case of fulminant type 1 diabetes mellitus after influenza B infection. *Diabetes Res Clin Pract* 2008; **79:** e8–e9.
9. Kobayashi T, Itoh T, Kosaka K, Sato K, Tsuji K. Time course of islet cell antibodies and beta-cell function in non-insulin-dependent stage of type I diabetes. *Diabetes* 1987; **36:** 510–517.
10. Kobayashi T, Nakanishi K, Sugimoto T, *et al.* Maleness as risk factor for slowly progressive IDDM. *Diabetes Care* 1989; **12:** 7–11.
11. Kobayashi T. Subtype of insulin-dependent diabetes mellitus (IDDM) in Japan: slowly progressive IDDM–the clinical characteristics and pathogenesis of the syndrome. *Diabetes Res Clin Pract* 1994; **24** (Suppl.): S95–S99.
12. Kobayashi T, Tamemoto K, Nakanishi K, *et al.* Immunogenetic and clinical characterization of slowly progressive IDDM. *Diabetes Care* 1993; **16:** 780–788.
13. Tanaka S, Kobayashi T, Nakanishi K, *et al.* Association of HLA-DQ genotype in autoantibody-negative and rapid-onset type 1 diabetes. *Diabetes Care* 2002; **25:** 2302–2307.
14. Oberste MS, Maher K, Flemister MR, Marchetti G, Kilpatrick DR, Pallansch MA. Comparison of classic and molecular approaches for the identification of untypeable enteroviruses. *J Clin Microbiol* 2000; **38:** 1170–1174.
15. Nix WA, Berger MM, Oberste MS, *et al.* Failure to detect enterovirus in the spinal cord of ALS patients using a sensitive RT-PCR method. *Neurology* 2004; **62:** 1372–1377.
16. Nix WA, Oberste MS, Pallansch MA. Sensitive, seminested PCR amplification of VP1 sequences for direct identification of all enterovirus serotypes from original clinical specimens. *J Clin Microbiol* 2006; **44:** 2698–2704.
17. Lönnrot M, Korpela K, Knip M, *et al.* Enterovirus infection as a risk factor for beta-cell autoimmunity in a prospectively observed birth cohort: the Finnish Diabetes Prediction and Prevention Study. *Diabetes* 2000; **49:** 1314–1318.
18. Dierssen U, Rehren F, Henke-Gendo C, Harste G, Heim A. Rapid routine detection of enterovirus RNA in cerebrospinal fluid by a one-step real-time-PCR assay. *J Clin Virol* 2008; **42:** 58–64.
19. Verstrepen WA, Kuhn S, Kockx MM, van de Vyvere ME, Mertens AH. Rapid detection of enterovirus RNA in cerebrospinal fluid specimens with a novel single-tube real-time reverse transcription-PCR assay. *J Clin Microbiol* 2001; **39:** 4093–4096.
20. Aida K, Nishida Y, Tanaka S, *et al.* RIG-I- and MDA5-initiated innate immunity linked with adaptive immunity accelerates {beta}-cell death in fulminant type 1 diabetes. *Diabetes* 2011; **60:** 884–889.
21. Satoh T, Kato H, Kumagai Y, *et al.* LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proc Natl Acad Sci U S A* 2010; **107**(26): 1512–1517.
22. Nejentsev S, Walker N, Riches D, Egholm M, Todd JA. Rare variants of IFIH1, a gene implicated in antiviral responses, protect against type 1 diabetes. *Science* 2009; **324:** 387–389.

23. Yoneyama M, Kikuchi M, Matsumoto K, *et al.* Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 2005; **175:** 2851–2858.
24. Wang JP, Cerny A, Asher DR, Kurt-Jones EA, Bronson RT, Finberg RW. MDA5 and MAVS mediate type I IFN responses to Coxsackie B virus. *J Virol* 2010; **84:** 254–260.
25. Dogusan Z, García M, Flamez D, *et al.* Double-stranded RNA induces pancreatic beta-cell apoptosis by activation of the toll-like receptor 3 and interferon regulatory factor 3 pathways. *Diabetes* 2008; **57:** 1236–1245.
26. Negishi H, Osawa T, Ogami K, *et al.* A critical link between Toll-like receptor 3 and type II interferon signaling pathways in antiviral innate immunity. *Proc Natl Acad Sci U S A* 2008; **105:** 20446–20451.
27. Tanaka S, Endo T, Aida K, Kobayashi T. Longitudinal changes in beta-cell function in the patients with slowly progressive insulin-dependent diabetes mellitus (SPIDDM). *J Jap Diab Soc* 2008; **51** (Suppl. 1): S–316 (Abstract).