expressed in the infiltrating T cells.

CXCL10 expression is induced by interferon gamma, so the first candidate of mechanisms of the induction of this chemokine may be interferon gamma from infiltrating T (Th1) cells, since insulitis is the main lesion of this type of diabetes. Viral infection to beta cells is one of the other candidate mechanisms of induction of CXCL10, because viral infection is not necessarily associated in the pathoetiology in the development of autoimmune type 1 diabetes.

It is very important that the expression of CXCL10 is beta cell specific, because beta cells are selectively destroyed in autoimmune type 1 (type 1A) diabetes. It is a likely hypothesis that beta cell specific infection by some kind of virus induces interferon gamma and subsequently CXCL10 in beta cells. There has been no report of the virus with specific affinity to beta cells, but coxsackie B4 virus have high affinity to islet cells and induced CXCL10 in islet cells *in vitro* [12]. Second hypothesis is that virus infects beta cells and other islet cells but interferon gamma is more strongly inducing CXCL10 in beta cells. CXCL10 is induced through the interferon gamma receptor, the Jak-Stat signaling pathway and nuclear factor-kappa B [13]. These factors may be responsible for the exclusive expression of CXCL10 in beta cells of insulitis lesions.

The other CXCL10 positive cells than beta cells were not identified in this study, though the other islet cells were unlikely from our present results.

In the infiltrating T cells of beta cell positive islets shown in this study, CXCR3, a receptor for CXCL10, also contributes to beta cell-specific damage in type 1 diabetes because it is not expressed in exocrine pancreas or beta cell-deficient islets. T cells would migrate to beta cells *via* the CXCR3-CXCL10 interaction in insulitis lesions.

The initial factor that promotes interferon gamma production has not been clarified; however, this cytokine would be secreted from islet cells or islet-infiltrating cells, especially Th1 cells in insulitis lesions [14]. Once activated, infiltrating cells would produce interferon gamma and make a vicious cycle with beta cells that secrete CXCL10, and which would further recruit CXCR3- positive T cells to insulitis lesions.

CXCL10 not only mediates leukocyte recruitment, but also drives T cell proliferation to antigenic stimulation and interferon gamma secretion in response to antigenic challenge [15]. CXCR3 positive T cell-CXCL10 positive beta cell interaction is prerequisite for beta cell-specific damage.

Serum levels of CXCL10 were significantly higher in patients with newly diagnosed type 1 diabetes and correlated with levels of GAD reactive CD4-positive T cells [8], also indicating the importance of CXCR3-CXCL10 interaction.

We did not examine whether CXCR3-positive T cells were CD4- or CD8- positive or not. However, we have reported that insulitis is predominantly composed of CD8-positive T lymphocytes in the pancreas biopsy specimens from newly diagnosed type 1 diabetes patients [16]. Therefore, it seems that infiltrating CXCR3-positive T cells in islets might be CD8-positive T cells.

It has been recently reported that CXCL10 expression were observed in beta cells, alpha cells, and other islet cells, which were infiltrated by CXCR3-positive CD8-positive T cells in three cases of fulminant type 1 diabetes in Japanese [17]. CXCL10 and CXCR3 expressions were also reported in a case of virus-induced diabetes and three cases of type 1A diabetes in Caucasian [18]. We identified the expression of CXCL10 and CXCR3 in insulitis lesion of five Japanese patients with type 1A diabetes. These findings suggest the underlying common mechanism of beta cell death both in fulminant and type 1A diabetes.

We have previously reported that Fas-Fas ligand system and cytokines such as tumor necrosis factor alpha and interleukin 1 beta would play important roles in the destruction of beta cells in type 1A diabetes [11]. Our present results would add new and third insight to the mechanism of beta cell death in human type 1 diabetes.

In conclusion, this study clearly identified the local expression of CXCL10 and CXCR3 in the inflamed islets of autoimmune type 1 diabetes.

Acknowledgements

We would like to thank Fumie Katsube for their excellent technical assistance. This study was supported in part by a Grant-in Aid from the Japanese Society for the Promotion of Science (KAKENHI 19790641, 19591087, 19591069), a grant from The Naito Foundation (2007), a grant from Takeda Science Foundation (2007) and a Health and Labour Sciences Research Grant on Research on Intractable Diseases from the Japanese Ministry of Health, Labour and Welfare.

References

1. Atkinson MA, Eisenbarth GS (2001) Type 1 diabetes: new perspectives on disease pathogenesis and treatment. *Lancet* 358:221-229. Erratum in: *Lancet* 358: 766.
2. Rotondi M, Chiovato L, Romagnani S, Serio M, Romagnani P (2007) Role of chemokines in endocrine autoimmune diseases. *Endocr Rev* 28: 492-520.
3. Taub DD, Lloyd AR, Conlon K, Wang JM, Ortaldo JR, Harada A, Matsushima K, Kelvin DJ, Oppenheim JJ (1993) Recombinant human interferon-inducible protein 10 is a chemoattractant for human monocytes and T lymphocytes and promotes T cell adhesion to endothelial cells. *J Exp Med* 177: 1809-1814.
4. Romagnani P, Lasagni L, Annunziato F, Serio M, Romagnani S (2004) CXC chemokines the regulatory link between inflammation and angiogenesis. *Trends Immunol* 25: 201-209.
5. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR (1998) The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. *J Clin Invest* 101: 746-754.
6. Shigihara T, Oikawa Y, Kanazawa Y, Okubo Y, Narumi S, Saruta T, Shimada A (2006) Significance of serum CXCL10/IP-10 level in type 1 diabetes. *J Autoimmun* 26: 66-71.
7. Rhode A, Pauza ME, Barral AM, Rodrigo E, Oldstone MB, von Herrath MG, Christen U (2005) Islet-specific expression of CXCL10 causes spontaneous islet infiltration and accelerates diabetes development. *J Immunol* 175: 3516-3524.
8. Shimada A, Morimoto J, Kodama K, Suzuki R, Oikawa Y, Funae O, Kasuga A. Saruta T, Narumi S (2001) Elevated serum IP-10 levels observed in type 1 diabetes. *Diabetes Care* 24: 510-515.
9. Frigerio S, Junt T, Lu B, Gerard C, Zumsteg U, Hollander GA, Piali L (2002) Beta cells are responsible for CXCR3-mediated T-cell infiltration in insulitis. *Nat Med* 8: 1414-1420.
10. van Halteren AG, Karbol MJ, Mulder A, Roep BO (2005) Homing of human autoreactive T cells into pancreatic tissue of NOD-scid mice. *Diabetologia* 48: 75-82.
11. Uno S, Imagawa A, Okita K, Sayama K, Iwahashi H,Yamagata K, Tamura S, Matsuzawa Y, Hanafusa T, Miyagawa J, Shimomura I (2007) Macrophages and dendritic cells infiltrating islets with or without bets cells produce tumor necrosis factor-alpha in patients with recent-onset type1 diabetes. *Diabetologia* 50: 596-601.
12. Berg AK, Korsgren O, Frisk G (2006) Induction of the chemokine interferon-gamma-inducible protein-10 in human pancreatic islets during enterovirus infection. *Diabetologia* 49: 2697-2703.
13. Liu D, Cardozo AK, Darville MI, Eizirik DL (2002) Double-stranded RNA cooperates with interferon-gamma and IL-1 beta to induce both chemokine expression and nuclear factor-kappa B-dependent apoptosis in pancreatic beta-cells: potential mechanisms for viral-induced insulitis and beta-cell death in type 1 diabetes mellitus. *Endocrinology* 143: 1225-1234.
14. Rabinovitch A, Suarez-Pinzon WL, Sorensen O, Bleackley RC, Power RF (1995) IFN-gamma gene expression in pancreatic islet-infiltrating mononuclear cells correlates with autoimmune diabetes in nonobese diabetic mice. *J Immunol* 154: 4874-4882.
15. Romagnani S (1997) The Th1/Th2 paradigm. *Immunol Today* 18: 263-266.
16. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M, Kawata S, Tarui S, Kono N, Matsuzawa Y (1993) Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J Clin Invest* 92: 2313-2322.
17. Tanaka S, Nishida Y, Aida K, Maruyama T, Shimada A, Suzuki M, Shimura H, Takizawa S, Takahashi M, Akiyama D, Arai-Yamashita S, Furuya F, Kawaguchi A, Kaneshige M, Katoh R, Endo T, Kobayashi T (2009) Enterovirus infection, CXC chemokine ligand 10 (CXCL10), and CXCR3 circuit: a mechanism of accelerated beta-cell failure in fulminant type 1 diabetes. *Diabetes* 58: 2285-2291.
18. Roep BO, Kleijwegt FS, van Halteren AG, Bonato V, Boggi U, Vendrame F, Marchetti P, Dotta F (2010) Islet inflammation and CXCL10 in recent-onset type 1 diabetes. *Clin Exp Immunol* 159: 338-343.

Endogenous insulin secretion even at a very low level contributes to the stability of blood glucose control in fulminant type 1 diabetes

Saeko Shibasaki[1], Akihisa Imagawa[1,2]*, Jungo Terasaki[1], Toshiaki Hanafusa[1]

ABSTRACT

Fulminant type 1 diabetes is characterized by almost complete β-cell destruction, resulting in scarce insulin secretion. In the present study, we aimed to clarify clinical features related to serum C-peptide levels measured by a high sensitivity method, chemiluminescent enzyme immunoassay, in 12 patients with fulminant type 1 diabetes. Serum C-peptide was detected (0.007–0.10 nmol/L) in four patients and was not detected in eight patients. A negative correlation was observed between serum C-peptide levels and daily dosages of insulin ($P < 0.01$). The patients with detectable C-peptide showed a significantly lower *M*-value than those without ($P = 0.01$). In conclusion, our present results suggest that even very low levels of endogenous insulin secreting capacity can improve daily dosages of insulin and stabilize blood glucose levels. (J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00059.x, 2010)

KEY WORDS: Fulminant type 1 diabetes, Serum C-peptide, *M*-value

INTRODUCTION

Type 1 diabetes is characterized by insulin deficiency resulting from destruction of pancreatic β-cells and subclassified into type 1A (autoimmune) and type 1B (idiopathic) diabetes[1]. We previously reported a novel subtype of type 1B diabetes that we referred to fulminant type 1 diabetes[2]. In fulminant type 1 diabetes, remarkably acute and almost complete β-cell destruction occurs and nearly no insulin secretion remains, even just after the disease onset[3].

Measurement of serum C-peptide level is effective for assessing the ability of endogenous insulin secretions. In particular, it is valuable to presume residual β-cell capability for type 1 diabetic patients. With a conventional method, enzyme immunoassays (EIA), low levels (usually <0.07 nmol/L [0.2 ng/mL]) of serum C-peptide are difficult to detect. However, we can now detect up to 0.003 nmol/L (0.01 ng/mL) serum C-peptide levels using a high sensitivity method, chemiluminescent enzyme immunoassay (CLEIA). It enables us to know more precise levels of serum C-peptide in patients who have almost no insulin secretion, such as patients with fulminant type 1 diabetes[4].

In the present study, we evaluated serum C-peptide levels measured by CLEIA and clarified the clinical features of fulminant type 1 diabetic patients based on residual endogenous insulin secretions.

[1]First Department of Internal Medicine, Osaka Medical College, Takatsuki, and
[2]Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Suita, Japan
*Corresponding author. Akihisa Imagawa Tel.: +81-6-6879-3732 Fax: +81-6-6879-3739
E-mail address: aimagawa@endmet.med.osaka-u.ac.jp
Received 22 March 2010; revised 8 June 2010; accepted 14 July 2010

MATERIALS AND METHODS

Patients and Samples

We studied 12 patients with fulminant type 1 diabetes (5 males and 7 females). These patients were diagnosed as having fulminant type 1 diabetes between 1988–2006, and had been followed in our hospital for more than 0.5 years since the disease onset. The diagnosis of fulminant type 1 diabetes was established according to the inclusion criteria proposed by the committee of the Japan Diabetes Society[5]. Namely: (i) the presence of ketosis or ketoacidosis within a week after the onset of hyperglycemic symptoms; (ii) urinary C-peptide excretion <10 μg/day or fasting serum C-peptide level <0.10 nmol/L (0.3 ng/mL) and peak serum C-peptide level <0.17 nmol/L (0.5 ng/mL) after glucagon (1 mg) or a meal load soon after disease onset; and (iii) plasma glucose level ≥16.0 mmol/L (288 mg/dL) and HbA_{1c} level <8.5% at first visit.

Patients' characteristics were as follows: they were aged 22–78 years (44.2 ± 18.4), duration of the disease was 0.8–19 years (4.8 ± 5.2), body mass index (BMI) was 18.5–24.8 (20.8 ± 1.9), HbA_{1c} was 5.7–10.4% (7.2 ± 1.5), and glycoalbumin was 19.0–32.5% (25.1 ± 4.1). GAD_{65} antibody was negative in all patients. Eight of the 12 patients were receiving multiple daily injections and three patients were receiving continuous subcutaneous insulin infusion. One patient was treated with biphasic insulin analog crystallized with protamine twice a day. Every patient was usually followed every month in our outpatient clinic and insulin doses were adjusted for targeting nearly normal glucose levels (HbA_{1c} < 6.5%, fasting glucose level <5.6 mmol/L [100 mg/dL], 120-min postprandial glucose level <7.8 mmol/L [140 mg/dL]).

The data of HbA_{1c}, glycoalbumin, bodyweight and daily dosages of insulin were measured every 9 months (from January to

September in 2006) and expressed as mean of 9 months' measurements. Seven-point (fasting and 120 min postprandial for each meal and bedtime) capillary blood glucose concentrations were measured in seven of the 12 patients for at least two days, and mean *M*-value was calculated in each patient. *M*-value is a logarithmic transformation of the deviation of blood glucose from a selected standard. We used 5.6 mmol/L (100 mg/dL) as the selected standard. The deviation index (δ) of each individual glucose value (γ) is first calculated as $\delta = (10 \times \log[\gamma/5.0])^3$. *M*-value is the average of all the individually calculated deviation indices ($\sum\delta/n$)[6].

Serum C-peptide levels were measured by CLEIA (LUMIPULSE f, lumipulse C-peptide; Fuji-revio, Tokyo, Japan) and two different EIA, AIA-21 (E test C-peptide; TOSOH, Tokyo, Japan) and ECLusys 2010 (eclusis C-peptide; Roche Diagnostics, Basel, Switzerland) simultaneously by using the same serum samples. Serum C-peptide level was also followed for 9 months by CLEIA, and the maximal value was determined in each patient.

The present study was carried out with patients' approvals and informed consent was obtained from every patient.

Statistical Analysis

The significance of differences between the two groups was evaluated by Pearson's correlation coefficient and *M*-value was evaluated by Mann–Whitney *U*-test. Data are presented as mean ± SD. $P < 0.05$ was considered statistically significant.

RESULTS

Maximal concentrations of serum C-peptide evaluated by CLEIA were 0.02–0.10 nmol/L (0.06–0.29 ng/mL) in four patients and <0.003 nmol/L (0.01 ng/mL) in eight patients.

Serum C-peptide concentrations of the former four patients ranged from 0.007 to 0.10 nmol/L (0.02–0.29 ng/mL) and those of the latter eight patients were always less than 0.003 nmol/L throughout the study period (Figure 1). In contrast, serum C-peptide was detectable in just two patients (0.09–0.10 nmol/L, respectively) by conventional EIA.

Patients who kept more serum C-peptide levels needed significantly fewer daily dosages of insulin ($P < 0.01$; Figure 2). The patients with detectable serum C-peptide levels showed a significantly lower *M*-value (median 2.94, range 0.96–6.15, $n = 4$) than those without detectable serum C-peptide levels (median 16.62, range 5.36–27.10, $n = 3$; $P = 0.01$).

There were no significant correlations between HbA_{1c}, glycoalbumin, BMI, duration of disease and serum C-peptide levels.

DISCUSSION

In the present study, we clarified a significant negative correlation between serum C-peptide levels and daily dosages of insulin. The patients who had more residual endogenous insulin secreting capacity needed fewer daily dosages of insulin injection. In addition, the patients whose serum C-peptide levels were <0.003 nmol/L had a significantly higher *M*-value than the patients whose serum C-peptide levels were 0.007–0.10 nmol/L. These results suggest that even very low endogenous insulin secreting capacity would improve the doses of required insulin and stability of blood glucose controls in patients with fulminant type 1 diabetes. Fukuda *et al.*[7] have reported a negative

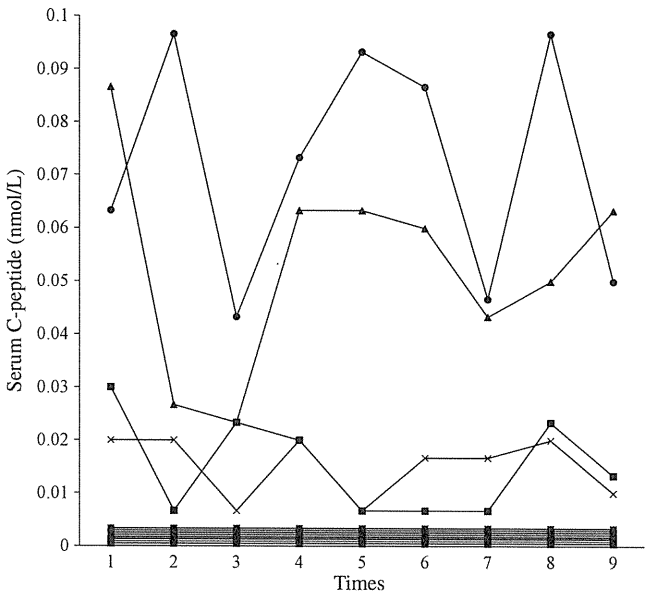

Figure 1 | Transition of serum C-peptide levels during the study period. Detectable serum C-peptide levels in 4 patients (●, ▲, ■, ×) ranged from 0.007 to 0.10 nmol/L, however serum C-peptide levels in eight patients were always <0.003 nmol/L.

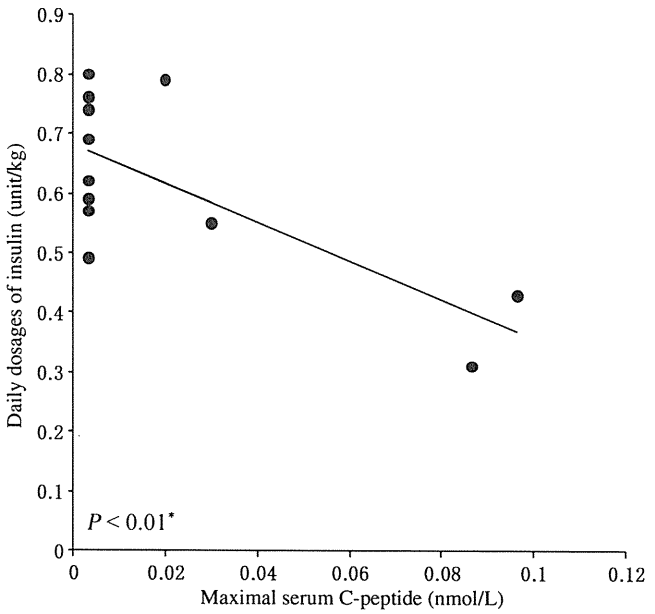

Figure 2 | Relationship between maximal serum C-peptide level (nmol/L) and daily dosages of insulin (unit/kg). Residual insulin secreting capacity shown as serum C-peptide level and daily dosages of insulin indicated a significant negative correlation (*$P < 0.01$).

correlation between endogenous insulin secreting capacity and degree of blood glucose instability in 20 patients with type 1 diabetes whose fasting serum C-peptide levels were 0.01–0.13 nmol/L. In the present study, compared with the report from Fukuda *et al.*, not fasting but maximal postprandial C-peptide levels were 0.02–0.10 nmol/L or less, showing that a negative correlation was observed even in the patients with lower levels of serum C-peptide.

Second, serum C-peptides were detectable and ranged from 0.007 to 0.10 nmol/L in four patients with fulminant type 1 diabetes throughout the follow-up period of 9 months. Pathophysiology of fulminant type 1 diabetes is known as almost complete β-cell destruction around the disease onset, resulting in nearly no C-peptide secretion. However, our results also suggest that endogenous insulin secreting capacity would be preserved by intensive insulin therapy, as shown in data from the Diabetes Control and Complications Trial[8], even in patients with fulminant type 1 diabetes. It is important because preserving β-cell function, even at a low level, could help to stabilize blood glucose levels in patients with fulminant type 1 diabetes.

In conclusion, our present results suggest that even very low levels of serum C-peptide could reduce daily dosages of insulin and stabilize blood glucose controls in fulminant type 1 diabetes.

ACKNOWLEDGEMENTS

We thank Michiyo Horie, Kazuhito Ueda (Central Laboratory, Osaka Medical College Hospital), and Hiroaki Matsumoto and Tetsuya Hiraiwa (First Department of Internal Medicine, Osaka Medical College) for their excellent assistance. This study was supported in part by a Grant-in Aid from the Japanese Society for the Promotion of Science (KAKENHI 19790641, 19591087, 19591069, 21591184) and Labour Sciences Grants on Research on Intractable Diseases by the Ministry of Health, Labour and Welfare. The authors declare that there is no conflict of interest associated with this manuscript.

REFERENCES

1. American Diabetes Association. Diagnosis and classification of diabetes mellitus. *Diabetes Care* 2008; 31: S55–S60.
2. Imagawa A, Hanafusa T, Miyagawa J, *et al.* 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. Imagawa A, Hanafusa T, Uchigata Y, *et al.* Fulminant type 1 diabetes: a nationwide survey in Japan. *Diabetes Care* 2003; 26: 2345–2352.
4. Horie M, Ueda K, Shibasaki S, *et al.* The assessment of low-dose serum C-peptide secretion clarified by chemiluminescent enzyme immunoassay and its clinical effectiveness. *J Japan Diab Soc* 2008; 51: 639–644 (in Japanese).
5. Hanafusa T, Imagawa A. Fulminant type 1 diabetes: a novel clinical entity requiring special attention by all medical practitioners. *Nat Clin Pract Endocrinol Metab* 2007; 3: 36–45.
6. Murase Y, Imagawa A, Hanafusa T, *et al.* Fulminant type 1 diabetes as a high risk group for diabetic microangiopathy – a nationwide 5-year-study in Japan. *Diabetologia* 2007; 50: 531–537.
7. Fukuda M, Tanaka A, Tahara Y, *et al.* Correlation between minimal secretory capacity of pancreatic beta-cells and stability of diabetic control. *Diabetes* 1988; 37: 81–88.
8. The Diabetes Control and Complications Trial Research Group. Effect of intensive therapy on residual beta-cell function in patients with type 1 diabetes in the diabetes control and complications trial. A randomized, controlled trial. *Ann Intern Med* 1998; 128: 517–523.

Correlation of glycated albumin but not hemoglobin A_{1c} with endogenous insulin secretion in fulminant type 1 diabetes mellitus

Masafumi Koga[1]*, Jun Murai[1], Hiroshi Saito[1], Soji Kasayama[2], Tetsuro Kobayashi[3], Akihisa Imagawa[4], Toshiaki Hanafusa[5], the Members of the Japan Diabetes Society's Committee on Research on Type 1 Diabetes†

ABSTRACT

Aims/Introduction: Fulminant type 1 diabetes mellitus (FT1DM) develops as a result of very rapid and almost complete pancreatic β-cell destruction. We hypothesized that in patients with FT1DM who have less endogenous insulin secretion, disease progression is more rapid, and thus glycated albumin (GA) levels are lower. This study was designed to prove this hypothesis.

Materials and Methods: The present study included 42 patients with FT1DM (24 men, 18 women) in whom glycated hemoglobin (HbA_{1c}), GA and daily urinary C-peptide (CPR) were measured at the time of diagnosis. Patients with complications, such as liver disease, kidney disease, anemia, or who were pregnant were excluded.

Results: Urinary CPR (log transformed) was not correlated with HbA_{1c} ($R = 0.168$, $P = 0.287$), but was positively correlated with GA ($R = 0.336$, $P = 0.030$). It was weakly, but not significantly, correlated with GA/HbA_{1c} ratio ($R = 0.281$, $P = 0.072$). In patients with GA < 24.0%, urinary CPR was significantly lower than in patients with GA ≥ 24.0%. In addition, in patients with GA/HbA_{1c} ratio <3.8, urinary CPR was significantly lower than in patients with GA/HbA_{1c} ratio ≥ 3.8.

Conclusions: Our findings suggest that in patients with FT1DM, GA at the time of diagnosis was correlated with endogenous insulin secretion. GA < 24.0% at the time of diagnosis is predictive for less endogenous insulin secretion in patients with FT1DM.

(J Diabetes Invest, doi: 10.1111/j.2040-1124.2010.00050.x, 2010)

KEY WORDS: Glycated albumin, Fulminant type 1 diabetes mellitus, C-peptide

INTRODUCTION

Fulminant type 1 diabetes mellitus (FT1DM) is a novel subtype of type 1 diabetes (T1DM) reported in 2000 by Imagawa *et al.* FT1DM develops as a result of very rapid and almost complete pancreatic β-cell destruction[1,2]. FT1DM has been shown to account for approximately 20% of Japanese patients with T1DM[2]. At the time of onset of FT1DM, plasma glucose (PG) is markedly elevated, in contrast to glycated hemoglobin (HbA_{1c}), which out of proportion to PG, is normal or only slightly elevated. Therefore, the recommended diagnostic criteria for FT1DM include a very rapid onset of diabetic ketoacidosis (DKA) together with low serum or urinary C-peptide (CPR; which reflects decreased endogenous insulin secretion), marked hyperglycemia and a relatively low HbA_{1c} to PG ratio[3,4].

In diabetes, as compared with non-diabetes, glycation of several proteins is known to be increased, thus suggesting that some play a role in the onset and progression of chronic diabetes complications[5]. Among these glycated proteins, HbA_{1c} is widely used clinically as an index of glycemic control[6,7]. Meanwhile, glycated albumin (GA) has also become accepted as another index of glycemic control. Because the half-life of serum albumin is shorter than that of erythrocytes, GA reflects PG levels over a shorter period (approximately 2 weeks)[8,9]. Thus, GA reflects more acute glycemic changes[10]. FT1DM is characterized by acutely elevated PG, so we have shown that whereas HbA_{1c} is only slightly elevated, GA is relatively elevated, reflecting acutely elevated PG[11].

Individual differences are seen in the degree of residual endogenous insulin secretion among FT1DM patients. We hypothesized that in patients with FT1DM who have less endogenous insulin secretion, disease progression is more rapid, and thus levels of HbA_{1c} and GA are lower. The present study was designed to prove this.

[1]Department of Internal Medicine, Kinki Central Hospital, Hyogo, [2]Department of Medicine, Nissay Hospital, [3]Third Department of Internal Medicine, University of Yamanashi School of Medicine, Yamanashi, [4]Department of Metabolic Medicine, Osaka University Graduate School of Medicine, and [5]First Department of Internal Medicine, Osaka Medical College, Osaka, Japan

*Corresponding author. Masafumi Koga Tel.: +81-72-781-3712 Fax: +81-72-779-1567 E-mail address: koga_m@kich.itami.hyogo.jp

†Hiroshi Ikegami, Hideichi Makino, Taro Maruyama, Akira Shimada, Yasuko Uchigata, Hiromi Iwahashi, Ikki Shimizu, Eiji Kawasaki, Kazuma Takahashi, Takuya Awata, Yumiko Kawabata, Masao Nagata, Haruhiko Osawa, Minoru Okubo, Hiroshi Kajio, Hisashi Kamoi, Jo Sato, Shoichiro Tanaka, Koji Nakanishi, Sumie Fujii, Satoshi Murao, and Kinichiro Miura.

Received 1 March 2010; revised 19 April 2010; accepted 4 June 2010

MATERIALS AND METHODS

Patients

Among FT1DM patients identified by a survey research committee or already reported, we enrolled 42 patients in whom HbA_{1c}, GA and daily urinary CPR were measured when FT1DM was diagnosed (24 men, 18 women; mean age 43.1 ± 15.3 years; Table 1). FT1DM was diagnosed based on criteria by a Japanese T1DM Survey Research Committee[3,4]. In some patients with a urinary CPR exceeding 10 μg/day, the diagnosis of FT1DM was based on serum CPR levels (fasting serum CPR < 0.3 ng/mL, or serum CPR < 0.5 ng/mL after glucagon injection or meal load soon after disease onset). Because of possible effects on measured values of HbA_{1c} and GA, patients with complications, such as anemia, liver disease, kidney disease, or who were pregnant were excluded from the present study.

Laboratory Methods

Plasma glucose was determined using the glucose-oxidase method. HbA_{1c} was measured by high performance liquid chromatography (HPLC), with calibration using Japan Diabetes Society (JDS) Lot 2[12]. Serum GA was determined by enzymatic methods using albumin-specific protease, ketoamine oxidase and an albumin assay reagent (Lucica GA-L; Asahi Kasei Pharma, Tokyo, Japan)[13,14]. Glycated albumin was hydrolyzed to amino acids by an albumin-specific proteinase, and then oxidized by ketoamine oxidase to produce hydrogen peroxide, which was measured quantitatively. Glycated albumin level was calculated as the percentage of GA relative to total albumin, which was measured in the same serum sample using a new bromocresol purple method[13].

Statistical Analysis

All data are shown as means ± SD. To correct for skewed distributions, urinary CPR excretion concentrations were logarithmically transformed. In statistical analyses, urinary CPR was assigned at 0.1 μg/day when it was undetectable levels (13 patients). For statistical analysis, the Mann–Whitney *U*-test was used to compare the two groups. To analyze the effects of explanatory variables on urinary CPR, univariate regression analysis was carried out using StatView software (Version 5.0 for Windows, Abacus Concepts, Berkeley, CA, USA). A *P*-value of <0.05 was considered to be statistically significant.

Table 1 | Clinical characteristics of study patients with fulminant type 1 diabetes mellitus

Number	42
Sex (male/female)	24/18
Age	43.1 ± 15.3 years
Body mass index	21.2 ± 2.7 kg/m^2
Plasma glucose	825 ± 327 mg/dL
HbA_{1c}	6.1 ± 0.8%
Glycated albumin	24.1 ± 4.5%
GA/HbA_{1c} ratio	4.0 ± 0.5
Urinary C-peptide	3.1 (<0.1–14) μg/day
Fasting serum C-peptide	0.15 ± 0.16 ng/mL
Duration from onset	3.8 ± 2.4 days

Data represent number, mean ± SD, or median (range).
GA, glycated albumin; HbA_{1c}, glycated hemoglobin.

RESULTS

Urinary CPR (log transformed) was not correlated with HbA_{1c} (R = 0.168, P = 0.287), but was positively correlated with GA (R = 0.336, P = 0.030) in the study patients (Figure 1). Urinary CPR was weakly, but not significantly, correlated with GA/HbA_{1c} ratio (R = 0.281, P = 0.072). In patients with HbA_{1c} < 6.2% (n = 24), compared with those with HbA_{1c} ≥ 6.2% (n = 18), urinary CPR did not significantly differ (2.9 ± 3.2 *vs* 3.9 ± 3.7 μg/day; P = 0.322; Figure 2). By contrast, in patients with GA < 24.0% (n = 24), compared with those with GA ≥ 24.0% (n = 18), urinary CPR was significantly lower (2.3 ± 2.9 *vs* 4.7 ± 3.7 μg/day; P = 0.027). In addition, in patients with GA/HbA_{1c} ratio < 3.8 (n = 12), compared with those with GA/HbA_{1c} ratio ≥ 3.8 (n = 30), urinary CPR was significantly lower (1.4 ± 2.4 *vs* 4.1 ± 3.5 μg/day; P = 0.022; Figure 2).

Fasting serum CPR was not associated with HbA_{1c} (R = 0.253, P = 0.155) and GA (R = 0.247, P = 0.166) in 33 patients. Serum CPR after the glucagon injection was not associated with HbA_{1c} (R = 0.415, P = 0.077) and GA (R = 0.397, P = 0.092) in 19 patients who had glucagon stimulation test.

DISCUSSION

The present study found no correlation between urinary CPR and HbA_{1c} in FT1DM patients, but urinary CPR was significantly correlated with GA and GA/HbA_{1c} ratio. In FT1DM, because of acute destruction of pancreatic β-cells, insulin secretion is depleted, and DKA rapidly develops. The diagnostic criteria for FT1DM include a low (<10 μg/day) urinary CPR, known index of endogenous insulin secretion[3,4], and indeed, most study patients had values <10 μg/day. However, among the patients, the values were distributed over a range from undetectable or very low up to 14 μg/day. We were able to postulate that in patients with undetectable urinary CPR, DKA rapidly develops, whereas in patients with even minimal residual pancreatic β-cell function, the time to onset of DKA is longer.

In contrast to urinary CPR, serum CPR before and after the glucagon stimulation test showed no significant association with HbA_{1c} and GA. These results suggest that urinary CPR rather than serum CPR might more accurately reflect endogenous insulin secretion in FT1DM patients.

It is suggested that GA, which more so than HbA_{1c} reflects acute PG changes, is higher in FT1DM patients in whom time to development of DKA is longer after the onset of hyperglycemic symptoms. In contrast, because HbA_{1c} is only slightly elevated with acute rises in PG, HbA_{1c} values do not reflect well the time to development of DKA. Because GA, compared with

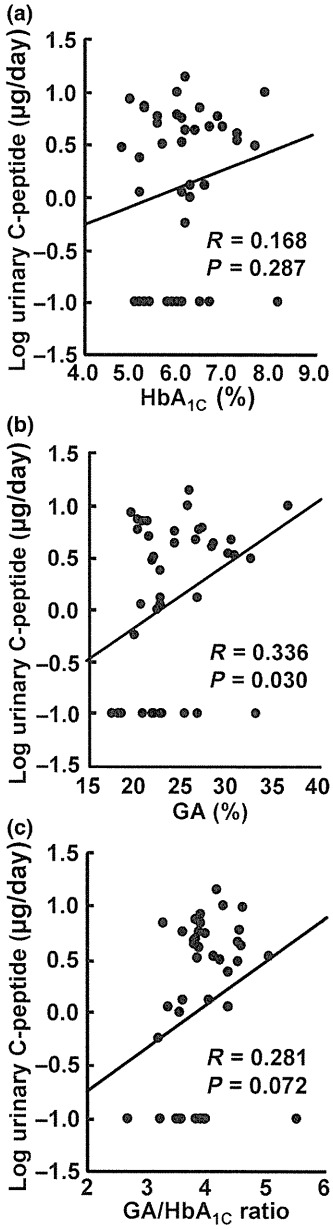

Figure 1 | Correlation between urinary C-peptide (CPR) and (a) glycated hemoglobin (HbA_{1c}), (b) glycated albumin (GA) and (c) GA/HbA_{1c} ratio. Urinary CPR was assigned at 0.1 μg/day when it was undetectable levels.

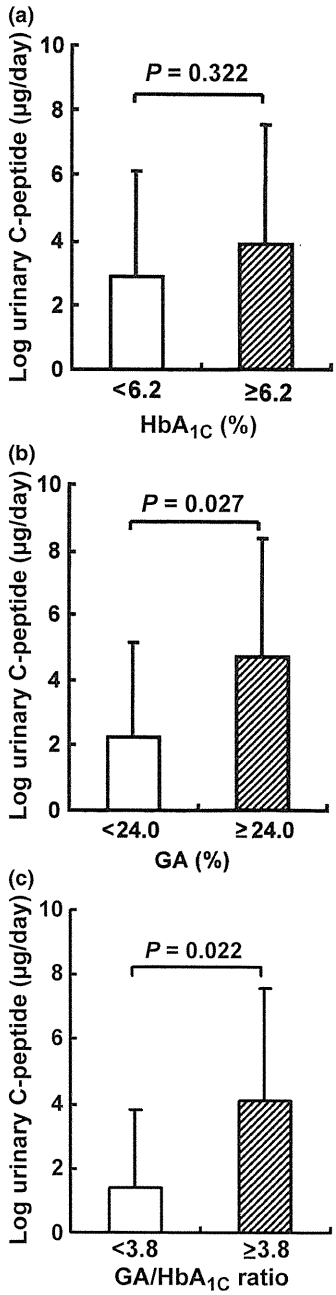

Figure 2 | Comparison of urinary C-peptide between (a) patients with glycated hemoglobin (HbA_{1c}) < 6.2% (n = 24) and patients with HbA_{1c} ≥ 6.2% (n = 18), (b) patients with GA < 24.0% (n = 24) and patients with GA ≥ 24.0% (n = 18), and (c) patients with GA/HbA_{1c} ratio < 3.8 (n = 12) and patients with GA/HbA_{1c} ratio ≥ 3.8 (n = 30).

HbA_{1c}, markedly increases with acute rises in PG, as seen in FT1DM, the GA/HbA_{1c} ratio is high before treatment of FT1DM[11]. Meanwhile, when PG acutely decreases, GA markedly decreases, whereas HbA_{1C} only slightly decreases, so the GA/HbA_{1c} ratio is lower[9]. In the present study, GA was well correlated with urinary CPR in FT1DM patients, suggesting the usefulness of GA as indexes of short-term glycemic changes. On the contrary, HbA_{1c} has been shown to be inadequate to reflect short-term changes in PG in FT1DM patients.

We found significant correlations of GA with urinary CPR. In addition, cut-off values of GA of 24.0% and GA/HbA_{1c} ratio of 3.8 were found to discriminate between urinary CPR levels in the study patients. These data suggest that GA and the GA/HbA_{1c} ratio are set relatively higher with longer disease duration until development of DKA after symptom onset in patients who had relatively higher endogenous insulin secretion. However, we failed to show the correlation of estimated disease duration with GA and urinary CPR (data not shown).

Conversely, the estimated disease duration based on the medical history might be unreliable. FT1DM patients sometimes do not present with typical symptoms, such as thirst, polydipsia and polyuria; they can also present with non-specific symptoms, such as fatigue[15]. Therefore, estimated disease duration diagnosed by physicians might be inaccurate. By referring GA, actual disease duration in FT1DM might be able to be calculated. In the future, the relationship of the disease duration with GA and urinary CPR should be evaluated in FT1DM patients.

In conclusion, our findings suggest that GA at the diagnosis of FT1DM was correlated with patients' endogenous insulin secretion. GA < 24.0% or GA/HbA_{1c} ratio < 3.8 at the time of diagnosis is predictive for less endogenous insulin secretion, and thus might indicate difficulties in obtaining good glycemic control. More intensive diabetes management would be necessary in FT1DM patients with GA < 24.0% or GA/HbA_{1c} ratio < 3.8 at the time of diagnosis.

ACKNOWLEDGEMENT

None of the authors have conflicts of interest to declare.

REFERENCES

1. Imagawa A, Hanafusa T, Miyagawa J, *et al.* 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.
2. Imagawa A, Hanafusa T, Uchigata Y, *et al.* Fulminant type 1 diabetes: a nationwide survey in Japan. *Diabetes Care* 2003; 26: 2345–2352.
3. Hanafusa T, Imagawa A, Iwahashi H, *et al.* Report of Japan Diabetes Society Committee on fulminant type 1 diabetes mellitus research. epidemiological and clinical analysis and proposal of diagnostic criteria. *J Japan Diab Soc* 2005; 48(Suppl. 1): A1–A13. (In Japanese).
4. Imagawa A, Hanafusa T. Pathogenesis of fulminant type 1 diabetes. *Rev Diabet Stud* 2006; 3: 169–177.
5. Cohen MP. Nonenzymatic glycation: a central mechanism in diabetic microvasculopathy? *J Diabet Complications* 1998; 2: 214–217.
6. Koenig RJ, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin Alc in diabetes mellitus. *N Engl J Med* 1976; 295: 417–420.
7. Bunn HF, Gabbay KH, Gallop PM. The glycosylation of hemoglobin: relevance to diabetes mellitus. *Science* 1978; 20: 21–27.
8. Tahara Y, Shima K. Kinetics of HbA1c, glycated albumin, and fructosamine and analysis of their weight functions against preceding plasma glucose level. *Diabetes Care* 1995; 18: 440–447.
9. Takahashi S, Uchino H, Shimizu T, *et al.* Comparison of glycated albumin (GA) and glycated hemoglobin (HbA1c) in type 2 diabetic patients: usefulness of GA for evaluation of short-term changes in glycemic control. *Endocr J* 2007; 54: 139–144.
10. Kohzuma T, Koga M. Lucica GA-L glycated albumin assay kit. *Mol Diagn Ther* 2010; 14: 49–51.
11. Tominaga M, Makino H, Yoshino G, *et al.* Japanese standard reference material for JDS Lot 2 haemoglobin A1c. I: comparison of Japan Diabetes Society-assigned values to those obtained by the Japanese and USA domestic standardization programmes and by the International Federation of Clinical Chemistry reference laboratories. *Ann Clin Biochem* 2005; 42: 41–46.
12. Kouzuma T, Usami T, Yamakoshi M, *et al.* An enzymatic method for the measurement of glycated albumin in biological samples. *Clin Chim Acta* 2002; 324: 61–71.
13. Kouzuma T, Uemastu Y, Usami T, *et al.* Study of glycated amino acid elimination reaction for an improved enzymatic glycated albumin measurement method. *Clin Chim Acta* 2004; 346: 135–143.
14. Koga M, Murai J, Saito H *et al.*. Serum glycated albumin to hemoglobin A_{1C} ratio can distinguish fulminant type 1 diabetes mellitus from type 2 diabetes mellitus. *Ann Clin Biochem* DOI: 10.1258/acb.2010.009234
15. Imagawa A, Hanafusa T. Fulminant type 1 diabetes as an important exception to the new diagnostic criteria using HbA_{1c}-response to the International Expert Committee. *Diabetologia* 2009; 52: 2464–2465.

Clinical Biochemistry 43 (2010) 1265–1267

Contents lists available at ScienceDirect

Clinical Biochemistry

journal homepage: www.elsevier.com/locate/clinbiochem

Case Report

Serum 1,5-anhydroglucitol levels in patients with fulminant type 1 diabetes are lower than those in patients with type 2 diabetes

Masafumi Koga [a,*], Jun Murai [a], Hiroshi Saito [a], Mikio Mukai [a], Soji Kasayama [b], Akihisa Imagawa [c], Toshiaki Hanafusa [d]

[a] Department of Internal Medicine, Kinki Central Hospital, Hyogo, Japan
[b] Department of Medicine, Nissay Hospital, Osaka, Japan
[c] Department of Metabolic Medicine, Osaka University Graduate School of Medicine, Osaka, Japan
[d] First Department of Internal Medicine, Osaka Medical College, Osaka, Japan

ARTICLE INFO

Article history:
Received 30 April 2010
Received in revised form 20 July 2010
Accepted 5 August 2010
Available online 12 August 2010

Keywords:
1,5-AG
Fulminant type 1 diabetes mellitus
HbA1c

ABSTRACT

Objectives: We investigated clinical relevance of serum 1,5-anhydroglucitol (1,5-AG) levels in fulminant type 1 diabetes mellitus (FT1DM) patients, because 1,5-AG is known to reflect short term glycemic control.

Design and methods: Subjects comprised 7 patients with FT1DM and 32 patients with type 2 diabetes mellitus (T2DM) with HbA1c <8.5%. All of them have never been treated for diabetes.

Results: HbA_{1C} showed no significant difference between both groups. On the other hand, serum 1,5-AG levels were significantly lower in the FT1DM patients than in the T2DM patients. Serum 1,5-AG levels were<5.0 μg/ml in 6 of 7 (86%) FT1DM patients, compared with only 1 of 32 (3%) T2DM patients.

Conclusions: Serum 1,5-AG levels were lower in the FT1DM patients than in the T2DM patients. Serum 1,5-AG, but not HbA_{1C}, reflects short-term exacerbation of glycemia in patients with FT1DM.

Introduction

Fulminant type 1 diabetes (FT1DM) is a new subtype of type 1 diabetes first reported in 2000 by Imagawa et al. [1] and characterized clinically by acute and almost complete pancreatic β cell destruction without islet-related autoantibodies such as glutamic acid decarboxylase (GAD) antibody or insulinoma-associated antigen 2 (IA-2) antibody [1,2]. In Japanese patients, FT1DM reportedly accounts for about 20% of all cases of type 1 diabetes with abrupt onset [2]. In contrast to plasma glucose (PG) levels, which are markedly elevated at clinical onset, HbA_{1C} levels are normal or only slightly elevated in FT1DM.

It is known that several types of protein glycation increase in diabetic patients compared with non-diabetic subjects. Some of these proteins play roles in the onset and progression of complications in chronic diabetes [3]. Among these glycated proteins, HbA_{1C} is widely used in clinical practice as a marker of glycemic control [4,5]. Since the average lifespan of erythrocytes is about 120 days, HbA_{1C} level reflects glycemic control state over the past 1–2 months. Glycated albumin (GA), as well as HbA_{1C}, has become used as another marker of glycemic control. Since half-life of albumin is shorter than that of erythrocytes, GA reflects short-term glycemic control (about 2 weeks) [6,7]. We have already suggested that GA/HbA_{1C} ratio is high in FT1DM patients, because HbA_{1C} only slightly elevates but GA markedly elevates due to rapid elevation of plasma glucose in these patients [8].

1,5-anhydroglucitol (1,5-AG) is a novel marker of glycemic control in diabetic patients, because urinary excretion of 1,5-AG increases and serum 1,5-AG decreases in the conditions of increased glucose in urine [9,10]. This is because reabsorption of 1,5-AG at kidney is antagonistic to that of glucose. Serum 1,5-AG levels changes faster than HbA_{1C} at the time of rapid deterioration in glycemic control, and serum 1,5-AG reflects postprandial hyperglycemia more correctly than HbA_{1C} [11]. In this study, we examined whether or not serum 1,5-AG reflects short term exacerbation of glycemia in patients with FT1DM.

Methods

Patients studied

Patients of this study were 7 patients with FT1DM whose HbA_{1C} and serum 1,5-AG were determined simultaneously at their initial visit from case reports. Control group consisted of 32 patients with type 2 diabetes (T2DM) whose HbA_{1C} was lower than 8.5%, because inclusion criteria for FT1DM was HbA_{1C}<8.5% at initial visit [1,2], selected from 558 subjects who underwent health examination at Kinki Central Hospital from July to August 2008 (Table 1). They showed no past history of diabetes treatment. Due to potential effects on HbA_{1C} and serum 1,5-AG values, exclusion criteria for both groups were: pregnancy; renal, hepatic, or hematologic disease; and steroid

* Corresponding author. Department of Internal Medicine, Kinki Central Hospital, Kuruma-zuka 3-1, Itami, Hyogo 664-8533, Japan. Fax: +81 72 779 1567.
E-mail address: koga_m@kich.itami.hyogo.jp (M. Koga).

0009-9120/$ – see front matter
doi:10.1016/j.clinbiochem.2010.08.001

Table 1
Clinical characteristics of FT1DM and T2DM.

	FT1DM	T2DM	*p*
n	7	32	
M/F	5/2	23/9	0.981
Age (years)	40.1 ± 13.6	56.0 ± 5.2	<0.0001
BMI (kg/m^2)	20.9 ± 0.5	24.8 ± 3.9	0.0344
PG (mg/dl)	945 ± 297[a]	114 ± 11[b]	–
PG (mmol/l)	52.5 ± 16.5[a]	6.3 ± 0.6[b]	–
HbA_{1C} (%)	5.9 ± 0.7	6.0 ± 0.5	0.7316
1,5-AG (μg/ml)	3.3 ± 1.4	13.5 ± 5.4	<0.0001
Duration from onset (day)	2.0 ± 1.3	–	

[a] Randomly measured plasma glucose.
[b] Fasting plasma glucose.

therapy. Diagnosis of FT1DM and T2DM was based on previously reported criteria. The institutional review board approved this study, and all T2DM patients as control group provided written informed consent.

Laboratory methods

Plasma glucose was determined using the glucose-oxidase method. HbA_{1C} was measured by high performance liquid chromatography (HPLC), with calibration using Japan Diabetes Society (JDS) Lot 2 [12]. Serum 1,5-AG was measured using Lana 1,5AG Auto Liquid Kit using an enzymatic method (Nippon Kayaku, Tokyo, Japan), as described previously [13]. Intra- and inter-assay coefficients of variation for 1,5-AG measurement were 1.7% and 1.2%, respectively.

Statistical analysis

Data are shown as means ± SD. For statistical analyses, unpaired Student's *t*-test was used to compare two groups. To evaluate the relationship between HbA_{1C} and 1,5-AG levels, single linear univariate regression analyses was performed. The Stat View computer program (Version 5.0 for Windows; Abacus Concepts and Berkeley, CA) was used for all statistical analyses. A p value of <0.05 was considered statistically significant.

Results

Table 1 shows clinical characteristics of the study patients. Mean age was significantly younger in FT1DM patients (40.1 ± 13.6 years) than in T2DM patients (56.0 ± 5.2 years; $p<0.0001$). Mean body mass index (BMI) was significantly lower in FT1DM patients (20.9 ± 0.5 kg/m^2) than in T2DM patients (24.8 ± 3.9 kg/m^2; $p=0.0344$). In the FT1DM patients, mean duration from the onset of hyperglycemic symptoms to the start of insulin therapy was 2.0 ± 1.3 days.

In patients with FT1DM at initial diagnosis, randomly measured PG was markedly elevated [945 ± 297 mg/dl (52.5 ± 16.5 mmol/l); range, 509–1195 mg/dl (28.3–66.4 mmol/l)]. In patients with T2DM, fasting PG was 114 ± 11 mg/dl (6.3 ± 0.6 mmol/l) [range, 95–136 mg/dl (5.3–7.6 mmol/l)]. HbA_{1C} showed no significant difference between the groups (5.9 ± 0.7% vs. 6.0 ± 0.5%; $p=0.7316$). On the other hand, serum 1,5-AG levels in the FT1DM patients was significantly lower than that in the T2DM patients (3.3 ± 1.4 μg/ml vs. 13.5 ± 5.4 μg/ml; $p<0.0001$) (Table 1).

In the T2DM patients, there was significant inverse correlation between HbA_{1C} and serum 1,5-AG ($R=-0.621$, $p=0.0002$) (Fig. 1). On the other hand, serum 1,5-AG levels in the FT1DM patients were remarkably low regardless of HbA_{1C} levels. Serum 1,5-AG levels were<5.0 μg/ml in 6 of 7 (86%) FT1DM patients, compared with only 1 of 32 (3%) T2DM patients (Fig. 1).

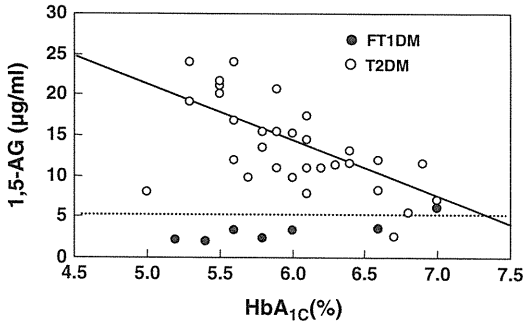

Fig. 1. Relationship between serum 1,5-AG and HbA_{1C}. The figure shows relationship between serum 1,5-AG and HbA_{1C} in the treatment-naive FT1DM patients (closed circle) and the T2DM patients (open circle). The dotted line represents serum 1,5-AG 5.0 μg/ml. The solid line represents the regression line of serum 1,5-AG and HbA_{1C} in the T2DM patients.

Discussion

The diagnosis of FT1DM is clinically important, as initiation of insulin treatment is crucial to avoiding abrupt deterioration and poor prognosis [1,2]. It has been reported that about 10% of FT1DM patients showed randomly measured PG levels ≤ 400 mg/dl (22.2 mmol/l) [14]. By contrast, randomly measured PG in patients with T2DM sometimes exceeds 288 mg/dl (16 mmol/l), which is a diagnostic level for FT1DM. In these cases, FT1DM is not easily distinguishable from T2DM. Clinical indicators other than PG levels are thus needed for differentiating FT1DM from T2DM.

When plasma glucose exceeds threshold of reabsorption at kidney, urinary glucose not reabsorbed suppresses reabsorption of 1,5-AG via sodium glucose cotransporter 4 (SGLT4) at proximal tubules of kidney [9,10]. When glycemic control is improved and urine glucose disappears, the glucose-dependent suppression of 1,5-AG reabsorption disappears, and most of 1,5-AG can be reabsorbed at renal tubules. It is known that pooled serum 1,5-AG is elevated by 0.3 μg/ml/day with 1,5-AG taken from meal [15]. When glycemic control state is poor, urine glucose prevents reabsorption of 1,5-AG, resulting in rapid decrease of serum 1,5-AG. This decrease occurs faster than its increase during improvement of glycemic control, as above [16].

Based on these facts, we investigated whether or not serum 1,5-AG reflects short term exacerbation of glycemia in patients with FT1DM. As far as we have searched, this study is the first report on serum 1,5-AG in FT1DM patients. As a result, serum 1,5-AG levels were lower than the reference level (< 14.0 μg/ml) in all FT1DM patients at initial visit, and markedly low in most of them. When 5.0 μg/ml was used as a cut-off value of serum 1,5-AG, sensitivity was 86% and specificity was 97%, for diagnosis of FT1DM. This finding indicates that serum 1,5-AG, but not HbA_{1C}, reflects short term exacerbation of glycemia in patients with FT1DM.

GA, another glycemic control marker, is known to reflect acute short term changes in plasma glucose, similar to 1,5-AG [6,7]. The rate of change is almost similar for 1,5-AG and GA, when glycemic control is improved. As mentioned, serum 1,5-AG is more sensitive to deterioration than to improvement of glycemic control. Serum 1,5-AG changes faster than GA in FT1DM patients in which plasma glucose elevates rapidly. Thus, it is serum 1,5-AG can reflects shorter term of hyperglycemia than GA in patients with FT1DM. We have already reported that measurement of GA is useful to discern FT1DM from T2DM. When 3.2 is used as a cut-off value of GA/HbA_{1C} ratio, sensitivity was 97% and specificity was 98% for diagnosis of FT1DM [8]. In the present study, sensitivity of serum 1,5-AG was slightly lower than that of GA, contrary to our expectation. The limited number of patients included in this study (7 cases) might have affected the result. It is necessary to examine the utility of 1,5-AG in identifying the

presence of short-term exacerbation of glycemia in patients with FT1DM within an extended number of subjects.

Conflicts of interest statement

None of the authors have conflicts of interest to declare.

References

[1] 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. N Engl J Med 2000;342:301–7.
[2] Imagawa A, Hanafusa T, Uchigata Y, et al. Fulminant type 1 diabetes: a nationwide survey in Japan. Diab Care 2003;26:2345–52.
[3] Cohen MP. Nonenzymatic glycation: a central mechanism in diabetic microvasculopathy? J Diabet Complications 1998;2:214–7.
[4] Koenig RJ, Peterson CM, Jones RL, Saudek C, Lehrman M, Cerami A. Correlation of glucose regulation and hemoglobin A1c in diabetes mellitus. N Engl J Med 1976;295:417–20.
[5] Bunn HF, Gabbay KH, Gallop PM. The glycosylation of hemoglobin: relevance to diabetes mellitus. Science 1978;20:21–7.
[6] Tahara Y, Shima K. Kinetics of HbA1c, glycated albumin, and fructosamine and analysis of their weight functions against preceding plasma glucose level. Diab Care 1995;18:440–7.
[7] Takahashi S, Uchino H, Shimizu T, et al. Comparison of glycated albumin (GA) and glycated hemoglobin (HbA1c) in type 2 diabetic patients: usefulness of GA for evaluation of short-term changes in glycemic control. Endocr J 2007;54:139–44.
[8] Koga M, Murai J, Saito H, et al. Serum glycated albumin to hemoglobin A1C ratio is a suitable index for diagnosis of fulminant type 1 diabetes mellitus. Ann Clin Biochem 2010;47:313–7.
[9] Yamanouchi T, Tachibana Y, Sekino N, et al. Origin and disposal of 1, 5-anhydroglucitol, a major polyol in the human body. Am J Physiol 1992;263:E268–73.
[10] Yamanouchi T, Akanuma H, Nakamura T, Akaoka I, Akanuma Y. Reduction of plasma 1, 5-anhydroglucitol (1-deoxy-glucose) concentration in diabetic patients. Diabetologia 1988;31:41–5.
[11] Yamanouchi T, Ogata N, Tagaya T, et al. Clinical usefulness of serum 1, 5-anhydroglucitol in monitoring glycaemic control. Lancet 1996;347:1514–8.
[12] Tominaga M, Makino H, Yoshino G, et al. Japanese standard reference material for JDS Lot 2 haemoglobin A1c. I: Comparison of Japan Diabetes Society-assigned values to those obtained by the Japanese and USA domestic standardization programmes and by the International Federation of Clinical Chemistry reference laboratories. Ann Clin Biochem 2005;42:41–6.
[13] Koga M, Murai J, Saito H, et al. Close relationship between serum concentrations of 1, 5-anhydroglucitol and uric acid in non-diabetic male subjects implies common renal transport system. Clin Chim Acta 2009;410:70–3.
[14] Hanafusa T, Imagawa A, Iwahashi H, et al. Epidemiological and clinical analysis and proposal of diagnostic criteria. J Jpn Diab Soc 2005;48(1):A1–A13 (In Japanese).
[15] Yamanouchi T, Akanuma H, Asano T, Konishi C, Akaoka I, Akanuma Y. Reduction and recovery of plasma 1, 5-anhydro-D-glucitol level in diabetes mellitus. Diabetes 1987;36:709–15.
[16] Yamanouchi T, Akanuma Y. Serum 1, 5-anhydroglucitol (1, 5-AG): new clinical marker for glycemic control. Diabetes Res Clin Pract 1994;24:S261–8.

Endocrine Journal 2010, **57** (3), 211-219

ORIGINAL

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

SAEKO SHIBASAKI [1], AKIHISA IMAGAWA [1),2)], SISKO TAURIAINEN [3)], MORIO IINO [4)], MAARIT OIKARINEN [3)], HITOSHI ABIRU [4)], KEIJI TAMAKI [4)], HIROAKI SEINO [5)], KATSUHIRO NISHI [6)], IZUMI TAKASE [6)], YOSHIKATSU OKADA [7)], SAE UNO [2)], YUKO MURASE-MISHIBA [1)], JUNGO TERASAKI [1)], HIDEICHI MAKINO [8)], IICHIRO SHIMOMURA [2)], HEIKKI HYÖTY [3),9)] AND TOSHIAKI HANAFUSA [1)]

[1)] *First Department of Internal Medicine, Osaka Medical College, Takatsuki 569-8686, Japan*
[2)] *Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, Suita 565-0871, Japan*
[3)] *Department of Virology, Medical School, University of Tampere, FIN-33520 Tampere, Finland*
[4)] *Department of Forensic Medicine and Molecular Pathology, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan*
[5)] *Diabetic Center, Ota-Nisinouchi Hospital, Koriyama 963-8558, Japan*
[6)] *Department of Legal Medicine, Shiga University of Medical Science, Otsu 520-2192, Japan*
[7)] *Department of Pathology, Osaka Medical College, Takatsuki 569-8686, Japan*
[8)] *Diabetes Research Center, Takanoko Hospital, Matsuyama 790-0925, Japan*
[9)] *Department of Microbiology, Center for Laboratory Medicine, Tampere University Hospital, FIN-33521 Tampere, Finland*

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

Key words: Insulitis, Toll-like receptor, Enterovirus

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

A nationwide survey identified that this variant accounts for approximately 20 % of acute-onset type 1 diabetes cases in Japan [3]. Recently, 30.4 % of adult-onset type 1 diabetes was classified as fulminant type 1 diabetes in Korea according to the Japanese crite-

Received Sep. 30, 2009; Accepted Nov. 30, 2009 as K09E-291
Released online in J-STAGE as advance publication Dec.12, 2009
Correspondence to: Akihisa IMAGAWA M.D., Ph.D., FACP. Department of Metabolic Medicine, Graduate School of Medicine, Osaka University, 2-2-B5, Yamadaoka, Suita 565-0871, Japan.
E-mail: aimagawa@endmet.med.osaka-u.ac.jp

Table 1. Patients' characteristics

	Age(yr)/Sex	Disease duration (days)*	Blood glucose (mg/dL)	HbA_{1c} (%)	Serum C-peptide (ng/mL)	GADAb/ IA-2Ab/ ICA	*HLA DRB1- DQB1*
1	29/Male	3+3	1585	5.8	undetectable	(-)/(-)/(-)	*0502**/-*
2	35/Male	3+0	1300	7.5	0.08	(-)/(-)/(-)	*0405-0401/1405-0503*
3	47/Male	5+0	660	6.2	0.39	(-)/(-)/(-)	*0404-0402/0802-0302*

*Disease duration refers to the period with hyperglycemic symptoms before the diagnosis of diabetes plus that with the treatment for diabetes. Patient 2 and 3 were diagnosed as having diabetes after death. ***DRB1* not available.

ria [5]. Several cases have been reported, not only in other Asian populations, but also in Caucasian populations [6, 7].

The destruction of beta cells was confirmed by virtually no C-peptide secretion in fulminant type 1 diabetes; however, the mechanism of beta cell death is largely unknown. For example, it remains controversial whether insulitis, mononuclear cell infiltration to the pancreatic islets, is characteristics of fulminant type 1 diabetes [3, 9, 10]. In addition, fulminant type 1 diabetes is different from classical type 1A diabetes with respects to the human leukocyte antigen (HLA)-DR/DQ and cytotoxic T lymphocyte antigen (CTLA)-4 gene polymorphisms [11, 12].

The possible involvement of viral infections has been suggested in the pathogenesis of fulminant type 1 diabetes. Flu-like symptoms are common and were exhibited just before the onset of overt diabetes in 71.2 % of patients diagnosed with fulminant type 1 diabetes [4]. IgA antibody titres to enterovirus common antigen were significantly higher in patients with recent-onset fulminant type 1 diabetes than in those with type 1A diabetes and in controls [13]. Finally, several cases have been reported in which the onset of diabetes was accompanied by a reactivation or an infection of human herpesvirus-6 and -7, herpes simplex virus, coxsackie A4, A5, A6 and B3 viruses, influenza B virus, mumps virus, rotavirus, Epstein-Barr virus and cytomegalovirus in fulminant type 1 diabetes [4, 6, 14-16]. However, there has been no direct evidence so far, for the pathogenic role of viruses in beta cell destruction.

To clarify the pathogenesis of fulminant type 1 diabetes with special reference to insulitis and viral infection, we investigated pancreatic autopsy specimens obtained from the patients who died soon after the disease onset.

Materials and Methods

Patients and samples

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

In all three patients, postmortem dissections were performed, and we examined the pancreatic tissues. Patient 1 was diagnosed with diabetic ketoacidosis and died after 3 days of treatment. Patients 2 and 3 were diagnosed with diabetes after death. The direct causes of death of patient 2 and 3 were unknown at the time of autopsies. They ranged in age from 29 to 47 years, and the duration of disease was 3 to 6 days. Their blood glucose, HbA_{1c} and serum C-peptide levels were 660 to 1585 mg/dL, 5.8 to 7.5 % and less than 0.39 ng/mL, respectively. GAD_{65} antibody, IA-2 antibody and islet cell antibodies (ICA) were negative in all three patients. Patient 2 and 3 possessed HLADR4-DQ4 haplotype that is susceptible to fulminant type 1 diabetes (Table 1) [4]. The pancreatic tissues were fixed in 10 % formalin and embedded in paraffin. The normal pancreatic tissues of six individuals were examined as non-diabetic control samples. These individuals were free from pancreatic diseases.

Immunohistochemistry and morphometry

Formalin-fixed paraffin embedded pancreatic sections cut at a thickness of 4 μm were deparaffinized and rehydrated using xylene and graded descending series of alcohol. After washing in distilled water once for 5 min, the slides only for natural killer (NK) cells were exposed to microwave pretreatment in a target retrieval solution (Dako Japan, Kyoto, Japan) at 100 °C for 15 min to enhance antigenisity. Endogenous

peroxidase activity was blocked for all sections using ice-cold 3 % H_2O_2/methanol for 30 min. All slides were incubated for 30 min in 10 % normal serum. The slides were then incubated at room temperature for 1 hour with guinea pig anti-insulin antibody (1:1000; Dako Japan), rabbit anti-glucagon antibody (1:1000; Linco Research, Ellisville, MO, USA), rabbit anti-human CD3 antibody (1:100, Dako Japan), monoclonal mouse anti-human CD68 antibody (1:100, KP1, Dako Japan), monoclonal mouse anti-human CD56 antibody (1:100, SNCL-CD56-1B6, Novocastra, Newcastle, UK), goat anti-human Toll-like receptor (TLR) 3 antibody (1:200, Santa Cruz Biotechnology, Santa Cruz, CA, USA), goat anti-human TLR7 antibody (1:100, Santa Cruz) and monoclonal mouse anti-human TLR9 antibody (1:200, 26C593, Imgenex, San Diego, CA, USA). As a negative control for TLR9 antibody, the same concentration of mouse IgG1 (Dako, Japan) was applied as a primary antibody. As negative controls for TLR3 and TLR7, those antibodies incubated with 10-fold higher concentration of TLR3 and TLR7 blocking peptides (Santa Cruz) were applied. Then slides were incubated with secondary antibodies; mouse or rabbit Envision kit/HRP (Dako Japan), Elite ABC mouse or goat IgG kit (Vector Laboratories, Burlingame, CA, USA), following the manufacturers' instructions. Finally, antibody binding was detected using 3', 5'-diaminobenzidine (DAB) (Dako Japan). All the washes were performed in PBS (pH 7.4). Sections were counterstained with methyl green or hematoxylin and were mounted in oil mounting medium (Mount-quick, Tokyo, Japan) before microscopy (BH-2, Olympus, Tokyo, Japan).

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

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

In situ hybridization

The used method was modified from a previously published method [18, 19] and has been described in detail by Oikarinen *et al.* [20]. An enterovirus-specific oligonucleotide probe designed to hybridize with the conserved 5' non-coding sequence was used (sequence from 5' to 3'GAA ACA CGG ACA CCC AAA GTA GTC GGT TCC GCT GCR GAG TTR CCC RTT ACG ACA) to detect all known enterovirus types. The probe was 3'end –labelled with digoxigenin using a kit (DIG oligonucleotide tailing kit; Roche Diagnostics, Welwyn Garden City, UK). A 10 pmol sample of the probe was used for one labelling reaction. Hybridization was performed using earlier published conditions [20]. The amount of probe in the hybridization cocktail was 250 ng, the hybridization time was 3 hour. Binding of the probes was revealed by anti-digoxigenin antibody, which was conjugated with alkaline phosphatase. This enzyme together with

Table 2. Results of the histological analysis

	Beta cell area	Alpha cell area	CD3+ insulitis	CD68+ insulitis	TLR3+/ CD3+ cells	TLR3+/ CD68+ cells
Fulminant						
No. 1	0.00259	0.0153	79.4	93.5	90.0	78.0
No. 2	0.00412	0.0430	78.9	94.7	27.0	92.0
No. 3	0.000970	0.0252	48.0	89.6	71.0	84.0
Mean±SD	0.00256±0.00158*	0.0278±0.0140*	68.8±18.0*	92.6±2.7*	62.7±32.3	84.7±7.0
Control (n=6)						
Mean±SD	1.745±0.336	0.266±0.049	2.8±1.9	1.3±1.2		

Islet cell areas are shown in percentage of insulin- and glucagon-positive cell area in total observed area. The results of insulitis and TLR3+ cells are shown in percentage. *$P<0.001$ *vs.* controls

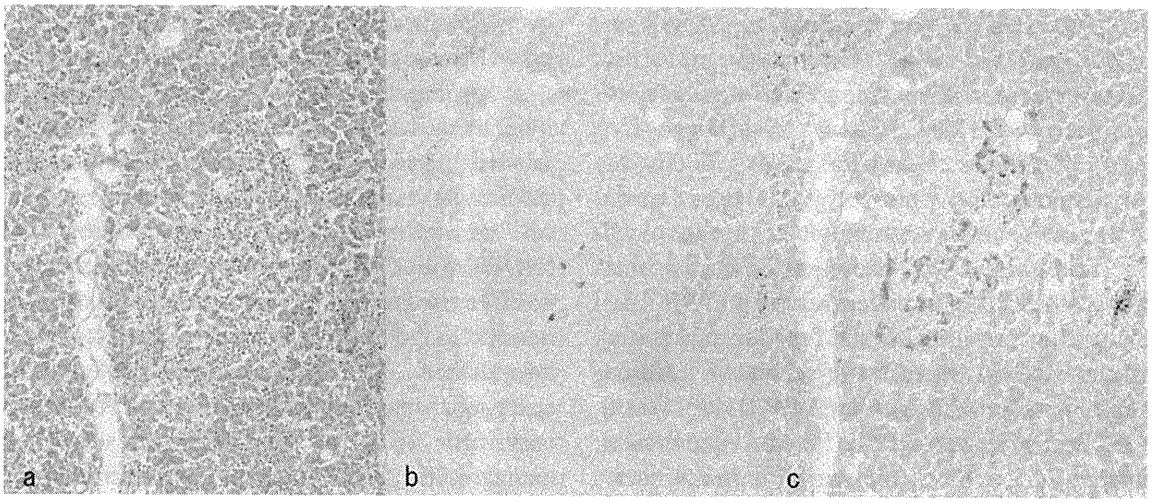

Fig. 1. Microphotographs of consecutive sections stained with H&E (a) and antibodies to insulin (b) and glucagon (c) in case 1 as a representative. Massive mononuclear cell infiltration was detected in and around the islet as well as in exocrine tissue by H&E stain. Decreased numbers of insulin+ cells and glucagon+ cells were seen in the islet. Original magnification: x300.

its substrate, nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate yields an insoluble purple precipitate, which can be detected using a light microscope. Enterovirus-infected and mock-infected green monkey kidney cells were used as controls.

Statistical analysis

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

Results

Insulin- and glucagon-positive cell regions

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

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

We detected the infiltration of CD3+ cells and CD68+ cells in and around the islets as well as in the exocrine pancreas in all patients with fulminant type 1 diabetes. Insulitis were seen both in insulin-positive islets and insulin-negative islets (Figure 1, 2). CD56+ cells were not detected in mononuclear cells at all. Insulitis infiltrated by CD3+ cells was observed in 68.8±18.0 % (mean±SD) of islets examined in fulminant type 1 diabetes but only in 2.8±1.9 % in control subjects ($P<0.001$). Insulitis infiltrated by CD68+

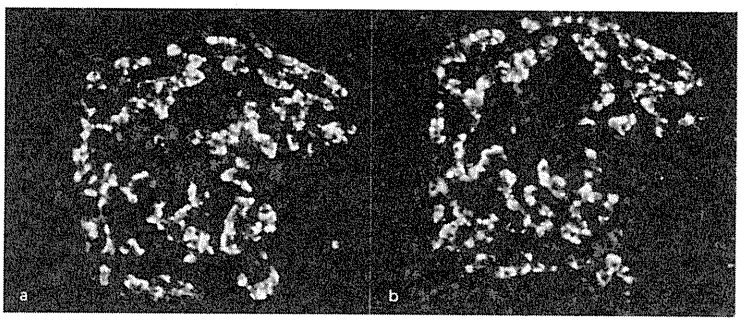

Fig. 2. Microphotographs of double staining sections for CD3/CD68 and glucagon in case 1 as a representative. CD3+ cells (red; a) and CD68+ cells (red; b) are infiltrating in and around the islet (green represents glucagon). Original magnification: x500.

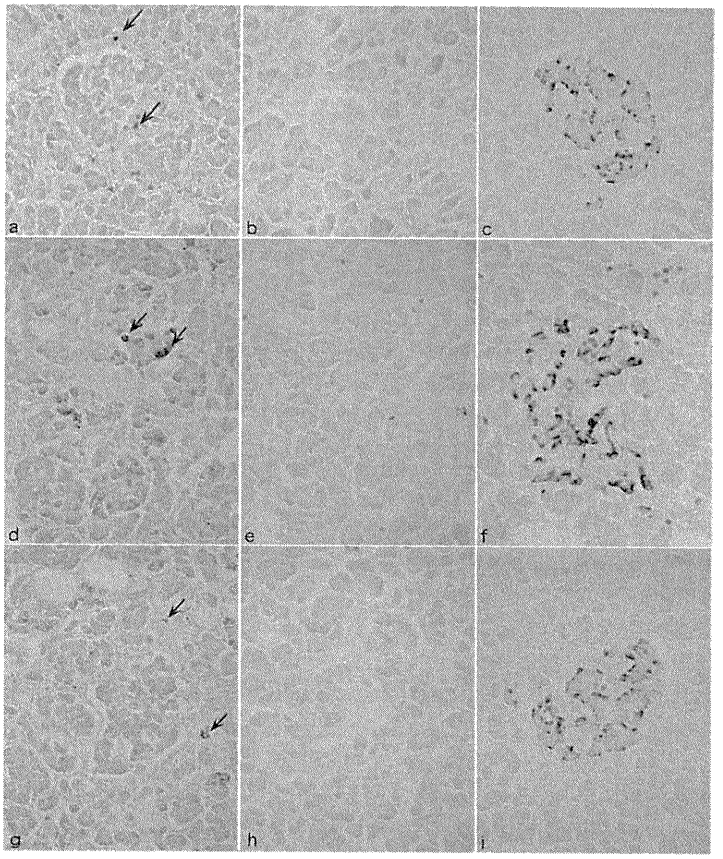

Fig. 3. Microphotographs of consecutive sections stained with antibodies to TLRs and controls for each other. The expressions of TLR3, its negative control and glucagon for patient 2 (a-c), TLR7, its negative control and glucagon for patient 3 (d-f), TLR9, its negative control and glucagon for patient 3 (g-i) in the consecutive sections are shown. Glucagon staining indicates the islet area. TLR3, TLR7 and TLR9 were expressed in mononuclear cells located both inside and outside an islet. Arrows (a, d, g) represent positive cells. Original magnification: x 200.

cells was observed in 92.6±2.7 % in fulminant type 1 diabetes but only in 1.3±1.2 % in control subjects (*P<0.001*) (Table 2).

Expression of TLRs

We detected the expression of TLR3, TLR7 and TLR9 in all patients with fulminant type 1 diabetes (Figure 3). Double staining method revealed that TLR3 was positive in 84.7±7.0 % of macrophages and 62.7±32.3 % of T cells (Table 2). TLR3-positive macrophages and T cells were detected both in the islets and exocrine areas (Figure 4). No positive cells were

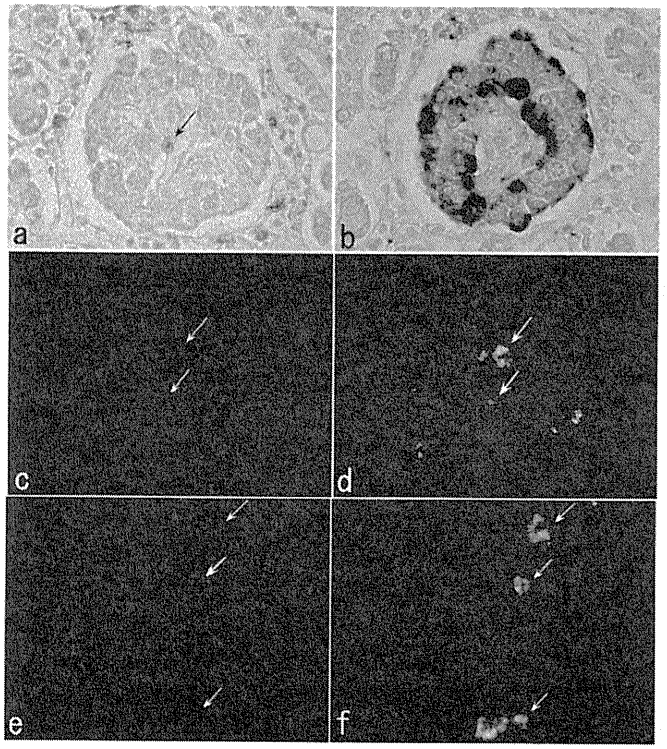

Fig. 4. Microphotographs of consecutive sections stained with antibodies to insulin (a), glucagon (b), CD3 (red; c), TLR3 (green, d), CD68 (red; e) and TLR3 (green; f) in case 2. Microphotographs of (c)(d) and (e)(f) are the same sections and visualized by a double staining method. Insulin+ and glucagon+ cells are detected respectively (a, b). The expression of TLR3 was detected in islet-infiltrating T cells and macrophages. Arrows (a), (c, d) and (e, f) represent insulin+ cell, TLR3+/CD3+ cells and TLR3+/CD68+ cells respectively. Original magnification: x 600.

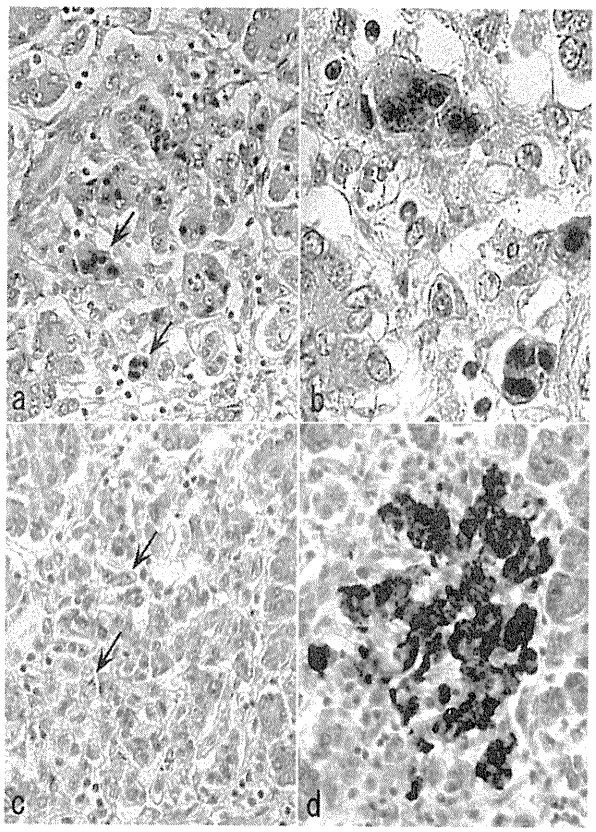

Fig. 5. Microphotographs of consecutive sections of enterovirus RNA (a, b), insulin (c) and glucagon (d) in Case 1. Enterovirus RNA was detected in the islets with insulin of case 1 but not in cases 2 and 3. No enterovirus RNA was detected in the exocrine pancreas. Arrows (a) (c) represent positive cells. Original magnification: x 500 (a, c, d), x 1500 (b).

detected in the control slides for any of the proteins TLR3, TLR7 or TLR9.

Expression of enterovirus RNA

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

Discussion

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

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

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

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

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

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

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

Acknowledgements

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

References

1. American Diabetes Association (2008) Diagnosis and classification of diabetes mellitus. *Diabetes Care* 31: S55-60.
2. Imagawa A, Hanafusa T, Miyagawa J, Matsuzawa Y for the Osaka IDDM Study Group. (2000) A novel subtype of type 1 diabetes mellitus characterized by a rapid onset and an absence of diabetes-related antibodies. *N Engl J Med* 342: 301-307.
3. Imagawa A, Hanafusa T, Uchigata Y, Kanatsuka A, Kawasaki E, Kobayashi T, Shimada A, Shimizu I, Toyoda T, Maruyama T, Makino H (2003) Fulminant type 1 diabetes: a nationwide survey in Japan. *Diabetes Care* 26: 2345-2352.
4. Hanafusa T, Imagawa A (2007) Fulminant type 1 diabetes: a novel clinical entity requiring special attention by all medical practitioners. *Nat Clin Pract Endocrinol Metab* 3: 36-45.
5. Cho YM, Kim JT, Ko KS, Koo BK, Yang SW, Park MH, Lee HK, Park KS (2007) Fulminant type 1 diabetes in Korea: high prevalence among patients with adult-onset type 1 diabetes. *Diabetologia* 50: 2276-2279.
6. Chiou CC, Chung WH, Hung SI, Yang LC, Hong HS (2006) Fulminant type 1 diabetes mellitus caused by drug hypersensitivity syndrome with human herpesvirus 6 infection. *J Am Acad Dermatol* 54: S14-S17.
7. Moreau C, Drui D, Arnault-Ouary G, Charbonnel B, Chaillous L, Cariou B (2008) Fulminant type 1 diabetes in Caucasians: a report of three cases. *Diabetes Metab* 34: 529-532.
8. Sayama K, Imagawa A, Okita K, Uno S, Moriwaki M, Kozawa J, Iwahashi H, Yamagata K, Tamura S, Matsuzawa Y, Hanafusa T, Miyagawa J, Shimomura I (2005) Pancreatic beta and alpha cells are both decreased in patients with fulminant type 1 diabetes: a morphometrical assessment. *Diabetologia* 48: 1560-1564.
9. Tanaka S, Kobayashi T, Momotsu T (2000) A novel subtype of type 1 diabetes mellitus. *N Engl J Med* 342: 1835-1838.
10. Yamazaki M, Hayashi T (2002) Rapid-onset type 1 diabetes mellitus without pancreatic exocrine dysfunction. *Ann Intern Med* 137: 145-146.
11. Imagawa A, Hanafusa T, Uchigata Y, kanatsuka A, Kawasaki E, Kobayashi T, Shimada A, Shimizu I, Maruyama T, Makino H (2005) Different contribution of class II HLA in fulminant and typical autoimmune type 1 diabetes mellitus. *Diabetologia* 48: 294-300. *Erratum in: Diabetologia* (2008) 51: 524-526.
12. Kawasaki E, Imagawa A, Makino H, Uga M, Abiru N, Hanafusa T, Uchigata Y, Eguchi K (2008) Differences in the contribution of CTLA4 gene to susceptibility to fulminant and type 1A diabetes in Japanese patients. *Diabetes Care* 31: 1608-1610.
13. Imagawa A. Hanafusa T, Makino H, Miyagawa J, Juto P (2005) High titres of IgA antibodies to enterovirus in fulminant type-1 diabetes. *Diabetologia* 48: 290-293.
14. Sano H, Terasaki J, Tsutsumi C, Imagawa A, Hanafusa T (2008) A case of fulminant type 1 diabetes mellitus after influenza B infection. *Diabetes Res Clin Pract* 79: e8-9.
15. Goto A, Takahashi Y, Kishimoto M, Nakajima Y, Nakanishi K, Kajio H, Noda M (2008) A case of fulminant type 1 diabetes associated with significant elevation of mumps titers. *Endocr J* 55: 561-564.
16. Hanafusa T, Imagawa A, Iwahashi H, Uchigata Y, Kanatsuka A, Kawasaki E, Kobayashi T, Shimada A, Shimizu I, Maruyama T, Makino H (2008) Report of the Japan Diabetes Society's committee on research on fulminant type 1 diabetes mellitus: Analysis of antiviral antibodies at disease onset. *J Japan Diab Soc* 51 :531-536.
17. Itoh N, Hanafusa T, Miyazaki A, Miyagawa J, Yamagata K, Yamamoto K, Waguri M, Imagawa A, Tamura S, Inada M, Kawata S, Tarui S, Kono N, Matsuzawa Y (1993) Mononuclear cell infiltration and its relation to the expression of major histocompatibility complex antigens and adhesion molecules in pancreas biopsy specimens from newly diagnosed insulin-dependent diabetes mellitus patients. *J Clin Invest* 92: 2313-2322.
18. Oikarinen M, Tauriainen S, Honkanen T, Oikarinen S, Vuori K, Kaukinen K, Rantala I, Mäki M, Hyöty H (2008) Detection of enteroviruses in the intestine of type 1 diabetic patients. *Clin Exp Immunol* 151: 71-75.
19. Oikarinen M, Tauriainen S, Honkanen T, Vuori K, Karhunen P, Vasama-Nolvi C, Oikarinen S, Verbeke C, Blair GE, Rantala I, Ilonen J, Simell O, Knip M, Hyöty H (2008) Analysis of pancreas tissue in a child positive for islet cell antibodies. *Diabetologia* 51: 1796-1802.
20. Hohenadl, C, Klingel, K, Mertsching, J, Hofschneider PH, Kandolf R (1991) Strand-specific detection of enteroviral RNA in myocardial tissue by in situ hybridization. *Mol Cell Probes* 5: 11-20.
21. Ylipaasto P, Kutlu B, Rasilainen S, Passchaert J, Salmera K, Teerijoki H, Korsgren O, Lahesmaa R, Hovi T, Eizirik DL, Otonkoski T, Roivainen M (2005) Global profiling of coxsackievirus- and cytokine-induced gene expression in human pancreatic islets *Diabetologia* 48: 1510-1522.
22. Hultcrantz M, Hühn MH, Wolf M, Olsson A, Jacobson S, Williams BR, Korsgren O, Flodström-Tullberg M (2007) Interferons induce an antiviral state in human pancreatic islet cells. *Virology* 367: 92-101.
23. Komatsuda A, Wakui H, Iwamoto K, Ozawa M,