Table 1: Demography and ocular findings of NMO and MS patients

	NMO (n = 15)	MS (n = 20)	*P* value
Sex (male/female)	0/15	5/15	0.057
Age at onset	36.0 ± 10.9	29.2 ± 8.8	0.038
Duration of disease (years)	14.4 ± 8.8	11.5 ± 9.9	0.216
Number of total relapses	10.1 ± 4.8	6.3 ± 6.4	0.007
Number of ON relapses	3.4 ± 3.2	1.7 ± 0.8	0.004
EDSS score	5.1 ± 2.5	2.6 ± 1.6	0.009
Brain lesion, % (n)	60 (9)	90 (18)	0.051
Spinal lesion, % (n)	100 (15)	60 (12)	0.005
LESCL, % (n)	87 (13)	10 (2)	<0.001
Anti-AQP4 antibody, % (n)	100 (15)	0	<0.001

NMO = neuromyelitis optica; MS = multiple sclerosis; ON = optic neuritis; EDSS = expanded disability status scale; LESCL = longitudinally extensive spinal cord lesions.

recognized as a typical visual field defect pattern of ON in MS [17]. In this study, all MS patients experienced central scotoma, with 90% showing central scotoma with every ON attack. On the contrary, 53% of NMO patients showed central scotoma with every ON attack, and the remaining 47% of patients experienced non-central scotoma. Moreover, 13% of NMO patients did not experience central scotoma during the course of their disease. Of the non-central scotoma patterns, altitudinal hemianopia was most frequent. Since altitudinal hemianopia was not recognized in MS patients, this visual field defect may be characteristic of ON for patients with NMO.

ON is the initial manifestation of NMO in 77% of patients. In 30% of NMO patients, the initial attack of ON led to blindness, with only 43% of patients completely recovering from the first attack. Compared with MS patients, NMO patients had a significantly higher rate of bilateral ON (70% versus 19%) [18]. Although the optic nerve is mainly affected in both NMO and MS, the pathogenesis of ON in NMO might differ from that of MS. Compared to MS, the study using optical coherence tomography indicated a thinner overall average retinal nerve fiber layer, suggesting widespread axonal injury in the affected optic nerves in NMO [19].

Pathologically, NMO shares with MS a pattern of focal demyelination, inflammation, scar formation, and axonal destruction, but NMO also has an intense perivascular response, prominent necrosis, and cavitation, which are not seen in MS [9]. IgG, IgM, and products of complement activation are deposited in a perivascular pattern in NMO, suggesting a pathogenic role involving autoantibodies [9]. Blood vessels within demyelination spinal lesions of NMO are thickened and hyalinized [20]. Active lesions exhibit tissue swelling, infiltrating polymorphonuclear macrophages, active microglia, demyelination, axonal loss, prominent necrosis, and variable degrees of

Table 2: Visual field defect patterns of NMO and MS patients

	NMO	MS	*P* value
Total number of ON relapses	51	33	
Visual field defects			
Central scotoma (%)	39 (76)	31 (94)	0.041
Non-central scotoma			
Altitudinal (%)	5 (10)	0 (0)	0.151
Quadrant (%)	3 (6)	0 (0)	0.276
Three quadrant (%)	2 (4)	1 (3)	1
Hemianopia (%)	1 (2)	1 (3)	1
Bitemporal hemianopia (%)	1 (2)	0 (0)	1

NMO = neuromyelitis optica; MS = multiple sclerosis; ON = optic neuritis.

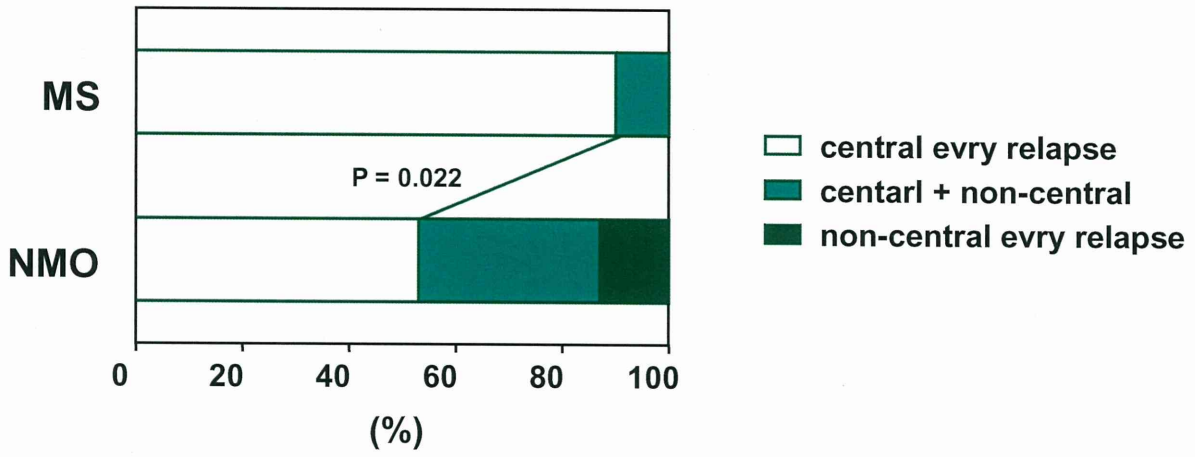

Figure 1 Comparison of visual field defects during the disorders. Ninety percent of MS patients showed central scotoma every time, but 54% in NMO (p = 0.022). In NMO patients, 33% of patients showed both central scotoma and non-central scotoma, and 13% of patients showed non-central scotoma every time. MS = multiple sclerosis; NMO = neuromyelitis optica.

perivascular inflammation with prominent eosinophils and products of their exocytosis. Chronic lesions show gliosis, cystic degeneration, cavitation, and atrophy [21]. These findings suggest that a humoral effector mechanism is initiated by binding of the NMO antibody at the blood-brain barrier (BBB).

Several studies have reported that areas of CNS inflammation correlate with expression pattern of AQP4 in NMO [22,23]. Expression of AQP4 in the brain and spinal cord is associated with astrocyte membranes that appose endothelial cell basal membranes. Astrocytes interact extensively with endothelial cells to maintain the CNS BBB, which normally limits the access of immune system effectors unless localized or distant events disrupt the BBB, thus allowing access of cellular or soluble immune effectors. AQP4 is also expressed by astrocytes that surround the optic nerve [24]. Since the optic nerve head is an area of the CNS where the BBB is more permissive, as evidenced by immunostaining for markers of intact BBB [25,26], tissues of the optic nerve might be more sensitive to AQP4 dysfunction mediated by anti-AQP4 antibodies [27]. Thus, in NMO, optic nerve lesions would have demyelination, axonal loss, and perivascular response, as seen in spinal cord lesions.

Central scotoma is recognized to be a typical visual field defect pattern of ON in MS. In this study, NMO patients showed higher incidence of non-central scotoma than MS patients (p = 0.022, Figure 1); altitudinal hemianopia was more common in non-central scotoma. An altitudinal visual field defect is suggestive of ischemic optic neuropathy, which occasionally is the result of posterior ciliary artery occlusion [28,29]. We suggest that ischemic mechanism mediated by anti-AQP4 antibody may play a role in ON for NMO patients. Pathological study demonstrated that vascular degeneration, such as thickened or hyalinized vessels, existed in the spinal cord lesions [21]. Recent study indicated that NMO patients showed more vascular changes, including attenuation of the peripapillary vascular tree and focal arteriolar narrowing as the retinal features of ON than MS patients [30]. These vascular changes may results from direct vascular inflammation mediated by anti-AQP4 antibody [30,31]. Therefore, the tissue organization of optic nerve cells, such as the vascular structures associated with the optic nerves, is thought to express AQP4, resulting in non-central scotoma, especially altitudinal hemianopia.

Although NMO is often fulminant and has a more negative outcome than MS [32], NMO responds to glucocorticoids, immunosuppressive agents, or plasmapheresis. Since monosymptomatic ON is often seen as being the first indication of an attack of NMO and MS, ophthalmoscopic examination, especially the visual field test, is helpful for diagnosis of NMO, and anti-AQP4 antibody should be checked to decide the most effective treatment [33].

Conclusion

NMO patients showed higher incidence of non-central scotoma than MS, and altitudinal hemianopia may be characteristic of ON occurring in NMO. As altitudinal hemianopia is highly characteristic of ischemic optic neuropathy, we suggest that an ischemic mechanism mediated by anti-aquaporin-4 antibody may play a role in ON in NMO patients.

Table 3: Clinical findings of 7 NMO patients with non-central scotoma

Patient No.	1	2	3	4	5	6	7
Sex/Age at onset	F/48	F/21	F/47	F/34	F/32	F/32	F/54
Duration of disease (years)	9	11	24	4	15	18	12
EDSS score	2.5	1	7	3.5	7	7.5	4
Number of total relapses	5	5	12	6	11	18	23
Number of ON relapses	3	2	2	2	3	4	12
Ocular pain	moderate	none	none	mild	mild	none	moderate
Optic disk in acute phase	normal	NE	NE	normal	normal	normal	normal
Course of ON	lt-altitudinal (inferior) rt-central lt-central	lt-three quadrant rt-altitudinal (inferior)	rt-altitudinal (superior) rt-central	lt-altitudinal (inferior) rt-quadrant	rt-altitudinal (superior) lt-central lt-central	bil-central lt-guadrant rt-hemianopia	rt-central lt-central: 2nd-3rd rt-quadrant lt-three quadrant rt-central lt-central rt-central: 8th-11th bitemporal
Outcome of ON	rt-recover lt-recover	rt-recover lt-recover	rt-recover	rt-recover lt-recover	rt-light perception lt-light perception	rt-recover lt-recover	rt-light perception lt-light perception

NMO = neuromyelitis optica; EDSS = expanded disability status scale; ON = optic neuritis; NE = not evaluated.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
HN performed analyses, collected data and wrote the manuscript. TH helped to draft the manuscript and collected data. MS, FK, JS, and TH helped to draft the manuscript. TT performed anti-AQP4 antibody assay. All authors read and approved the final manuscript.

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

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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.

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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.

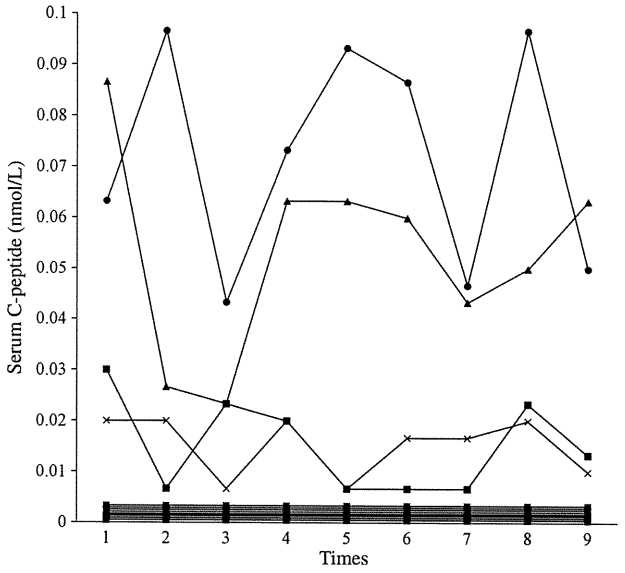

Figure 1 | Transition of serum C-peptide levels during the study period. Detectable serum C-peptide levels in 4 patients (●, ▲, ■, ×) ranged from 0.007 to 0.10 nmol/L, however serum C-peptide levels in eight patients were always <0.003 nmol/L.

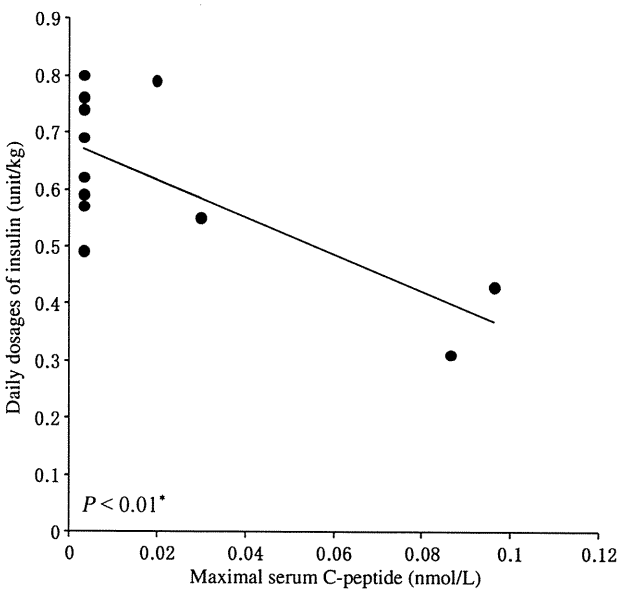

Figure 2 | Relationship between maximal serum C-peptide level (nmol/L) and daily dosages of insulin (unit/kg). Residual insulin secreting capacity shown as serum C-peptide level and daily dosages of insulin indicated a significant negative correlation (*$P < 0.01$).

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

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.

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Original Article

Switching to Aggressive Statin Improves Vascular Endothelial Function in Patients with Stable Coronary Artery Disease

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Aim: The clinical relevance of the suggested pleiotropic effects of hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) is controversial. Aggressive statins effectively reduce lipid levels, but whether their other effects are more powerful than those of regular statins is unknown.
Methods: We enrolled 32 patients (mean age, 65 y; male, 23) who had undergone coronary revascularization over 6 months previously and whose serum LDL cholesterol levels persisted at >100 mg/dL, regardless of pravastatin (10 mg/day). Before and 1 and 6 months after switching to atorvastatin (10 mg/day), we evaluated lipid profiles, including RLP-C (remnant-like particle cholesterol), high sensitive CRP (hsCRP), soluble CD40 ligand (sCD40L), TBARS (thiobarbituric acid reactive substances), and endothelial function determined from flow-mediated dilation (FMD) of the brachial artery.
Results: One month on atorvastatin lowered LDL cholesterol by 24% (131 to 100 mg/dL, $p<0.001$). In addition, RLP-C, sCD40L and hsCRP significantly decreased, whereas FMD did not change. After 6 months of this therapy, FMD significantly improved compared to baseline values (5.1 vs 3.6%, $p=0.04$). Changes in FMD and in total and RLP cholesterol significantly correlated. Moreover, FMD was remarkably improved in patients who achieved target LDL levels (<100 mg/dL).
Conclusions: Switching from a regular to an aggressive statin can improve endothelial function at 6 months in patients with previous coronary artery disease. This effect is suggested to be mainly due to the lipid-lowering effect. Achievement and maintenance of the target LDL level by switching statins is beneficial in the clinical setting.

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Key words; Pleiotropic effect, Cardiovascular diseases, Clinical study

Introduction

Numerous large trials have established that lowering low density lipoprotein (LDL)-cholesterol using hydroxymethylglutaryl coenzyme A reductase inhibitor (statin) can prevent cardiovascular disease[1, 2]. Statins apparently have pleiotropic properties other than the ability to lower LDL, such as anti-inflammatory effects, anti-platelet aggregation and so on[3, 4]; however, differentiating the pleiotropic and lipid-lowering effects of statins in the clinical setting is difficult because statins per se already lower LDL-cholesterol.

Many guidelines recommend targeting plasma LDL levels of <100 mg/dL for the secondary prevention of ischemic heart disease[5, 6]. In fact, the Japan Atherosclerotic Society (JAS) guidelines require LDL <100 mg/dL[7, 8]; however, the rates of achieving this target are only about 30% in Japan[9, 10] and in the United States[11]. Various reasons for this have been considered, such as low drug dosages, limited drug effectiveness, inappropriate choice of drug, drug tolerability, and poor patient compliance[9]. Recent reports indicate that the rates of target LDL achievement vary among statins and that targets are achieved more frequently with aggressive statins, such as atorvastatin, than regular statins, such as pravastatin or simvastatin[9, 10]. However, whether aggressive statins can exert more powerful pleiotropic effects than regular statins

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in addition to their lipid-lowering effect is obscure. In addition, few studies have investigated changes in pleiotropic effects after switching from regular to more aggressive statins. We examined this issue by switching from a regular to an aggressive statin and measuring various parameters, including endothelial function, in patients with coronary artery disease (CAD) who had not achieved LDL targets.

Methods

Study Population and Design

We enrolled 34 patients who had undergone coronary revascularization (percutaneous coronary intervention: PCI or coronary artery bypass graft: CABG) over 6 months previously and who had not achieved a serum LDL cholesterol target level of <100 mg/dL despite pravastatin (10 mg/day) administration. The patients were switched to atorvastatin 10 mg/day) for at least 6 months. Two of the 34 patients could not tolerate atorvastatin because of muscle pain; thus, we analyzed data from 32 patients who completed the study. The basic characteristics of these 32 patients are summarized in **Table 1**. Hypertension was defined as systolic or diastolic blood pressure ≥140 or ≥90 mmHg, respectively, or the current use of antihypertensive medication. Diabetes was defined as fasting glucose ≥126 mg/dL or the current use of antiglycemic medication. None of the patients currently smoked and 18 had smoked at some point during their lives.

All of the patients provided written, informed consent to participate in the study and the protocol conformed to the Declaration of Helsinki of 1975, as revised in 1983, and the guidelines provided by the Ethics Review Committee of Osaka Medical College. We performed blood tests and examined endothelial function before, 1 and 6 months after switching from pravastatin to atorvastatin.

Laboratory Examination

Blood was sampled between 8:00 and 9:00 am after an overnight fast. Fasting plasma glucose, hemoglobin A1c, and lipid profiles, namely, total-, high density lipoprotein (HDL)-cholesterol and triglycerides (TG), were measured in the fully accredited central laboratory of Osaka Medical College Hospital. LDL-cholesterol levels were calculated using the Friedewald equation. Sterols (campesterol and lathosterol) were analyzed by gas liquid chromatography according to the previously established method[12] and are expressed as ratios to serum cholesterol to standardize for variations in cholesterol concentrations[13]. High sensitive C-reactive protein (hsCRP) and remnant-like (RLP) cholesterol were analyzed using ELISA kits at SRL Inc. (Japan). Soluble CD40 ligand (sCD40L) was analyzed in duplicate using ELISA kits (R & D Systems Inc., Minneapolis, MN). Plasma malondialdehyde (MDA) was analyzed using TBARS (thiobarbituric acid reactive substance) assay kits (Cayman Chemical Co., Ann Arbor, MI). Pairs of samples from the same patient were measured in blinded fashion using the same assay kit. Inter- and intra-assay coefficients of variation were <7%.

Table 1. Baseline characteristics of patients in this study

Variables	N=32
Age (y.o.)	65.5 (39-79)
Sex (M/F)	26/6
Previous coronary artery disease (OMI/AP)	20/12
Revascularization (PCI/CABG)	25/7
Risk factors	
Hypertension	16
Diabetes	13
Ever-smoker	18

Flow-Mediated Dilation

Flow-mediated dilation (FMD) was measured according to the guidelines[14] 48 h after all vasodilators had been withdrawn. Briefly, after blood sampling from the cubital vein of the left hand, vasodilator responses in the right brachial artery were measured on B-mode ultrasound images using a 7.5-MHz transducer (HP-5500; Phillips Corp., Tokyo, Japan). Endothelium-dependent vasodilation of the brachial artery was assessed as flow-mediated vasodilation in reactive hyperemia after 5-min cuff occlusion at 250 mmHg. After 15-min bed-rest, endothelium-independent vasodilation was measured after a single 0.4 mg dose of nitroglycerin (NTG) delivered as a sublingual spray. Measurements were taken by two independent investigators (N.M. and T.O.) who were blinded to the identity of the subjects. Inter- and intra-observer variability for repeated FMD measurements was 0.10 ± 1.4 and 0.09 ± 1.2%, respectively.

Statistical Analysis

Data are expressed as the means ± SEM. Values before and after switching statins were compared using Student's paired *t*-test (**Table 2** and **Fig. 1**) and associations between parameters were examined using Spearman's correlation coefficient. Two groups were compared using analysis of variance (ANOVA) followed by post-hoc analysis or Student's unpaired *t*-test.

Table 2. Parameter changes by switching from pravastatin to atorvastatin

	Baseline	1 month	6 Month
total cholesterol (mg/dL)	214 ± 3.7	181 ± 4.0**	183 ± 3.1**
HDL cholesterol (mg/dL)	50.8 ± 1.8	51.6 ± 1.7	51.7 ± 1.8
TG (mg/dL)	175 ± 20.3	156 ± 18.0	153 ± 18.6
LDL cholesterol (mg/dL)	131 ± 3.6	100 ± 3.8**	100 ± 4.4**
campesterol (μg/100 mg cholesterol)	145 ± 7.6	183 ± 11**	177 ± 8.6**
lathosterol (μg/100 mg cholesterol)	76 ± 4.4	58 ± 4.3**	56 ± 4.2**
RLP cholesterol (mg/dL)	7.0 ± 0.99	5.3 ± 0.75*	5.5 ± 0.70*
glucose (mg/dL)	124 ± 6.8	121 ± 7.4	126 ± 6.8
hemoglobin A_{1C} (%)	6.3 ± 0.21	6.3 ± 0.21	6.3 ± 0.23
hsCRP (mg/L)	1.70 ± 0.48	0.71 ± 0.10*	0.79 ± 0.11*
sCD40L (mg/L)	1.1 ± 0.20	0.30 ± 0.060**	0.39 ± 0.096**
TBARS (nmol/mL)	7.3 ± 0.36	7.2 ± 0.40	7.2 ± 0.44
FMD (%)	3.6 ± 0.47	3.1 ± 0.29	5.1 ± 0.39**
NTG (%)	13.4 ± 1.1	14.1 ± 0.94	14.0 ± 1.0

*$p<0.05$, **$p<0.01$ vs. Baseline. Values are the mean ± SEM.

Data were analyzed using JMP software (NIH, Version 7.1). A value of $p<0.05$ was considered significant.

Results

Various parameters before and after switching to atorvastatin are shown in **Table 2**. The switch to atorvastatin decreased LDL-cholesterol by 24% at 1 and 6 months and 50% (16 of 32) of the patients achieved the target LDL value of < 100 mg/dL. Furthermore, atorvastatin decreased the cholesterol synthesis marker, lathosterol, by 26% and increased the cholesterol absorption marker, campesterol, with statistical significance. Levels of RLP cholesterol were also decreased, whereas neither TG nor HDL cholesterol had significantly changed at 1 month after switching.

Atorvastatin also decreased hsCRP and sCD40L levels at 1 and 6 months, but did not affect the oxidative stress marker, TBARS, whereas FMD did not change after 1 month but significantly increased at 6 months, although the extent of the changes differed among individual patients (**Fig. 1**). Endothelium-independent dilation, determined as vasodilation with NTG, did not change throughout the study period.

Our analysis of correlations between changes in FMD and in various parameters using Spearman analysis revealed that changes in total and RLP cholesterol significantly and inversely correlated with those in FMD (**Table 3**). Other parameter changes did not correlate with those in FMD. We also analyzed correlations between changes in FMD and individual parameters before switching (**Table 4**). Interestingly,

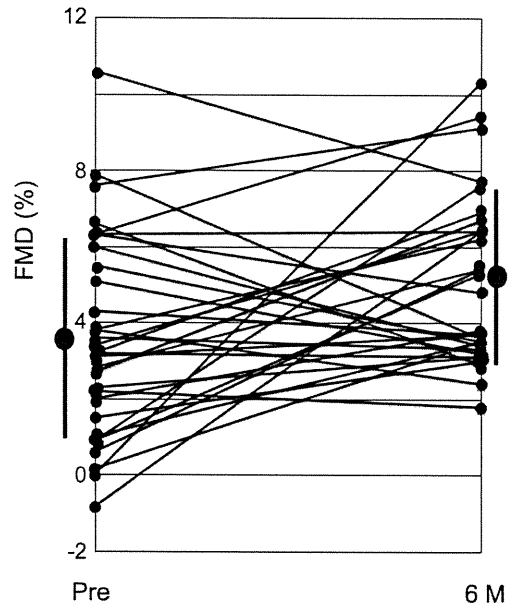

Fig. 1. Changes in FMD among individual patients.

Paired *t*-test shows significant increase in FMD between before and 6 months (Mo) after switching statins. FMD, flow-mediated dilation.

baseline levels of campesterol, a marker of cholesterol absorption, tended to correlate inversely with changes in FMD ($r=-0.31$, $p=0.080$).

We assigned the patients to groups with low (1.8–3.8%, 16 patients) or high (>4.8%, 16 patients) FMD values 6 months after switching to atorvastatin. The extent of the decrease in TG from baseline to 6

Table 3. Correlations between changes in FMD and various parameters

δFMD vs.	Correlation Coefficient	*p* value
δ total cholesterol	−0.40	0.023
δ HDL cholesterol	0.11	0.057
δ triglyceride	−0.33	0.064
δ LDL cholesterol	−0.17	0.35
δ RLP cholesterol	−0.41	0.019
δ campesterol	0.15	0.40
δ lathosterol	−0.18	0.32
δ glucose	−0.18	0.31
δ hemoglobin A1c	0.03	0.88
δ hsCRP	−0.056	0.76
δ sCD40L	−0.16	0.37
δ NTG	0.15	0.42

Table 4. Correlations between various parameters at baseline and changes in FMD

δFMD vs.	Correlation Coefficient	*p* value
total cholesterol	0.19	0.29
HDL cholesterol	0.010	0.95
triglyceride	0.37	0.10
LDL cholesterol	−0.14	0.46
RLP cholesterol	0.32	0.10
campesterol	−0.31	0.081
lathosterol	0.17	0.36
glucose	0.11	0.28
hemoglobin A1c	0.090	0.75
hsCRP	−0.040	0.84
sCD40L	0.18	0.33
NTG	0.070	0.72

Table 5. Comparison of parameters between groups by FMD or LDL cholesterol at final examination

parameter	low FMD (1.8–3.8)	high FMD (4.8<)	*p* value
1Mo LDL	107 ± 5.3	93.9 ± 5.3	0.096
6Mo NTG	12.2 ± 1.4	15.8 ± 1.4	0.067
δ TG	15.3 ± 21.3	61.1 ± 21.3	0.017
parameter	LDL < 100	LDL > 100	*p* value
6Mo total cholesterol	171 ± 4.4	189 ± 3.2	0.0018
6Mo FMD	5.77 ± 0.62	4.37 ± 0.42	0.036
δ LDL cholesterol	40.5 ± 4.2	20.9 ± 6.3	0.0075
δ FMD	2.65 ± 0.81	0.27 ± 0.76	0.020

months significantly differed between groups (**Table 5**). In addition, vasodilation with NTG tended to be high in patients with high FMD.

We compared various parameters between groups of patients according to whether they achieved the target LDL level of < 100 mg/dL ($n = 16$ each) 6 months after switching to atorvastatin. Other than lipid profiles, both the FMD values and changes in FMD were significantly greater in patients with low, than with high LDL levels (**Table 5**).

Discussion

The major finding of the present study is that switching from a regular to an aggressive statin improved FMD at 6 months in patients with coronary artery disease. Although various pleiotropic parameters, such as sCD40L and hsCRP, were changed at 1 month, as well as lipid profiles, a longer period was required for FMD improvement. Changes in FMD correlated with those in lipids, but not with sCD40L nor hsCRP.

Several lines of evidence suggest that forearm FMD can predict cardiovascular events[15-18] and that statin therapy can improve FMD in the short term, even after as little as 3 days[19-21]; however, these studies included patients who had not received prior statin therapy. Few studies have examined changes in FMD after switching statins and a study of a small patient cohort found no difference in FMD between patients already on a regular statin who switched to an aggressive type, or continued with the regular statin[22]. Kitta *et al.* reported more recently that persistently impaired FMD after 6 months of optimized therapy, including statins, is associated with a poor outcome in patients with CAD[23]. We found here that switching from pravastatin to atorvastatin improved FMD at 6 months, but not at 1 month. As far as we know, this is the first report to show that endothelial function can be improved by switching from a regular to an aggressive statin. We believe that this finding could have a significant impact in the clinical setting.

Switching to atorvastatin decreased hsCRP and sCD40L, indicating that an aggressive statin confers further pleiotropic and lipid-lowering effects more effectively than a regular statin. Both sCD40L and hsCRP might correlate with future cardiovascular events[24, 25]. Various reports have shown that statins can decrease the levels of these biomarkers but whether these effects correlate with the lipid-lowering effects of each statin remains controversial. For example, while atorvastatin (80 mg) and simvastatin (40 mg) similarly decreased sCD40L levels in a manner that did not correlate with LDL-lowering effects in the ASAP study[26], rosuvastatin decreased sCD40L within 12 weeks whereas simvastatin did not[27]. Although atorvastatin (80 mg) decreased hsCRP levels more efficiently than pravastatin (40 mg) in the REVERSAL trial, a correlation between lipid- and hsCRP-lowering effects was not determined[28]. These findings were obtained from patients who had not previously been administered statins. The present results indicated that switching from a regular to an aggressive statin can further reduce hsCRP or sCD40L levels in the clinical setting, which is especially meaningful for secondary prevention of cardiac events.

Whereas FMD was restored 6 months after switching to atorvastatin, overall vasodilation with NTG did not change throughout the present study; however, because all of our patients had previous ischemic heart disease, their capacity for arterial dilation might have been impaired in addition to endothelial function. Some investigators have pointed out that smooth muscle dysfunction or changes in vascular structure impair vasodilation with NTG[29]. Our comparison of patients separated into groups based on FMD values above or below 4.8% showed that those with a lower FMD tended to have poor vasodilatation with NTG (12.2 ± 1.4 vs $15.8 \pm 1.4\%$, $p=0.067$). These findings suggest that a poor FMD is partly associated with smooth muscle dysfunction.

Switching to an aggressive statin decreased LDL (and total) cholesterol, RLP, hsCRP and sCD40L, all of which might improve FMD. Among various parameters, the absolute change in FMD correlated with total and RLP cholesterol (**Table 3**). In addition, the amount of the decrease in serum TG attained by switching to atorvastatin was greater among patients with a better (>4.5%) than a poorer FMD. Others have shown that atorvastatin, but not pravastatin, can decrease TG and RLP levels[30]. Thus, the mechanism through which switching from pravastatin to atorvastatin improved FMD in the present study seemed to be mainly the strong lipid-lowering effect of atorvastatin. This is supported by a recent report that cholesterol lowering is more important than the pleiotropic effects of statins for endothelial function in CAD patients[31].

Various guidelines, including JAS, have recommended target LDL levels of < 100 mg/dL for secondary CAD prevention[5-8]. The rationale for this target is based upon the outcomes of various randomized clinical trials[2]. Our patients who achieved this target had a better FMD at 6 months after switching ($p=0.036$) and a more improved FMD ($p=0.020$) than those who did not. Thus, our study also implies the value of achieving and maintaining target LDL levels in the clinical setting.

More precise data have been published about cholesterol homeostasis, such as cholesterol absorption or synthesis in the clinical setting[32-35], since phytosterols, such as campesterol, or cholesterol precursors, such as lathosterol, can be measured and used as markers of cholesterol absorption and cholesterol synthesis, respectively. The present study showed that switching from regular to aggressive statins decreases cholesterol synthesis but increases cholesterol absorption. That atorvastatin increases markers of cholesterol absorption more than regular statin has already been established[36], but few studies have investigated changes in cholesterol absorption after switching statins. Cholesterol absorption markers can predict future cardiovascular events[37, 38]. Although we did not find a difference in FMD values associated with levels of cholesterol absorption, higher values of absorption markers before switching tended to correlate with less FMD improvement after switching to an aggressive statin ($r=-0.31$, $p=0.080$, **Table 4**). This suggests that a strategy to decrease absorption markers would alter FMD; however, more recent reports have shown that ezetimibe, a cholesterol absorption inhibitor, does not improve FMD, even though serum LDL levels decrease in a similar manner to those induced by statins that can also induce FMD improvement[39, 40]. Further studies are required to determine whether a strategy to modify cholesterol absorption would be of clinical benefit.

Although we recognize pravastatin and atorvastatin as regular and aggressive stains, respectively, we should also consider their different chemical properties: pravastatin is hydrophilic, while atorvastatin is lipophilic[41]. As a lipophilic statin seems to have more direct effects on cells other than hepatocytes[41], the differences in hydrophobicity might have affected the non-lipid effects in the present study. In this regard, further study using other statins, such as rosuvastatin, a hydrophilic aggressive statin, is required.

Limitations

This is a single center, nonrandomized study of a small patient cohort. In addition, we used 0.4 mg NTG to measure vasodilation. A recent report has recommended a lower NTG dose, such as 75 μg, because 0.4 mg is excessive as an internal control of FMD response[29].

Conclusion

In conclusion, switching from a regular to an aggressive statin can improve the endothelial function of patients with previous coronary artery disease at 6 months. This effect is suggested to be mainly due to lipid-lowering effects. Achievement and maintenance of the target LDL level by switching statins is beneficial in the clinical setting.

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ORIGINAL

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

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

Key words: Insulitis, Toll-like receptor, Enterovirus

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

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

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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

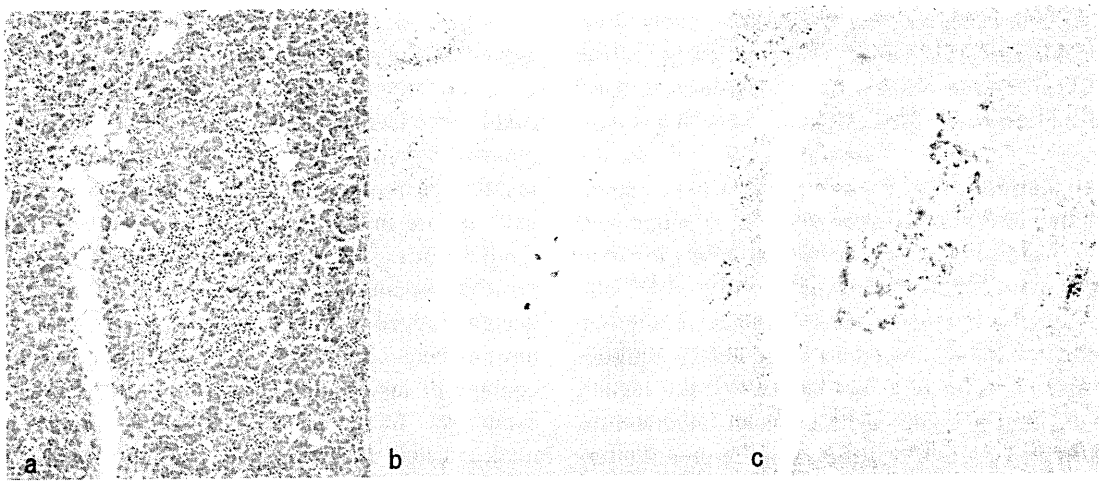

Fig. 1. Microphotographs of consecutive sections stained with H&E (a) and antibodies to insulin (b) and glucagon (c) in case 1 as a representative. Massive mononuclear cell infiltration was detected in and around the islet as well as in exocrine tissue by H&E stain. Decreased numbers of insulin+ cells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

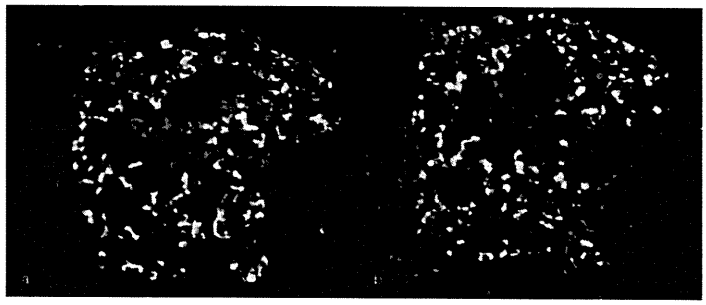

Fig. 2. Microphotographs of double staining sections for CD3/CD68 and glucagon in case 1 as a representative. CD3+ cells (red; a) and CD68+ cells (red; b) are infiltrating in and around the islet (green represents glucagon). Original magnification: x500.

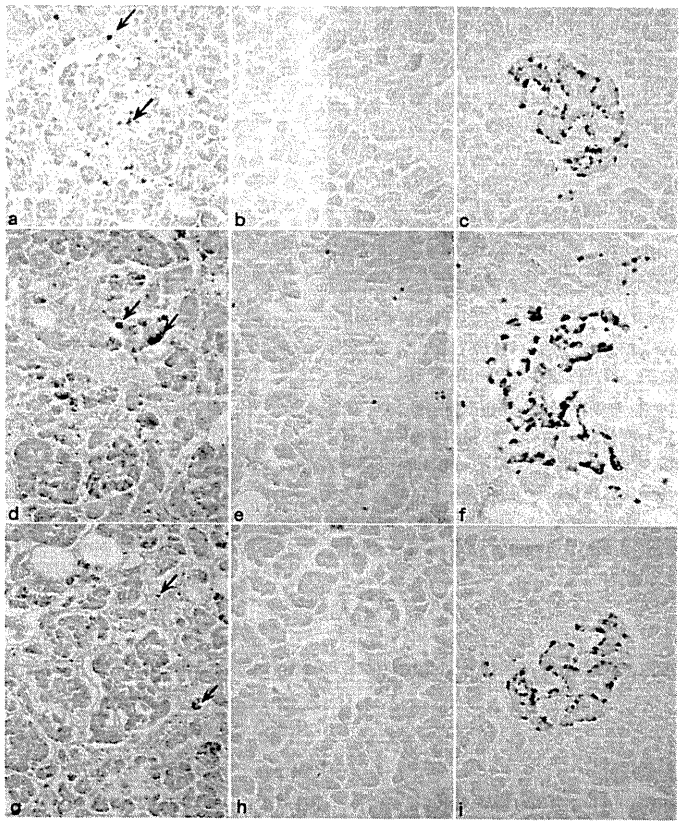

Fig. 3. Microphotographs of consecutive sections stained with antibodies to TLRs and controls for each other. The expressions of TLR3, its negative control and glucagon for patient 2 (a-c), TLR7, its negative control and glucagon for patient 3 (d-f), TLR9, its negative control and glucagon for patient 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

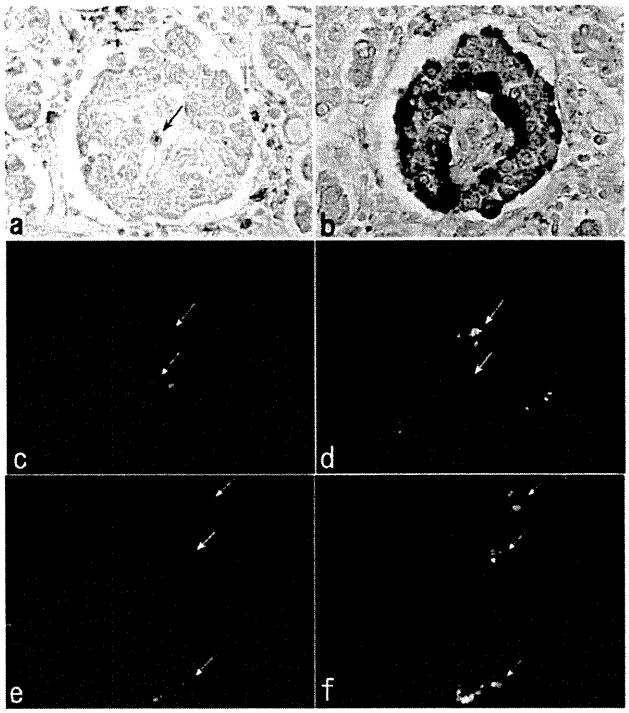

Fig. 4. Microphotographs of consecutive sections stained with antibodies to insulin (a), glucagon (b), CD3 (red; c), TLR3 (green, d), CD68 (red; e) and TLR3 (green; f) in case 2. Microphotographs of (c)(d) and (e)(f) are the same sections and visualized by a double staining method. Insulin+ and glucagon+ 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.

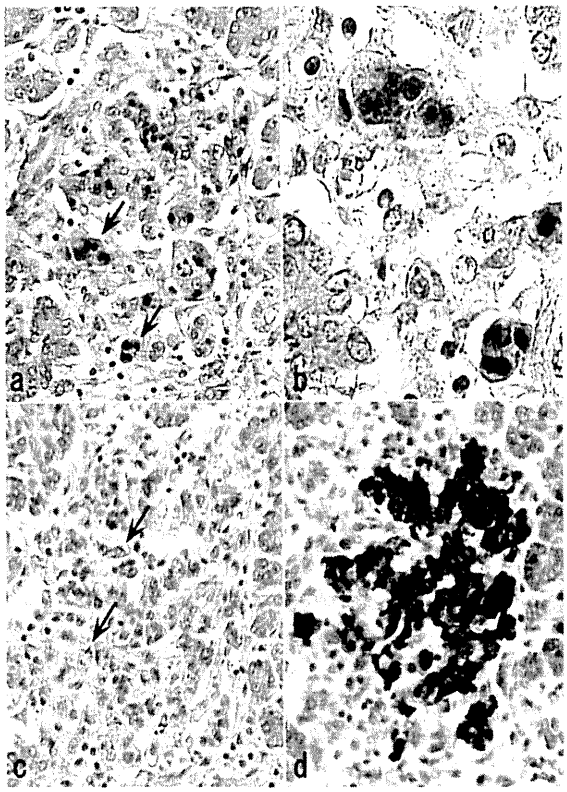

Fig. 5. Microphotographs of consecutive sections of enterovirus RNA (a, b), insulin (c) and glucagon (d) in Case 1. Enterovirus RNA was detected in the islets with insulin of case 1 but not in cases 2 and 3. No enterovirus RNA was detected in the exocrine pancreas. Arrows (a) (c) represent positive cells.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