adipose tissue. The phenomenon, so-called lipotoxicity, is one of the pathogenic mechanisms of insulin resistance associated with visceral obesity.[20] The 60-min post-prandial FFA level was reduced most commonly in patients with lower PAI-1 or AGE levels at baseline, indicating that mitiglinide may, therefore, be more effective in the early stages of diabetes with less advanced complications.

Mitiglinide therapy for 12 months significantly reduced urinary albumin excretion in the present study. It has previously been reported that mitiglinide prevents the increase in oxidative stress associated with post-prandial hyperglycaemia.[14] Thus, mitiglinide may reduce urinary albumin excretion by exerting an antioxidant effect. Furthermore, it has been reported that FFA-induced oxidative stress contributes to vascular endothelial dysfunction,[20] which suggests the possibility that mitiglinide reduces this FFA-induced vascular endothelial dysfunction, resulting in a reduction in urinary albumin excretion. Urinary albumin excretion was improved in patients with a higher HOMA-R level and lower PAI-1 level after 12 months of treatment in the present study. Thus, mitiglinide may have a tendency to reduce urinary albumin excretion in patients with increased insulin resistance and in those with impaired coagulation and fibrinolysis.

Treatment with mitiglinide did not significantly improve the levels of hs-CRP, adiponectin, TNF-α, PAI-1, AGE, RAGE or fibrinogen in the present study. Significant changes may not have been seen because these values were within normal ranges at baseline. Thus, further investigation in patients with aggravated arteriosclerosis is needed to clarify the anti-arteriosclerosis effects of mitiglinide.

In conclusion, mitiglinide significantly increased post-prandial insulin secretion and decreased post-prandial plasma glucose and FFAs, without causing weight gain or severe hypoglycaemia, suggesting that this agent may be indicated in the initial treatment of type 2 diabetes mellitus. Furthermore, mitiglinide showed promising pleiotropic effects, including an improvement in lipid metabolism, and may have a role in the prevention of vascular complications such as diabetic nephropathy in patients with type 2 diabetes.

Conflicts of interest

The authors had no conflicts of interest to declare in relation to this article.

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Effects of gliclazide on platelet aggregation and the plasminogen activator inhibitor type 1 level in patients with type 2 diabetes mellitus

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Abstract

Vascular complications are a common factor determining morbidity and mortality of diabetic patients. In vitro studies have revealed that gliclazide has antiplatelet activities. To clinically assess this action, we measured the effects of gliclazide on platelet activities and abnormal fibrinolysis in patients with type 2 diabetes mellitus. We studied 14 patients aged 38 to 72 years (9 men and 5 women) with type 2 diabetes mellitus who have been treated with glibenclamide in our hospital for more than 6 months. We switched from glibenclamide to gliclazide using the average ratio of the respective doses, 2.5 vs 40 mg. We titrated the dose of gliclazide to keep the glycemic control at the same level as the previous (glibenclamide) treatment. We measured 10 μmol/L serotonin–induced or 0.5 μmol/L adenosine diphosphate (ADP)–induced platelet aggregate formation by particle counting using light scattering at baseline and up to 6 months after the switch. After switching to gliclazide, platelet aggregate formation induced by serotonin was significantly reduced ($P < .05$, compared with the levels observed after glibenclamide treatment). The body mass index, fasting plasma glucose, immunoreactive insulin, homeostasis model assessment of insulin resistance, hemoglobin A_{1c} (HbA_{1c}), total cholesterol, triglycerides, high-density lipoprotein cholesterol, prothrombin time, activated partial thromboplastin time, fibrinogen, thrombin-antithrombin III complex, plasmin-α2-plasmin inhibitor complex, and plasma plasminogen activator inhibitor type 1 (PAI-1) were not changed. In the group with improved HbA_{1c} (n = 5), ADP-induced platelet aggregate formation and plasma PAI-1 level were significantly reduced ($P < .05$, compared with the group with aggravated HbA_{1c}, n = 9). Multiple regression analysis showed that percentage change of ADP-induced platelet aggregate formation (standardized $\beta = 0.540$, $P < .05$) was independently associated with percentage change of plasma PAI-1 level in addition to percentage change of HbA_{1c} (standardized $\beta = 0.657$, $P < .05$) ($R = 0.939$, $P < .05$) after switching to gliclazide. The other independent variants, like the final dose of gliclazide, homeostasis model assessment of insulin resistance, percentage change of prothrombin time, activated partial thromboplastin time, and total cholesterol, were not significantly associated with the percentage change of plasma PAI-1 level. These results indicate that gliclazide inhibits platelet aggregation via the serotonin pathway, independently of the metabolic control per se. Furthermore, in the patients with improved glycemic control, gliclazide could inhibit ADP-induced platelet aggregation and reduce PAI-I level. Taken together, the results show that gliclazide may be more useful for the prevention of diabetic vascular complications than glibenclamide.

1. Introduction

Atherosclerotic complications play a crucial role in the prognosis of type 2 diabetes mellitus (DM). It is fully recognized that long-term macrovascular complications are common factors determining morbidity and mortality in the diabetic population. The Diabetes Control and Complications Trial and UK Prospective Diabetes Study indicate a consistent relationship between hyperglycemia and the incidence of chronic vascular complications in type 1 and type 2 DM, respectively [1,2]. Platelet function in DM patients is enhanced and is correlated with both agonist-induced and spontaneous aggregation [3]. It is thought that long-term exposure to high glucose levels may enhance

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Table 1
Characteristics of enrolled patients

Age (y)	61.5 ± 2.6
Sex (M/F)	9/5
Height (cm)	165.3 ± 2.9
Weight (kg)	62.0 ± 3.1
BMI (kg/m^2)	22.5 ± 0.8
Duration of DM (y)	12.0 ± 1.8
HbA_{1c} (%)	7.4 ± 0.2
FPG (mg/dL)	165.0 ± 7.0
IRI (μU/mL)	7.8 ± 1.6
HOMA-R	3.0 ± 0.6

Data are expressed as mean ± SEM.

platelet function in DM patients. Moreover, rapid alterations of platelet aggregability in acute hyperglycemia have also been reported [4]. Intraplatelet serotonin (5-hydroxytryptamine; 5-HT) content is diminished and plasma levels of 5-HT are increased in DM patients [5].

This increase in plasma 5-HT may reflect enhanced release of platelet 5-HT by hyperactive platelets that may contribute to the pathogenesis of atherosclerosis. The measurement of 5-HT–induced platelet aggregation is therefore a useful method to evaluate the risk of diabetic complications in DM patients [5].

A technique for studying platelet aggregation by particle counting using light scattering may detect subtle changes in platelet activation [6]. Hypercoagulability and decreased fibrinolysis, including increased plasma plasminogen activator inhibitor type 1 (PAI-1) level, are often found and are considered to be risk factors of cardiovascular diseases and glucose intolerance, especially in patients with non–insulin-dependent DM [7]. Gliclazide is a second-generation sulfonylurea with the potency of free radical scavenger activity. Some studies have shown that gliclazide has beneficial effects on the hemorrheologic abnormalities seen in diabetic vascular disease [8-12].

To assess this clinically, we measured platelet activities and fibrinolysis in patients with type 2 DM treated with gliclazide; and we compared the results with those obtained in patients treated with glibenclamide.

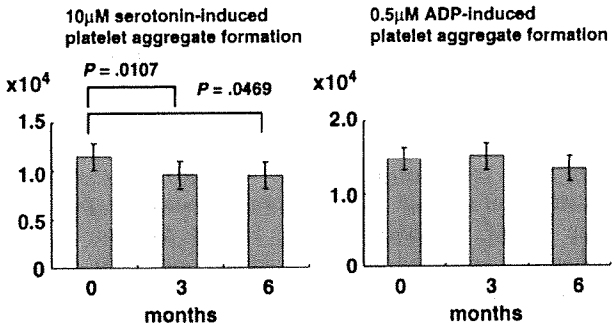

Fig. 1. Effects of gliclazide on platelet aggregation. Data are expressed as mean ± SEM.

2. Subjects and methods

2.1. Subjects

Fourteen patients with type 2 DM (9 men and 5 women; age [mean ± SEM], 61.5 ± 2.6) were randomly chosen as subjects. They were admitted to our metabolic ward between the years 2001 and 2002. Diagnosis of diabetes was based on World Health Organization 1998 criteria. All patients were treated with diet and glibenclamide. All the procedures in the study and the protection of the patients' private information were approved by the ethical committee of Hyogo College of Medicine. Informed consent was obtained from each patient before enrollment in the study.

2.2. Experimental protocol

We switched from glibenclamide to gliclazide using the average ratio of the respective doses, 2.5 vs 40 mg (1.25-20 mg in 2 patients, 2.5-40 mg in 6 patients, 5.0-80 mg in 2 patients, and 7.5-120 mg in 3 patients). We titrated the dose of gliclazide to keep the glycemic control at the same level as in the glibenclamide control. Patients' blood was assayed 3 times: before switching from glibenclamide to gliclazide and then 3 and 6 months after switching.

2.3. Blood sample preparation

Blood was collected in fasting condition on the respective mornings. Venous blood was drawn into 3.8% sodium citrate (1:9 vol/vol). Platelet-rich plasma (PRP) and platelet-poor plasma were obtained by centrifugation of the citrated blood at room temperature for 10 minutes at 150g and for 15 minutes at 3000g, respectively. The platelet count in PRP was adjusted to 2×10^{11}/L with platelet-poor plasma.

For the measurement of PAI-1, blood was centrifuged for 15 minutes at 3000g; and the supernatant was kept at −80 °C until assayed. Fasting plasma glucose (FPG), immunoreactive insulin (IRI), hemoglobin A_{1c} (HbA_{1c}), fasting serum concentrations of total cholesterol (T-Chol), triglycerides (TG), high-density lipoprotein cholesterol (HDL-Chol), prothrombin time (PT), activated partial thromboplastin time (APTT), fibrinogen (Fbg), thrombin-antithrombin III complex (TAT), and plasmin-α2-plasmin inhibitor complex (PIC) were also measured. Total cholesterol, TG, and HDL-

Table 2
Effects of gliclazide on metabolic factors

	Before	3 mo	6 mo
BMI (kg/m^2)	22.5 ± 0.8	22.3 ± 0.8	21.9 ± 0.8
HbA_{1c} (%)	7.4 ± 0.2	8.0 ± 0.3	7.7 ± 0.3
FPG (mg/dL)	165.0 ± 7.0	166.0 ± 9.9	170.0 ± 8.0
IRI (μU/mL)	7.8 ± 1.6	7.5 ± 1.5	7.4 ± 1.1
HOMA-R	3.0 ± 0.6	3.0 ± 0.8	3.0 ± 0.4
T-Chol (mg/dL)	205.0 ± 8.3	205.0 ± 8.7	201.0 ± 9.4
TG (mg/dL)	121.0 ± 22.1	102.0 ± 10.6	135.0 ± 19.3
HDL-Chol (mg/dL)	50.0 ± 2.6	50.0 ± 2.3	48.0 ± 1.8

Table 3
Effects of gliclazide on coagulation test and PAI-1

	Before	3 mo	6 mo
PT-INR	0.93 ± 0.01	0.92 ± 0.01	0.92 ± 0.02
APTT (s)	25.8 ± 0.7	25.8 ± 0.6	26.6 ± 0.4
Fbg (mg/dL)	300.0 ± 20.7	301.0 ± 9.5	340.0 ± 11.8
TAT (ng/mL)	50.2 ± 22.2	15.3 ± 5.4	25.8 ± 7.8
PIC (μg/mL)	0.8 ± 0.1	0.8 ± 0.1	1.3 ± 0.3
PAI-1 (ng/mL)	42.0 ± 5.6	35.4 ± 4.9	36.4 ± 5.3

Data are expressed as mean ± SEM. INR indicates international normalized ratio.

Chol were assayed using an autoanalyzer (JCA-BM 2250; Nihon Denshi, Akishima, Tokyo, Japan), while HbA_{1c} was measured by high-performance liquid chromatography (HLC-723G7 system; Tosoh, Tokyo, Japan). The subjects were then divided into 2 groups depending on whether their HbA_{1c} levels were improved or aggravated 6 months after switching from glibenclamide to gliclazide.

2.4. Platelet aggregation

Platelet aggregation was monitored with an AG10 aggregometer (Kowa, Tokyo, Japan) that determines the size and number of platelet aggregates based on particle counting using light scattering [6,13]. A laser beam (675 nm) is passed through a platelet suspension, and the intensity of light scattering provides information on the number and size of aggregates. Data were recorded as a 2-dimensional graph showing the change over time of total light intensity expressed as cumulative summation. The total light intensities of small aggregates were determined. Particles with an intensity of 25 to 400 mV represent small aggregates consisting of less than 100 platelets. Platelet-rich plasma (180 μL) was placed in a cuvette and incubated for 3 minutes at 37°C while rotating at 1000 rpm. Subsequently, 20 μL of 5-HT (final concentration, 10 μmol/L) or adenosine diphosphate (ADP) (0.5 μmol/L) was added; and the formation of platelet aggregates was monitored for 5 minutes. For this experiment, we determined the peak level of aggregate formation.

2.5. Statistical analysis

Values are presented as means ± SEM. Correlations were assessed using Spearman rank correlation test. Multiple regression analysis was performed to assess the combined influence of variables on percentage change of plasma PAI-1 levels. The Wilcoxon signed rank test or the Mann-Whitney U test were used for comparison. Differences were considered significant at $P < .05$. All the statistical analyses were performed using StatView J-5.0 software (SAS Institute, Berkeley, CA).

3. Results

3.1. Clinical characteristics of the patients

The clinical characteristics of the enrolled patients in the study are summarized in Table 1. The mean FPG was 165.0 ± 7.0 mg/dL (reference range, 70-110 mg/dL), and HbA_{1c} was 7.4% ± 0.2% (reference range, 4.0%-5.4%).

3.2. Change of platelet aggregation and metabolic factors after switching to gliclazide

After switching from glibenclamide to gliclazide, platelet aggregate formation induced by serotonin was significantly reduced (P = .0107, compared with glibenclamide treatment) after 3 months and (P = .0469, compared with glibenclamide treatment) after 6 months, although the ADP-induced platelet aggregate formation was not changed at all (Fig. 1). The switch from glibenclamide to gliclazide did not modify body mass index (BMI), FPG, IRI, homeostasis model assessment of insulin resistance (HOMA-R), HbA_{1c}, T-Chol, TG, and HDL-Chol (Table 2).

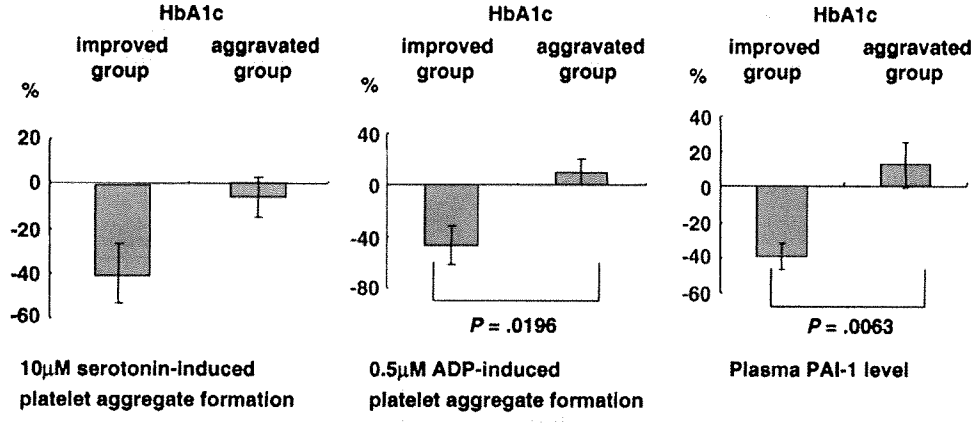

Fig. 2. Percentage change of platelet aggregate formations by 10 μmol/L serotonin or 0.5 μmol/L ADP and plasma PAI-1 level depend on the change of glycemic control after switching to gliclazide. Data are expressed as mean ± SEM.

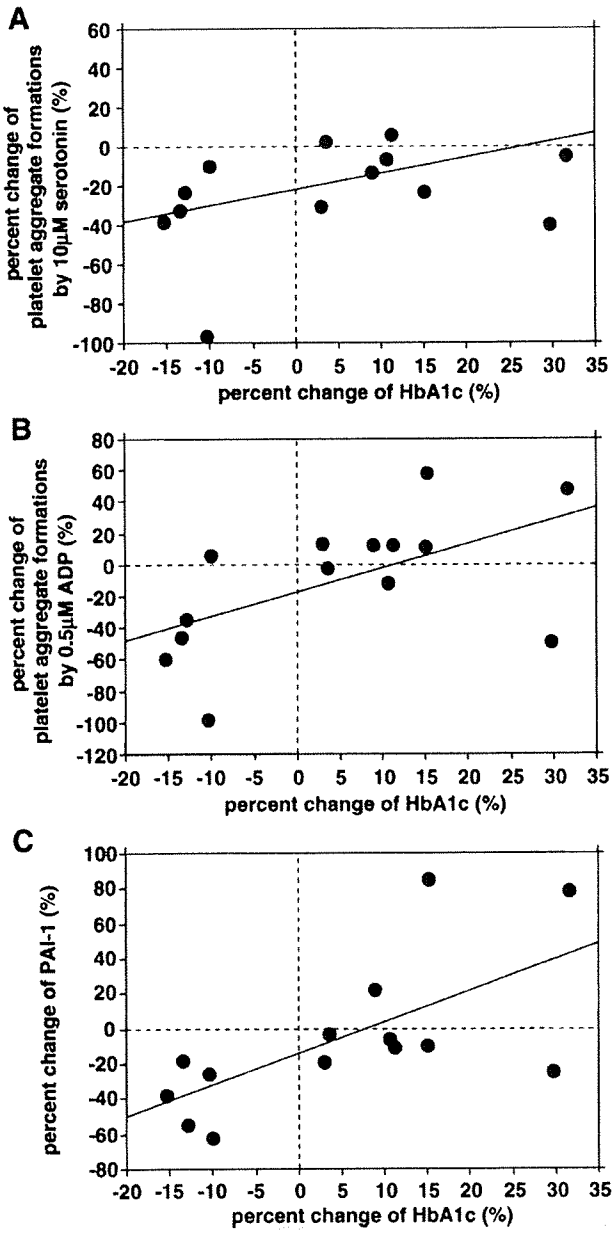

Fig. 3. A, Correlation between percentage change of HbA_{1c} and platelet aggregate formations by 10 μmol/L serotonin after switching to gliclazide (r = 0.39, P = .1712). B, Correlation between percentage change of HbA_{1c} and platelet aggregate formations by 0.5 μmol/L ADP after switching to gliclazide (r = 0.56, P = .0370). C, Correlation between percentage change of HbA_{1c} and PAI-1 after switching to gliclazide (r = 0.65, P = .0115).

3.3. Change of the levels of coagulation factors and PAI-1 after switching to gliclazide

After switching from glibenclamide to gliclazide, PT, APTT, Fbg, TAT, PIC, and PAI-1 were not changed significantly, although PAI-1 would tend to decrease (Table 3).

3.4. Relationship between platelet aggregate formation, plasma PAI-1, blood pressure, and glycemic control

At the end of the 6-month gliclazide treatment and compared with the group of patients with aggravated levels of HbA_{1c} (n = 9), patients with improved HbA_{1c} levels (n = 5) had significantly reduced ADP-induced platelet aggregate formation (P = .0196) and plasma PAI-1 levels (P = .0063) (Fig. 2).

Linear regression analysis showed that percentage change of HbA_{1c} correlated positively with both percentage change of platelet aggregate formation by 0.5μmol/L ADP (r = 0.56, P = .0370) (Fig. 3B) and percentage change of PAI-1 (r = 0.65, P = .0115) (Fig. 3C) after switching to gliclazide.

The mean systolic blood pressure (135.9 ± 3.9 mm Hg) in the group of patients with aggravated levels of HbA_{1c} was not significantly different from the mean systolic blood pressure (124.0 ± 9.2 mm Hg) in the group of patients with improved HbA_{1c} levels. The mean diastolic blood pressure (76.7 ± 2.4 mm Hg) in the former group was not significantly different from the mean diastolic blood pressure (70.2 ± 6.2 mm Hg) in the latter group. Percentage change of mean systolic blood pressure (−5.6% ± 4.3%) in the former group was not significantly different from percentage change of the mean systolic blood pressure (2.1% ± 2.5%) in the latter group. Percentage change of mean diastolic blood pressure (−2.0% ± 3.1%) in the former group was not significantly different from percentage change of the mean diastolic blood pressure (6.8% ± 2.7%) in the latter group.

3.5. Relationship between plasma PAI-1 and various factors

Multiple regression analysis showed that, after switching to gliclazide, the percentage change of ADP-induced platelet aggregate formation (r = 0.540, P = .0401) was independently associated with the percentage change of plasma PAI-1 level in addition to the percentage change of HbA_{1c} (r = 0.657, P = .0310) (R = 0.939, P = .0188) (Table 4). The other independent variants including the final dose of gliclazide, HOMA-R, percentage change of PT–international normalized ratio, APTT, and T-Chol were not significantly associated with percentage change of PAI-1.

Table 4
Multiple regression analysis with percentage change of plasma PAI-1 level

	Regression coefficient	SEM	Standardized regression coefficient	P
Percentage change of ADP-induced platelet aggregate formation	0.539	0.207	0.540	.0310
Percentage change of HbA_{1c}	1.809	0.645	0.657	.0401

4. Discussion

We found that platelet aggregate formation induced by 5-HT was significantly reduced after switching from glibenclamide treatment to gliclazide under the same conditions of metabolic control. Serum advanced glycation end products (AGEs) are significantly higher in DM subjects compared with healthy subjects [14], and our previous study indicated that enhancement of 5-HT–induced platelet aggregation in DM is dependent on the increased level of AGEs [15]. Gliclazide may therefore decrease the effect of AGEs on the enhancement of 5-HT–induced platelet aggregate formation in type 2 DM patients. When we switched from glibenclamide to gliclazide, BMI, FPG, IRI, HbA_{1c}, T-Chol, and TG were not changed at all. These results indicate that gliclazide inhibits platelet aggregation via the serotonin pathway, independently of the metabolic and/or glycemic control per se. Although gliclazide is a more potent ADP-induced platelet aggregation inhibitor than glibenclamide [16], ADP-induced platelet aggregate formation was not changed in our study when we switched from glibenclamide to gliclazide. We reported that ADP-induced platelet aggregation is increased by AGEs; but this increment is diminished by addition of sarpogrelate, a selective 5-HT receptor antagonist [15]. In the group with improved HbA_{1c}, ADP-induced platelet aggregate formation and plasma PAI-1 level were significantly reduced compared with the group with aggravated HbA_{1c}. Although a relationship between the level of blood pressure (particularly hypertensive levels) and platelet activation has been reported, there was no significant difference of hypertensive levels between the groups with aggravated and improved HbA_{1c} levels. The percentage change of ADP-induced platelet aggregate formation was independently associated with the percentage change of plasma PAI-1 level in addition to percentage change of HbA_{1c} after switching to gliclazide by multiple regression analysis. In some reports, an improved metabolic control of type 2 DM could significantly decrease the elevated concentrations of PAI-1. The decrement in PAI-1 is induced by drugs with dissimilar effects on insulin secretion (ie, glipizide gastrointestinal therapeutic system and metformin), emphasizing the important contribution that metabolic control has on this process [17].

Furthermore, in patients with improved glycemic control, gliclazide could inhibit ADP-induced platelet aggregation and PAI-1 level. Gliclazide rather than glibenclamide has been reported to attenuate the progression of carotid intima-media thickness in subjects with type 2 DM [18]. Furthermore, in a population-based case-control and follow-up study, the risk of myocardial infarction would appear to be higher among users of old sulfonylureas including glibenclamide (adjusted odds ratio, 2.07; 95% confidence interval, 1.81-2.37) than among users of new sulfonylureas including gliclazide (adjusted odds ratio, 1.36; confidence interval, 1.01-1.84) [19]. Recently, in the ADVANCE trial (Action in Diabetes and Vascular disease: preterAx and diamicroN modified release Controlled Evaluation), an intensive glucose-control strategy using gliclazide (modified release) and other drugs as required lowered the average HbA_{1c} value to 6.5% in a broad range of patients with type 2 DM and reduced the incidence of the combined primary outcome of major macrovascular or microvascular events [20]. We should have listed the limitations of the study in the interpretation of the results. All patients were switched from glibenclamide to gliclazide. Although we have claimed that the subjects were under the same conditions of metabolic control, at the end of the 6-month period, there was a group with aggravated levels of HbA_{1c} and a group with improved HbA_{1c} levels. To compare the 2 drugs, half the patients should have continued on glibenclamide; or, more practicable with the small number, a cross-over design could have been used. This would permit comparing the 2 drugs in the patients with matching HbA_{1c} levels.

In conclusion, the study results demonstrate that gliclazide inhibits serotonin-induced platelet aggregation independently of glycemic control, although being less effective on ADP-induced aggregation, and may have a better effect on the reduction of platelet aggregability than glibenclamide. Very importantly, this study supports previous results showing the reduction in platelet aggregability and reduction in PAI-1 level with the improvement in glycemic control. Therefore, gliclazide may be more useful for the prevention of diabetic vascular complications than glibenclamide via beneficial and pleiotropic effects on the hemorrheologic abnormalities.

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Role of Macrophages in the Development of Pancreatic Islet Injury in Spontaneously Diabetic Torii Rats

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Abstract: Spontaneously diabetic Torii (SDT) rats were established from Sprague-Dawley (SD) rat and are used as an animal model of type 2 diabetes mellitus. In the present study, the mechanism of the development of injury in the pancreas of these rats was examined focusing on the role of monocytes/macrophages. The number of lymphocytes and monocytes in the circulation of SDT rats increased with age, reaching a plateau at around 9 weeks of age and remaining at that level thereafter. The number of leukocytes in SDT rats was almost twice that of wild-type SD rats. Serum IL-18 levels began to increase at 8 weeks of age, forming a prominent peak at 9 weeks of age. In parallel with this, serum levels of NO_2/NO_3 showed an abrupt rise and decline. Spleen cells prepared from 9-week-old SDT rats expressed high levels of IFN-γ in response to IL-18, while those from 9-week-old wild-type SD rats did not. Immunohistochemical analysis revealed marked infiltration of $CD68^+$ cells in the islets of SDT rats. Treatment of SDT rats with Cl_2MDP-liposomes reduced the number of monocytes as well as levels of NO_2/NO_3 in the circulation. Consistent with this, the number of infiltrated $CD68^+$ cells in the islets was reduced in SDT rats treated with Cl_2MDP-liposomes. These results suggest that macrophages are involved in pancreatic islet injury in SDT rats through excess production of NO induced by IL-18 which increases transitorily at around 9 weeks of age.

Key words: Cl_2MDP-liposomes, IL-18, monocytes/macrophage, NO, SDT rat

Introduction

Male SDT rats, known as a model of non-obese type 2 diabetes, develop hyperglycemia without obesity at around 20 weeks of age and manifest nephropathy and ocular complications such as cataract and proliferative retinopathy [30]. At 8–10 weeks of age, SDT rats manifest microvascular abnormalities such as congestion and hemorrhage in pancreatic islets [20]. At around 9 weeks of age, invasion of inflammatory cells into the pancreas and destruction of β-cells are observed [20]. Fibrosis in the pancreas occurs starting at about 20 weeks of age

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[20]. The invasion of inflammatory cells into the pancreas continues throughout the life of SDT rats, and high levels of glucose are also maintained in the blood [30]. Interestingly, however, SDT rats live long, for more than 2 years, without fatal complications even in the absence of exogenous administration of insulin.

Although diabetes of SDT rats has been considered as type 2, the pancreas of SDT rats also manifests features of type 1 diabetes, such as vigorous cell invasion [20]. Type 1 diabetes mellitus is an autoimmune disease that results in destruction of insulin-producing β-cells in the pancreatic islets. A variety of inflammatory cells and mediating molecules including cytokines and oxygen radicals are involved in the destruction of islet β-cells [19]. In non-obese diabetic (NOD) mice, used widely as a model of type 1 diabetes, macrophages play a role in the initiation and progression of autoimmune diabetes. Macrophages accumulate around the islets at 4–5 weeks of age prior to the peri-insular concentration of lymphocytes and later infiltrate into the islets [14, 28]. It has been shown that excess nitric oxide (NO) production by macrophages present continuously in the pancreas of NOD mice mediates β-cell damage [2, 3, 5, 16]. It has also been shown that excess production of NO by macrophages is induced by IFN-γ, produced by lymphocytes in response to stimulation by IL-18 and IL-12 [4]. Histological studies show that the islets of SDT rats are invaded by various inflammatory cells, but whether these cells mediate β-cell injury, as in NOD mice, has not been investigated.

Macrophages play a role in the pathogenesis of various diseases as well as in host defense. Macrophage depletion can be achieved by systemic injection of liposomes containing clodronate [37]. Clodronate belongs to the family of bisphosphonates (BPs), bone-seeking agents that are potent inhibitors of osteoclasts. Like other BPs, clodronate has poor cell membrane permeability [27]. Liposomes are readily taken up by cells in the reticuloendothelial system, in particular by macrophages. Liposome-mediated delivery of clodronate inactivates and kills macrophages after effective phagocytosis [29] but is not toxic to nonphagocytic cells [37]. The action of liposomes is different from that of various immunosuppressant drugs which reduce leukocytes by production in bone marrow. In animal models, depletion of monocytes/macrophages using Cl_2MDP-liposomes has been shown to ameliorate immune thrombocytopenic purpura [1], vascular repair after mechanical arterial injury [7], pneumocystis pneumonia [17], and autoimmune hemolytic anemia [15].

In this study, we examined the possibility that macrophages may play a pivotal role in β-cell destruction in SDT rats and that systemic inactivation of macrophages might lead to attenuation of islet injury. The results show that depletion of macrophages by clodronate in liposome reduced pancreatic invasion of macrophages and destruction of islet β-cells.

Materials and Methods

Animals

Male SDT rats were provided by the animal control center of the SDT rat study group (Torii Pharmaceutical Company, Tokyo, Japan). Age-matched male SD rats were purchased from CLEA Japan Inc. (Tokyo, Japan). All experimental procedures were approved by the Animal Care Committee of Hyogo College of Medicine.

Blood cell count

Blood samples were collected from the cervical vein using heparinized syringes in tubes containing EDTA once a week. Red blood cells, leukocytes, and platelets were stained with hematoxylin-eosin (HE) and counted under a microscope.

Flow cytometory

Peripheral leukocytes were separated by density gradient centrifugation with Histopaque1083™ (Sigma Aldrich, St. Louis, MO, USA) and incubated with FITC-conjugated anti-rat CD8 and Gr-1 antibodies, phycoerythrin (PE)-conjugated anti-rat CD3 antibody, and cychrome-conjugated anti-rat CD4 and CD161a antibodies (BD Bioscience Pharmingen, San Jose, CA, USA) for 15 min at 4°C. Cells were washed 3 times with staining buffer (PBS containing 2% FCS and 0.05% NaN_3) and analyzed by flow cytometory (Becton Dickinson).

Assay of cytokines and NO metabolites

Serum levels of cytokines were measured by ELISA.

For the IL-18 assay, 96-well EIA plates were treated with 1 μg/ml of mouse anti-rat IL-18 antibody (R&D Systems, MN, USA) diluted with PBS for 16 h at 4°C, washed with PBS containing 0.5% Tween 20 and incubated with blocking solution (PBS containing 1% BSA) for 1 h at 37°C. Samples were loaded in the wells, kept at 37°C for 3 h, and incubated with 0.2 μg/ml of biotinylated anti-rat IL-18 antibody (R&D Systems) overnight at 4°C, and with 2.5 μg/ml of streptavidin-horseradish peroxidase (HRPO) for 30 min at 37°C. Then, 3,3', 5,5'-tetramethylbenzidine (TMB) (Sigma Chemical, St. Louis, MO, USA) solution was added (50 μl/well) and incubated for 30 min at room temperature in the dark to develop color. The reaction was stopped by adding stop solution (1N H_2SO_4), and OD values at 450 nm were measured by a microplate reader system (Bio-Rad, CA, USA). IL-18 concentrations were calculated using the MPM-III computer program (Bio-Rad). Serum IL-12 p40 and IL-12p70 were measured by an ELISA kit purchased from BioSource (CA, USA). Serum levels of IFN-γ, IL-4, IL-6, IL-10, and TNF-α were measured by Bio-Plex™ Suspension Array System (Bio-Rad, CA, USA). IFN-γ in the culture of splenocytes was assayed by an ELISA kit purchased from BioSource. NO metabolites were determined by a kit consisting of Griess reagents (Dojin Chemical Laboratory Institute, Kumamoto, Japan). NO_2 in the culture supernatant of leukocytes was assayed by the Griess method.

Cell cultures

Splenocytes isolated from 4- to 10-week old SD and SDT rats were cultured in RPMI1640 medium (Sigma Aldrich) supplemented with 10% FCS, 10 mM glutamine, 20 μM 2-mercaptoethanol, penicillin (100 U/ml), and streptomycin (100 μg/ml). Leukocytes prepared from peripheral blood were cultured in the same medium lacking phenol red (Sigma Aldrich). Cells were plated on 48- or 96-well culture dishes at a cell density of 1×10^6 cells/ml, and cultured with 100 ng/ml recombinant rat IL-18 (Glaxo Smith Kline Pharmaceuticals, PA, USA) and IL-12 (R&D Systems). The supernatants were collected and stored at –80°C until use.

Histological examination

The pancreases from 4- and 9-week-old SDT rats were fixed in 4% paraformaldehyde at 4°C overnight, embedded in paraffin, and cut into 3-μm sections. The sections were stained with HE for observation under a light microscope. For immunohistochemical analysis of CD68, a marker of macrophage, sections were treated with 0.1% trypsin for 5 min at room temperature after de-paraffinization. The sections were stained by the avidin-biotin complex (ABC) method using Vectastain ABC Kit (Vector laboratories, CA, USA). The primary antibody used was mouse anti-rat CD68 (1:200 in PBS containing 1% BSA) (Serotec Ltd., Oxford, UK). They were then incubated with biotinylated second antibodies for 30 min, and then with ABC reagent for 30 min. Positive reactions were visualized by developing color with peroxidase substrate solution containing 3,3'-diaminobenzidine tetrahydrochloride (DAB) (Zymed Laboratories, San Francisco, CA).

Treatment with Cl_2MDP-liposomes

Liposome encapsulated with clodronate (25 mg/kg) or PBS-liposomes were purchased from Katayama Chemical Industries Co., Ltd. (Osaka, Japan). Six-week-old SDT rats were intravenously injected with Cl_2MDP-liposomes (1 ml/rat) or PBS-liposomes as a control once a week for 3 weeks. Seven days after the final injection, all the rats were euthanized for flow cytometory analysis of blood cells and histological analysis of the pancreas. Pancreatic islets infiltrated with inflammatory cells were counted and expressed as a proportion of the total islets of the same pancreas. More than 50 pancreatic islets were examined for each rat.

Statistical analysis

Data are expressed as mean ± SE. The statistical significance of the difference between two means was evaluated using Student's *t*-test. In these tests, *P* values of <0.05 were considered to be significant.

Results

Age-dependent changes of leukocyte numbers in SD and SDT rats

The numbers of leukocytes in SDT and SD rats increased with age. The number of leukocytes in SDT rats was 6,888 ± 599 /μl at 4 weeks of age and 21,057

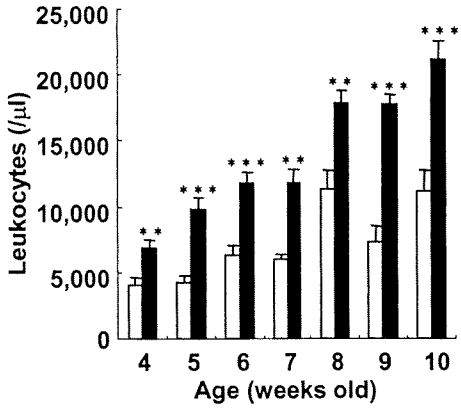

Fig. 1. Leukocytes in the blood in SD (open bars) and SDT (solid bars) rats at 4 to 10 weeks of age were counted. Data are expressed as the mean ± SE of 8 rats of each group. *P<0.05, **P<0.01, ***P<0.001, comparison between SD and SDT rats (Student's t-test).

± 1,436 /μl at 10 weeks of age. In SD rats, the leukocyte number was 4,063 ± 541 /μl at 4 weeks of age and 11,150 ± 1,574 /μl at 10 weeks of age. The number of leukocytes in SDT rats was twice as large as that in SD rats throughout the experimental period (Fig. 1). SDT rats exhibited significant leukocytosis throughout the experimental period as compared to SD rats.

Analysis of the cellular composition of blood lymphocytes by FACS

The number of $CD3^+CD4^+$ cells (helper/inducer T cells) in the circulation in SDT rats was 1,214 ± 227 /μl at 4 weeks of age and rose to 4,491 ± 332 /μl at 10 weeks of age. In SD rats, it was 994 ± 149 /μl at 4 weeks of age and 2,614 ± 352 /μl at 10 weeks of age (Fig. 2A). Thus, the number of $CD3^+CD4^+$ cells in SDT rats was about twice as large as that in SD rats. Similarly, the

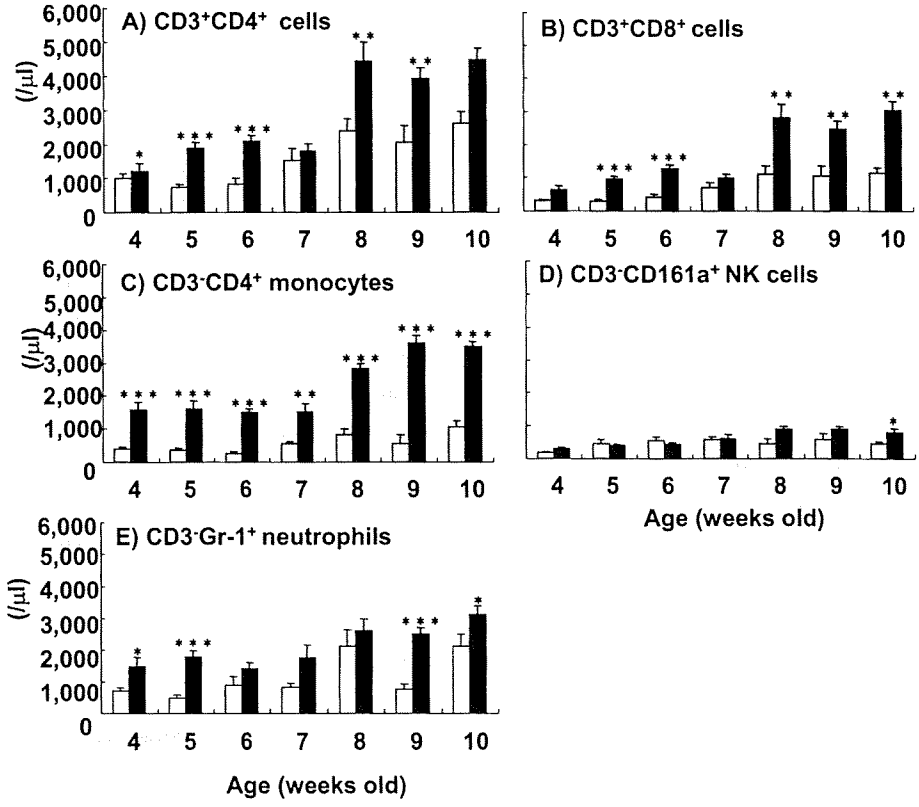

Fig. 2. Serum levels of $CD3^+CD4^+$ cells (A), $CD3^+CD8^+$ cells (B), $CD3^-CD4^+$ monocytes (C), $CD3^-CD161a^+$ NK cells (D), and $CD3^-Gr\text{-}1^+$ neutrophils (E) in SD (open bars) and SDT (solid bars) rats at 4 to 10 weeks of age were analyzed by flow cytometory. Data are expressed as the mean ± SE of 8 rats of each group. *P<0.05, **P<0.01, ***P<0.001, comparison between SD and SDT rats (Student's t-test).

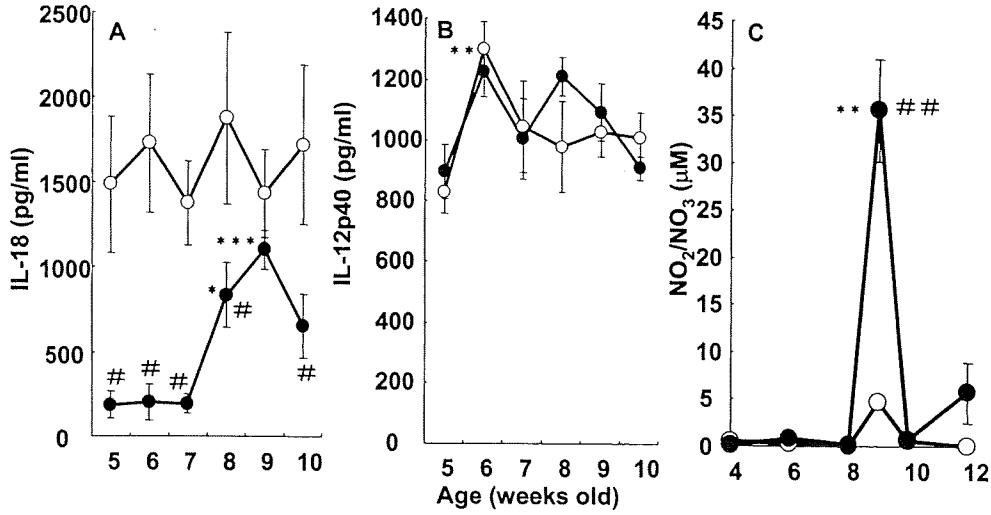

Fig. 3. Serum levels of IL-18 (A), IL-12p40 (B), and NO_2/NO_3 (C) were analyzed in SD and SDT rats of different ages. Open and closed circles indicate SD and SDT rats, respectively. Data are expressed as the mean ± SE of 8 rats. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, comparison with 4- or 5-week-old rats and $^{\#}P<0.05$, $^{\#\#}P<0.01$, comparison between SD and SDT rats (Student's *t*-test).

number of $CD3^+CD8^+$ cells (suppressor/killer T cells) was larger in SDT rats than in SD rats (Fig. 2B). The number of $CD3^-CD4^+$ monocytes in SDT rats was more than three times as large as that in SD rats (Fig. 2C). The number of $CD3^-CD161a^+$ NK cells was small throughout the experimental period (Fig. 2D). The number of $CD3^-Gr\text{-}1^+$ neutrophils in SDT rats was significantly larger than that in SD rats (Fig. 2E). Thus, $CD3^+CD4^+$ cells, $CD3^+CD8^+$ cells, $CD3^-CD4^+$ monocytes, and $CD3^-Gr\text{-}1^+$ neutrophils were significantly larger in number in SDT rats than in SD rats at 4 to 10 weeks of age.

Serum levels of IL-18, IL-12p40, IL-12p70, IFN-γ, IL-4, IL-6, IL-10, TNF-α, and NO metabolites in SD and SDT rats

Expression of IL-12p40, IL-18, IFN-γ, and iNOS is known to be augmented in NOD mice [12, 25, 26, 31]. We analyzed whether SDT rats also produce these molecules at high levels. Serum levels of IL-18 in SD rats ranged from 1,300 to 1,900 pg/ml throughout the experimental period (Fig. 3A); those in SDT rats ranged from 180 to 200 pg/ml at 5 to 7 weeks of age, increasing to 1,234 ± 314 pg/ml at 9 weeks, and then decreasing to 651 ± 190 pg/ml at 10 weeks. Thus, serum IL-18 formed a prominent peak at 9 weeks of age in SDT rats (Fig. 3A). Serum levels of IL-12p40 ranged from 800 to 1,300 pg/ml throughout the experimental period both in SD and SDT rats, and there was no significant difference between them (Fig. 3B). Serum levels of IL-12p70 were not detected throughout the experimental period both in SD and SDT rats (data not shown). There were also no significant differences in the levels of IFN-γ, IL-4, IL-6, IL-10, and TNF-α (data not shown).

Serum NO_2/NO_3 levels in SD rats were below 5.0 μM throughout the experimental period, while in SDT rats, they were less than 5.0 μM at 4 to 8 weeks of age, transiently increasing to 35.5 ± 5.4 μM at 9 weeks, and then decreasing to below 5.0 μM at 10 weeks (Fig. 3C).

IFN-γ production induced by IL-12 and IL-18 in splenocytes of SD and SDT rats

IFN-γ produced in the culture of splenocytes from 4- to 10-week-old SD and SDT rats in the presence of IL-12 was less than 50 pg/ml. In the presence of IL-18, levels of IFN-γ produced in the culture of splenocytes from 4- to 8-week-old SDT rats were 4,000 to 6,700 pg/ml, 11,623 ± 3,244 pg/ml in 9-week-old SDT rats, and were 2,001 ± 400 pg/ml in 10-week-old SDT rats. In the presence of IL-18, splenocytes from SDT rats of 9 weeks of age produced higher levels of IFN-γ than those from age-matched SD rats (Fig. 4A). Although a com-

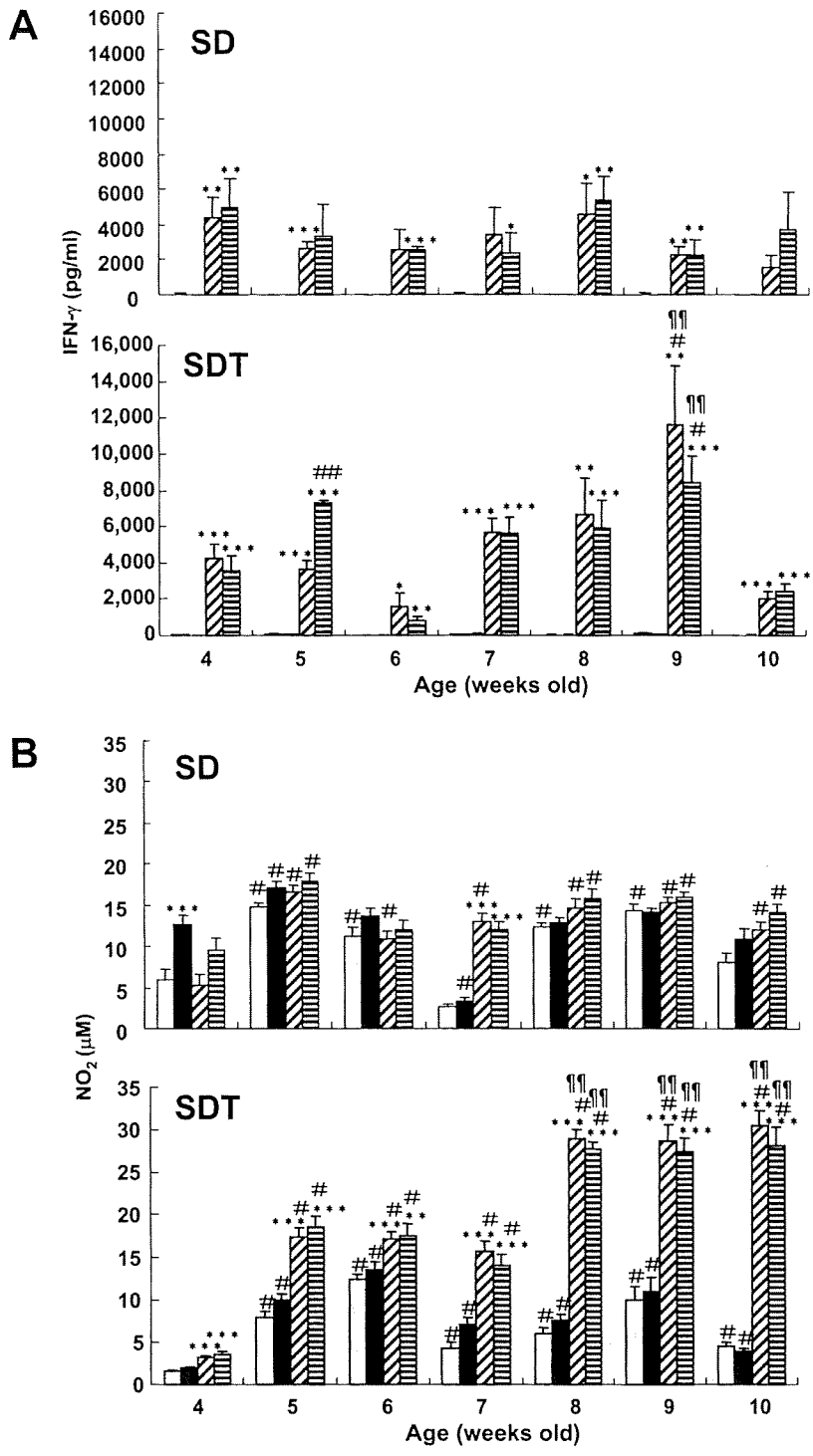

Fig. 4. Cytokine-induced IFN-γ production in splenocytes and NO_2 production in peripheral leukocytes isolated from SD and SDT rats *in vitro*. (A) SD and SDT splenocytes from SD and SDT rats of 4 to 10 weeks of age were cultured for 48 h with none (open columns), IL-12 (closed columns), IL-18 (shaded columns), and IL-12+IL-18 (hatched columns) and assayed for IFN-γ. Data are expressed as the mean ± SE (n=8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, comparison with none, and $^{\#}P<0.05$, $^{\#\#}P<0.01$, comparison with 4- week old SD and SDT rats, and $^{¶¶}P<0.01$, comparison between SD and SDT rats (Student's *t*-test). (B) SD and SDT peripheral leukocytes from SD and SDT rats of 4 to 10 weeks of age were cultured for 48 h with none (open columns), IL-12 (closed columns), IL-18 (shaded columns), and IL-12+IL-18 (hatched columns) and assayed for NO_2. Data are expressed as the mean ± SE (n=8). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, comparison with none, and $^{\#}P<0.05$, comparison with 4-week-old SD and SDT rats, and $^{¶¶}P<0.01$, comparison between SD and SDT rats (Student's *t*-test).

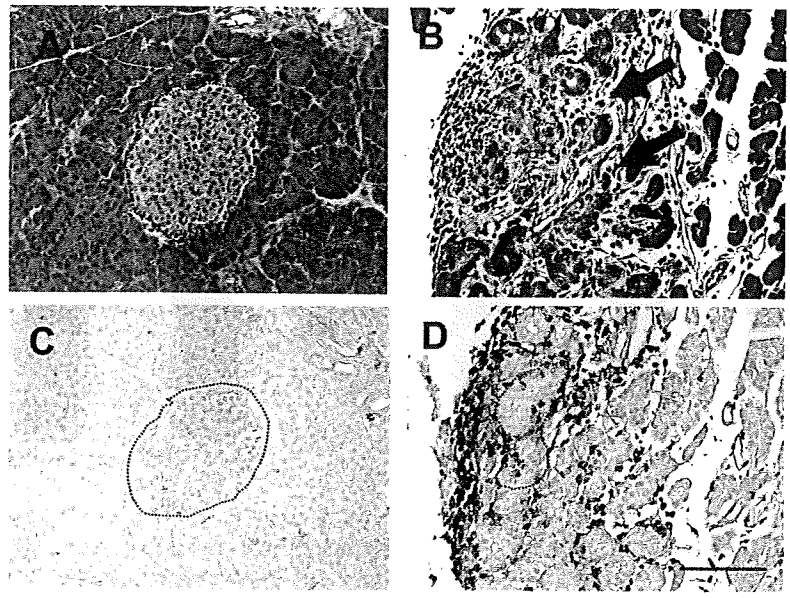

Fig. 5. Histological changes in the pancreatic islets of SDT rats. Paraffin-embedded sections of the pancreas of 4- and 9-week-old SDT rats were stained with HE or anti-CD68 antibody and examined under a microscope. (A) An intact pancreatic islet of a 4-week-old SDT rat (HE), (B) inflammatory cell infiltration (arrows) in the islet of a 9-week-old SDT rat (HE), (C) immunohistochemical staining of macrophages with CD68 (brown) in the islet of a 4-week-old SDT rats, (D) immunohistochemical staining of macrophages with CD68 (brown) in the islet of a 9-week-old SDT rats. (Scale bar =100 μm).

bination of IL-12 and IL-18 is known to synergistically upregulate IFN-γ production in lymphocytes, the combination failed to do so in this study. These results show that the ability to produce IFN-γ in response to IL-18 was augmented in the splenocytes of 9-week-old SDT rats.

NO_2 production in SD and SDT rats peripheral leukocytes induced by IL-12 and IL-18

In the presence of IL-12, NO_2 produced in the culture of leukocytes from SD and SDT rats was less than 17 μM, with no significant age-dependent differences (Fig. 4B). In the presence of IL-18, NO_2 production increased with age: 3.1 ± 0.3, 15.0–17.0, and 30.0 μM for 4-, 5- to 7-, and 8- to 10-week-old rats, respectively (Fig. 4B). NO_2 production was more efficiently induced by IL-18 in the splenocytes from SDT rats at around 9 weeks of age than in the cells from age-matched SD rats (Fig. 4B). A combination of IL-12 and IL-18 did not exhibit a synergistic effect on the production of NO_2 (Fig. 4B)

Histological examination of the pancreas of SDT rats

Histological examination of the pancreas showed that the islets of 4-week-old SDT rats were free from infiltration by inflammatory cells (Fig. 5A). However, in the pancreases of 9-week-old SDT rats, inflammatory cells were observed in and around the islets (Fig. 5B). Immunohistochemical analysis using antibody to CD68 (macrophage marker) showed vigorously infiltrating $CD68^+$ cells in the pancreas of 9-week-old SDT rats, while they were absent in the pancreases of 4-week-old rats (Figs. 5C and 5D).

Treatment with Cl_2MDP-liposomes reduced the number of leukocytes, monocytes, and levels of NO metabolites in the circulation and inhibited macrophage invasion in the islets

We examined the effect of removal of monocytes/macrophages with Cl_2MDP-liposomes on the number of monocytes in the circulation and on the infiltration of macrophages into the islets of 9-week-old SDT rats (Fig. 6). We found a significant reduction in the number of

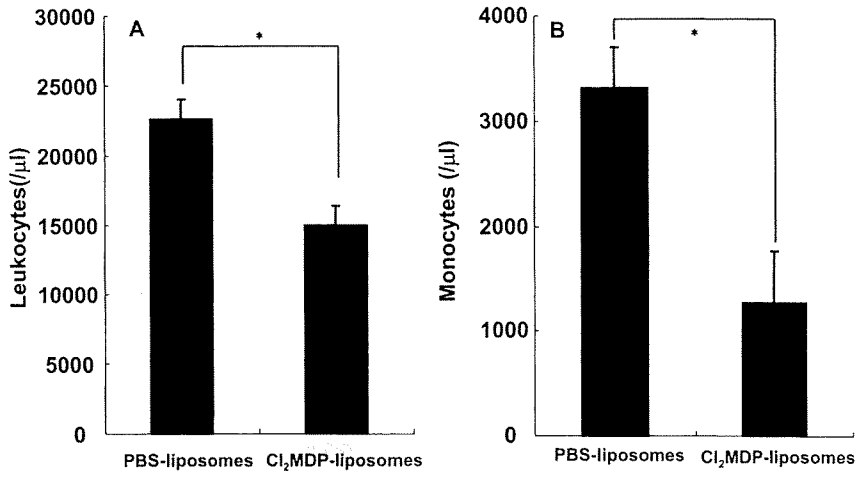

Fig. 6. Reduction of leukocytes and monocytes in SDT rats by treatment with Cl_2MDP-liposomes. (A) Leukocytes in SDT rats treated with PBS-liposomes (control) and Cl_2MDP-liposomes. (B) Monocytes in SDT rats treated with PBS-liposomes (control) and Cl_2MDP-liposomes. Data are expressed as the mean ± SE of three rats of each group. $*P<0.05$, comparison between PBS-liposomes and Cl_2MDP-liposomes (Student's *t*-test).

circulating leukocytes (Fig. 6A), monocytes in particular (Fig. 6B).

Cl_2MDP-liposome treatment also resulted in a reduction in the number of infiltrating macrophages in the pancreas of SDT rats. Treated rats showed essentially no fibrosis in and around the islets and much fewer infiltrating cells in the islets (Figs. 7B and 7E) as compared to control rats (Fig. 7A). In particular, only a few infiltrating $CD68^+$ cells were observed in the islets of treated rats (Fig. 7D). These results show that treatment of SDT rats with Cl_2MDP-liposomes improved infiltration of macrophages in the pancreas. Treatment of Cl_2MDP-liposomes also inhibited an increase of NO metabolites in serum of SDT rats. As expected from the histological results, Cl_2MDP-liposome treatment suppressed serum NO_2/NO_3 levels in 9-week-old SDT rats (Figs. 3C and 7F).

Discussion

It has been reported that higher white blood cell counts may predict the development of impaired fasting glucose or type 2 diabetes in middle-aged Japanese men [21]. For adults in the United States, raised leukocyte counts have been shown to be associated with type 2 diabetes onset [8]. However, the significance of this leukocytosis in relation to diabetes has not been elucidated. SDT rats have been considered to develop type 2 diabetes. In the present study, the roles of inflammatory cells in the destruction of β-cells in the pancreatic islets were examined. We found that SDT rats exhibited significant leukocytosis at 4 to 10 weeks of age in comparison to SD rats (Fig. 1). Leukocytes that increased in number in SDT rats at 8 to 10 weeks of age included $CD3^+CD4^+$ cells (Fig. 2A), $CD3^+CD8^+$ cells (Fig. 2B), $CD3^-CD161a^+$ NK cells (Fig. 2D), $CD3^-Gr\text{-}1^+$ neutrophils (Fig. 2E), and most notably $CD3^-CD4^+$ monocytes (Fig. 2C). Concomitantly, serum levels of IL-18 (Fig. 3A) and NO_2/NO_3 (Fig. 3C) increased in 9-week-old SDT rats. No such changes were observed in SD rats (Figs. 3A and 3C). However, in the presence of IL-18, splenocytes from SDT rats, particularly those at 9 weeks of age produced high levels of IFN-γ (Fig. 4A). Levels of NO_2 in leukocytes from SDT rats were also elevated in the presence of IL-18 (Fig. 4B). Serum NO_2/NO_3 levels were lower in 10-week-old SDT rats than those in 9-week-old SDT rats (Figs. 3A and 3C), which may reflect a rapid decrease in circulating IL-18 levels after 9 weeks of age. It has been demonstrated that excess NO production plays a crucial role in islet injury in NOD mice [6]. In

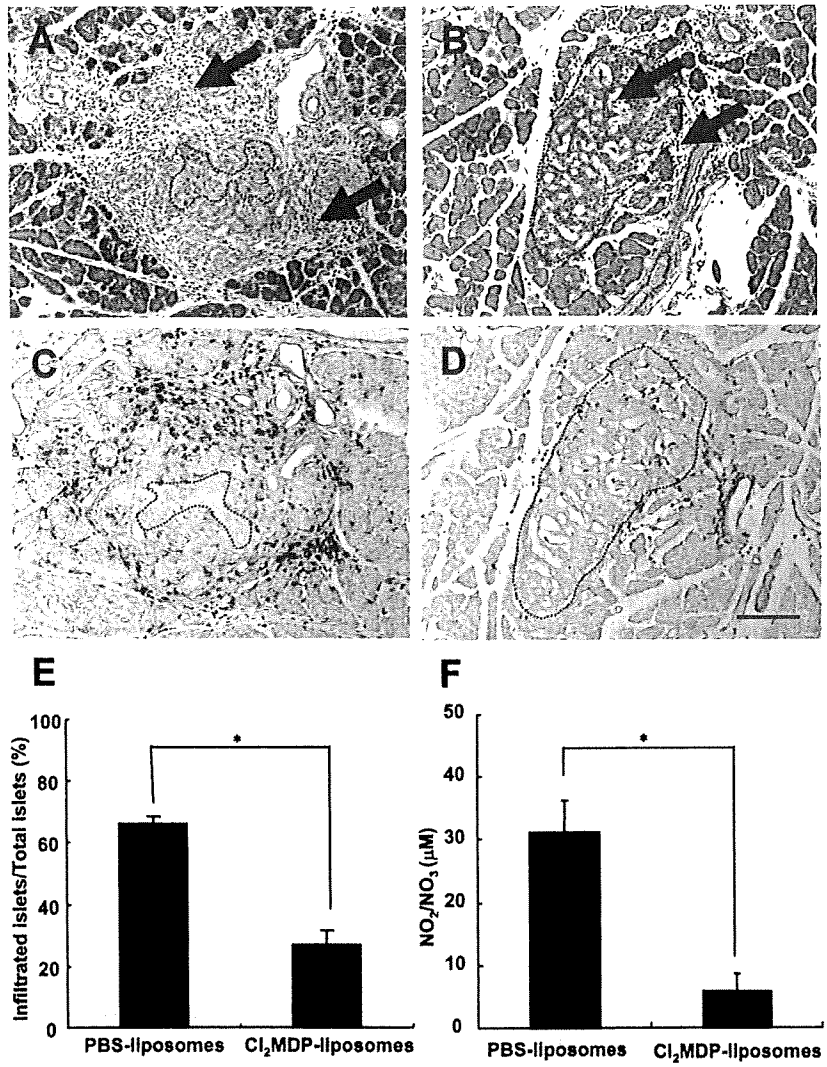

Fig. 7. Effect of Cl_2MDP-liposomes treatment on histological changes of the pancreatic islets of 9-week-old SDT rats and serum NO_2/NO_3 concentration. (A) An islet of a 9-week-old SDT rat treated with PBS-liposomes (HE), (B) An islet of a 9-week-old SDT rats treated with Cl_2MDP-liposomes (HE). Arrows show inflammatory cells. (C) Macrophages stained with CD68 (brown) in an islet of a 9-week-old SDT rat treated with PBS-liposomes (control). (D) Macrophages stained with CD68 (brown) in an islet of a 9-week-old SDT rat treated with Cl_2MDP-liposomes. (Scale bar =100 μm). (E) Comparison of inflammatory cell infiltration of pancreatic islets of 9-week-old SDT rats treated with PBS-liposomes (control) and Cl_2MDP-liposomes. Islets infiltrated with inflammatory cells were counted and expressed as a proportion of the total islets of the same pancreas. More than 50 pancreatic islets were analyzed per rat. (F) Concentration of serum NO_2/NO_3 in 9-week-old SDT rats treated with PBS-liposomes (control) and Cl_2MDP-liposomes. Data are expressed as the mean ± SE of three rats of each group. Data are expressed as the mean ± SE of 3 rats of each group. *P<0.05, comparison between PBS-liposomes and Cl_2MDP-liposomes (Student's t-test).

SDT rats, high levels of production of NO as well as those of IL-18 were transient and in parallel (Figs. 3A and 3C). The reason for this finding was not clear. However, it has been reported that IL-18, when administered to mice, causes production of a large amount of NO in an IFN-γ-dependent manner [4]. Since IL-18 stimulates

IFN-γ production, it is probable that IL-18 activates IRF-1 that is known to be activated via IFN-γ. In addition, IL-18 has been shown to activate NF-κB and NF-AT, which are essential for IFN-γ production [35]. Thus, IL-18 is likely to be involved in iNOS induction.

IL-12 is a proinflammatory cytokine. The biologically active IL-12 is a 70-kDa heterodimer (IL-12p70), with 40-kDa (p40) and 35-kDa (p35) subunits [10]. IL-12p40 is expressed and secreted in large excess over IL-12p70 [11, 24]. It has been demonstrated that IL-12p40 blocks the activities of IL-12p70, leading to the suggestion that IL-12p40 serves as a natural antagonist of IL-12p70 [9]. In this study, serum levels of IL-12p40 ranged from 800 to 1,300 pg/ml throughout the experimental period in SDT rats (Fig. 3B), and serum levels of IL-12p70 were not detected throughout the experimental period (data not shown), suggesting that the SDT rats were in an immune-suppressive condition.

It has been suggested that elevated levels of IL-18 predict the development of type 2 diabetes [34]. However, in young NOD mice developing type 1 diabetes, systemic administration of IL-18 promotes the development of diabetes [23] and IL-18 blockade with IL-18-binding protein (BP) delays its onset [39]. IL-18 also contributes to the injury of islets in diabetic mice induced by multiple low doses of streptozotocin (STZ) [22]. These results suggest that IL-18 is profoundly involved in the pathogenesis of type 1 diabetes through upregulation of IFN-γ and NO synthesis. The present study using SDT rats suggests that IL-18 is involved in the development of type 2 diabetes in a way similar to that of type 1 diabetes.

Masuyama *et al.* have reported the presence of inflammatory cells in and around the islets of 10-week-old SDT rats, suggesting that inflammatory cell infiltration is an important factor in islet destruction [20].

It has been shown that a large number of macrophages are present in the pancreas of neonatal NOD mice [36]. Macrophages are among the first cells to infiltrate the islets, and when fully activated can exert cytotoxicity against β-cells via excess production of TNF-α and reactive oxygen intermediates such as NO [18, 32, 33]. In this study, we found infiltration of macrophages in and around the islet of 9-week-old SDT rats (Fig. 5D). This occurred in parallel with raised serum levels of IL-18 and NO_2/NO_3, forming a prominent peak at around 9 weeks of age. These results suggest that infiltrating macrophages produce a large amount of NO by the induction of IL-18 and IFN-γ, resulting in damage to the islets. The mechanism of the progressive injury of the islets after 9 weeks of age was not explored in this study. Shinohara *et al.* have reported that infiltrating cells in and around the islets include lymphocytes and macrophages [30]. We speculate that the pancreatic islets of SDT rats may be injured by excess NO produced by macrophages at 9 weeks of age, and subsequently by infiltrating lymphocytes.

Cl_2MDP-liposomes are known as a potent anti-macrophage agent [38] which is useful for the treatment of various diseases in animal models [1, 13]. In this study, we found that treatment of 6-week-old SDT rats with Cl_2MDP-liposomes prevented infiltration of macrophages into the pancreas (Figs. 7D and 7E). At the same time, serum NO levels were low in the treated SDT rats as compared to control rats (Fig. 7F).

In summary, treatment with Cl_2MDP-liposomes decreased serum levels of NO_2/NO_3 and reduced invasion of macrophages in the pancreatic islets of SDT rats. This suggests that macrophages play an important role in pancreatic islet injury in SDT rats.

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