Original

Segmentation of the Pathophysiological Stages of Diabetic Changes in the *db/db* Mouse

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Abstract: The *db/db* mouse is one of the diabetes mellitus animal models and if the pathophysiological stages of diabetic changes in the mouse model could simulate the stages in human diabetes, the *db/db* mouse could be used to better evaluate drug candidates. Blood insulin, HbA1c levels and morphological features of pancreatic islets in *db/db* mice were evaluated to determine the pathophysiological stage. At 6 weeks of age, *db/db* mice showed the highest level of plasma insulin and lowest level of HbA1c, and histopathological examination revealed enlarged islets with a circular shape and hypertrophic islet cells. By 9 and 12 weeks of age, the plasma insulin levels had decreased to mid levels and HbA1c had increased to mid to high levels; histopathological examination at this time revealed two types of islets coexisting, enlarged circular islets and small irregular-shaped islets. By 15 and 22 weeks of age, plasma insulin had decreased further to low levels and HbA1c was at its highest level; the histopathological examination at this time revealed an increase in irregular-shaped and small islets. Based on blood insulin levels, HbA1c levels and histopathology findings in the *db/db* mice in this study, the clinical staging of diabetic changes were recognized. The pathophysiological stages of diabetes mellitus in this animal model were similar to the stages in humans. (J Toxicol Pathol 2009; **22**: 133–137)

Key words: *db/db* mouse, pancreas, histopathology, diabetes mellitus, insulin, HbA1c

Introduction

Diabetes mellitus is clinically classified into three stages based on the degree of insulin dependence. The stages are non-insulin requiring (NIR), insulin requiring for control of blood glucose (IRC) and insulin requiring for survival (IRS)[1–4]. A patients' pharmacologic treatment is selected based on these stages because combinations of different oral agents may be useful for controlling hyperglycemia before insulin therapy becomes necessary[3, 5]. At the NIR stage, adequate glycemic control can be achieved through weight reduction, exercise and/or oral glucose-lowering agents, and so individuals at this stage do not require insulin. Individuals at the IRC stage have some residual insulin secretion, but require exogenous insulin for adequate glycemic control, and can also survive without taking insulin. Individuals at the IRS stage with extensive *β*-cell destruction and therefore no residual insulin secretion require insulin for survival.

The C57BL/KsL *db/db* mouse (*db/db* mouse) is a diabetes mellitus animal model that is a spontaneous mutant strain of the C57BL/KsJ *db/db* mouse resulting from a point mutation of the downstream intron of the leptin receptor gene rendering it unresponsive to leptin[6–9]. Leptin is a peptide hormone secreted by adipocytes and is involved in eating behavior and energy homeostasis. So, this animal models expresses unrepressed eating behavior, becomes obese and develops severe insulin resistance associated with hyperinsulinemia and hypertriglyceridemia, followed by hyperglycemia peaking at 3–4 months of age[10]. Pancreatic islet *β*-cell mass is reduced as disease progresses, resulting in severe insufficiency of insulin secretion[11–14]. It has also been well shown by immunohistochemistry that a decrease in insulin levels of islets of db/db mice occurs at 18 weeks of age, without referring to the blood insulin levels, which is one of the most important biomarkers[6]. In spite of the extensive use of the *db/db* mouse in this field, there are no reports on the three clinical stages in the *db/db* mouse. However, if pathophysiological staging were possible in the *db/db* mouse, drug candidates for diabetes mellitus could be better evaluated in preclinical studies to selectively target a specific pathophysiological stage.

In this study, time course blood insulin and glycosylated hemoglobin (HbA1c) levels, the clinical

parameters for evaluation of the pathophysiological stages of diabetes mellitus in humans[3, 15], and morphological features of pancreatic islets in *db/db* mice were examined in order to determine the pathophysiological stage of the disease in the diabetic mouse model. Both the American Diabetes Association (ADA) and the American Association of Clinical Endocrinologists (AACE) recommend monitoring glycemic control using HbA1c as the parameter[15–18]. The major advantage of measuring HbA1c is that a specimen can be collected without regard to when the patient last ate[19].

Materials and Methods

Animals

Twenty five male *db/db* mice were purchased from Charles River Laboratories (Japan) and subjected to experimentation at 5 weeks of age. The animals were housed in cages in an animal room maintained at a temperature of 23 ± 2°C and a humidity of 55 ± 10%, with 14 to 16 air changes per hour and a 14-hour light and 10-hour dark cycle. The animals were given pelleted chow (CE-2; Clea Japan, Inc., Tokyo, Japan) and tap water *ad libitum*. All animal procedures were conducted in accordance with Chugai Pharmaceutical's *Guide for the Care and Use of Laboratory Animals*, and all experimental protocols were approved by the Institutional Animal Care and Use Committee.

Experimental design

The *db/db* mice were divided into 5 groups (n=5 per group), and the animals of each group were sacrificed by exsanguination under ether anesthesia at the age of 6, 9, 12, 15 or 22 weeks after their body weights were measured and blood samples were collected. Blood samples were obtained from the caudal vena cava for measurement of plasma insulin and HbA1c.

Plasma insulin levels were measured using ELISA (Institute of Biological Science, Inc., Yokohama, Japan), and the percentage of HbA1c was measured using an auto analyzer (Type 7170, Hitachi High-Technologies Corporation, Tokyo, Japan). The pancreas was removed from all necropsied animals, fixed in 20% neutral buffer formalin solution, embedded in paraffin wholly, sectioned longitudinally and stained with hematoxylin and eosin. Histopathological evaluation of pancreatic islets was performed under light microscopy.

Results

At 6 weeks of age, the mean body weight was 33.67 ± 0.39 g, the level of the plasma insulin was at its highest (16.82 ± 1.03 ng/mL) and the level of HbA1c was at its lowest (2.06 ± 0.14%) during the observation period (Fig. 1). Histopathological examination revealed enlarged, circular-shaped islets, which were consistent with hypertrophic islet cells having abundant cytoplasm and a large vacuole with a curved lucent region considered to be the Golgi apparatus[20]
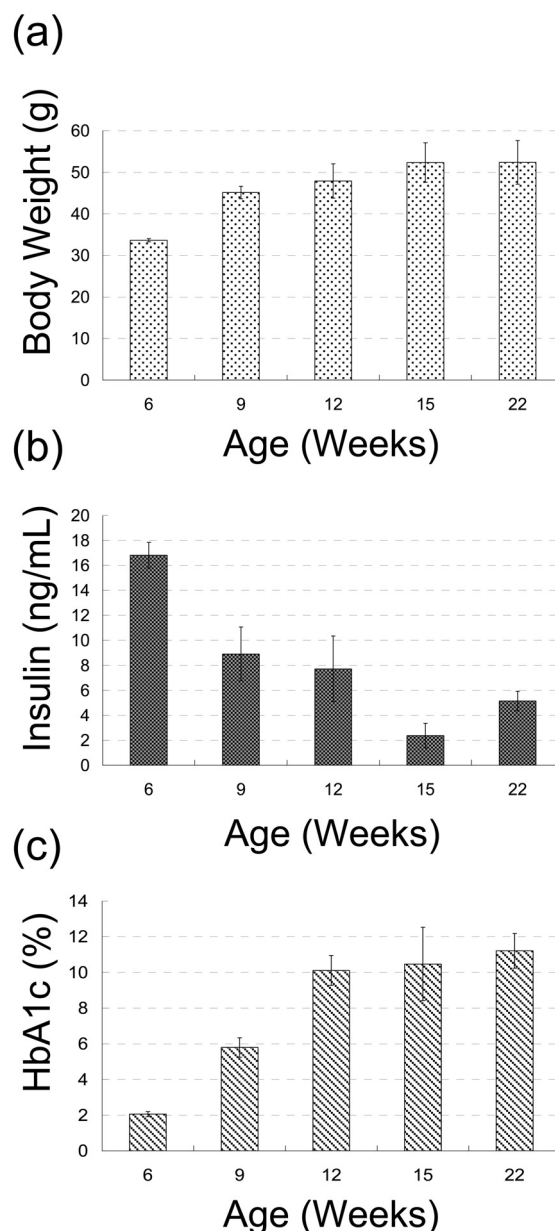

Fig. 1. Time course of changes of body weight (a), plasma insulin level (b) and HbA1c level (c) in the *db/db* mice. Each value represents the mean ± standard deviation.

(Fig. 2a).

At 9 and 12 weeks of age, the mean body weights were 45.13 g ± 1.50 and 47.93 g ± 4.14, respectively. The level of plasma insulin had decreased to mid levels (8.92 ± 2.14 ng/mL and 7.73 ± 2.61 ng/mL, respectively), and the level of HbA1c had increased to middle to high mean levels (5.79 ± 0.54% and 10.11 ± 0.84%; Fig. 1). In the histopathological examination, two types of islets were coexisting: enlarged islets similar to those observed in the 6-week-old animals (Fig. 2b) and small, irregular-shaped islets consisting of atrophic islet cells with acinar cells and spindle cells thought
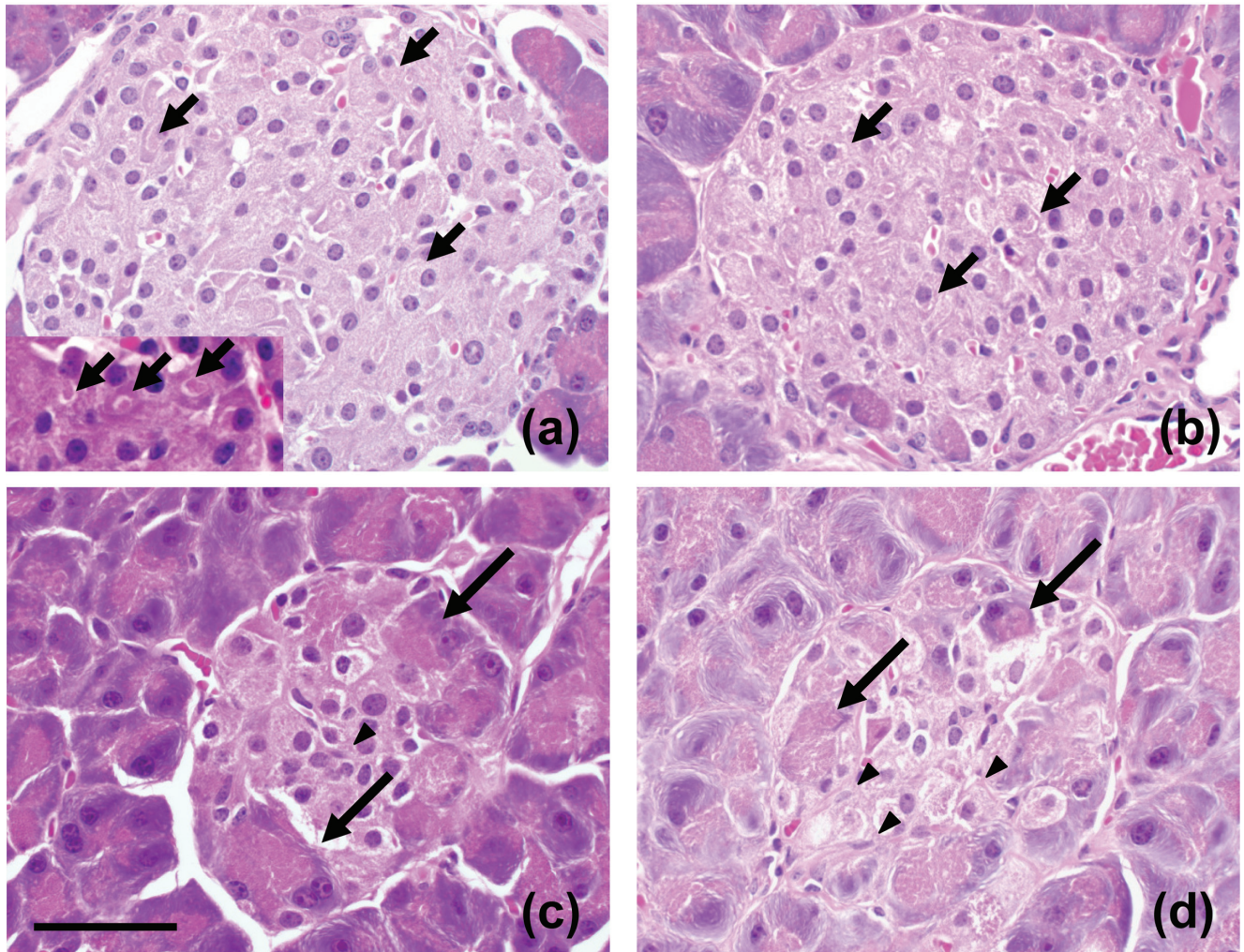
Fig. 2. The morphological features of pancreatic islets in *db/db* mice. Enlarged, circular islet at 6 weeks of age and islet cells with vacuoles (insert, × 600) (a), enlarged islet at 12 weeks of age (b), small irregular-shaped islet at 12 weeks of age (c) and a smaller and irregular-shaped islet at 22 weeks of age (d). Short arrows: large vacuoles considered to be Golgi apparatus. Long arrows: acinar cells, Arrowheads: spindle cells. Magnification: × 400. HE stain. Bar: 50 μm.

to be myofibroblasts[21] (Fig. 2c).

At 15 and 22 weeks of age, the mean body weights were 52.35 ± 4.75 g and 52.37 ± 5.24 g, respectively, the level of plasma insulin had decreased further to low mean levels (2.38 ± 0.99 ng/mL and 5.15 ± 0.78 ng/mL, respectively) and the level of HbA1c had increased to high mean levels (10.47 ± 2.06% and 11.21 ± 0.97%; Fig. 1). Histopathological examination revealed that the irregular-shaped islets had decreased further in size. Islet cells were even more atrophic than in the 12-week-old animals, and the presence of acinar cells and spindle cells was obvious (Fig. 2d).

Discussion

Staging of diabetes mellitus is clinically based on insulin dependence, namely non-insulin requiring (NIR), insulin requiring for control (IRC) of blood glucose and insulin requiring for survival (IRS)[1–4]. The purpose of this study was to determine the pathophysiological stage of diabetes mellitus in *db/db* mouse at various ages and discuss the relevance of the stages in the animal model to those in humans.

In patients at the NIR stage of diabetes mellitus, adequate glycemic control can be achieved with weight reduction, exercise and/or oral glucose-lowering agents. The patients in this stage have insulin secretion ability, and the glucose level is occasionally high but controllable. In this study, the 6-week-old *db/db* mice can be considered equivalent to being in the NIR stage of human diabetes mellitus patients according to the following results: the animals showed obesity, unlike the nonobese heterozygote (*db/+*) mouse[6], hypertrophic islet cells were observed in the histopathological examination and the findings well reflected blood parameters. These findings are considered to
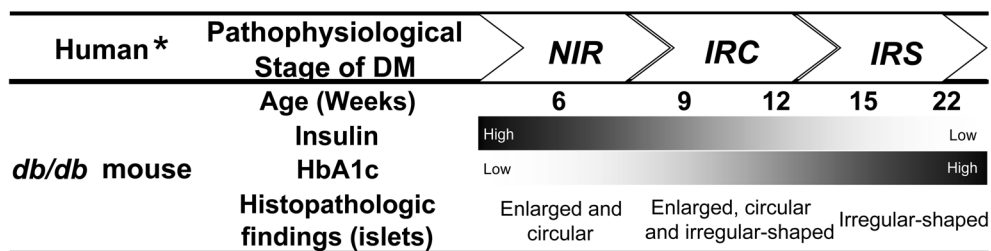
Human*	Pathophysiological Stage of DM	NIR		IRC		IRS	
db/db mouse	Age (Weeks)	6	9	12	15	22	
	Insulin	High				Low	
	HbA1c	Low				High	
	Histopathologic findings (islets)	Enlarged and circular	Enlarged, circular and irregular-shaped		Irregular-shaped		

Fig. 3. Pathophysiological stages of DM in humans and as represented in the *db/db* mouse. DM: Diabetes Mellitus. *The human column is quoted from reference 1–4.

be an insulin secretion reaction of islet cells caused by hyperphagia, a characteristic of the *db/db* mouse[6–14]. Based on the blood parameters of the animals at this stage, sufficient insulin secreting ability (the highest insulin level) was preserved to counteract high blood glucose levels.

At the IRC stage in human diabetes mellitus, patients have some residual insulin secretion but require exogenous insulin for adequate glycemic control. The findings in the *db/db* models at 9 and 12 weeks of age were equivalent to the IRC stage. The animals showed mid-level residual insulin secretion and mildly increased blood glucose level (mid levels of HbA1c). The histopathological findings showed some irregular-shaped islets, which correlates to the existence of acinar cells and spindle cells. It is known that the fibrotic distraction of islets is commonly observed in humans and several animal models of type 2 diabetes[21].

At 15 and 22 weeks, insulin secretion in the mice was very low (low levels of insulin), because islets were destructed (irregular-shaped and small), and the blood glucose levels had increased (high levels of HbA1c). In addition, the decrease of insulin in the islets of db/db mice at 18 weeks of age was shown well in a previous report[6], and the morphology of islet is similar to that in this study. Our results indicate that the sustained secretion of insulin induced β-cell destruction and that the ability to secrete insulin had almost disappeared. We consider these findings to be equivalent to the IRS stage in humans[21].

In the *db/db* mouse used in this study, three distinctive pathophysiological stages of diabetic change were identified as shown in Fig. 3. The results elucidate a time course relationship between the blood parameters and the morphology of pancreatic islets in the *db/db* mouse, where pathophysiological stages based on plasma insulin levels, HbA1c levels and histopathological findings are clearly distinguishable and characteristically reflect the stages of human diabetes mellitus. To our knowledge, this is the first report of a *db/db* mouse exhibiting pathophysiological stages similar to those in human diabetes mellitus. In the evaluation of new drug candidates for diabetes mellitus, indicating the time point in the life cycle of the disease would be very important, as the pharmacologic effects of a drug must be selected to match patient status[3, 5]. In addition, there is evidence that environmental factors contribute to the development of obesity and type 2 diabetes[22]. Being able to understand the pathophysiological stage of an animal model based on blood glucose and insulin would be beneficial.

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