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Probucol preserves pancreatic β-cell function through reduction of oxidative stress in type 2 diabetes

Shin-ichi Gorogawa, Yoshitaka Kajimoto *, Yutaka Umayahara, Hideaki Kaneto, Hirotaka Watada, Akio Kuroda, Dan Kawamori, Tetsuyuki Yasuda, Munehide Matsuhisa, Yoshimitsu Yamasaki, Masatsugu Hori

Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka Pref. 565-0871, Japan

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Abstract

Oxidative stress is induced under diabetic conditions and causes various forms of tissue damage in patients with diabetes. Recently, pancreatic β-cells have emerged as a putative target of oxidative stress-induced tissue damage and this seems to explain in part the progressive deterioration of β-cell function in type 2 diabetes. As a step toward clinical trial of antioxidant for type 2 diabetes, we investigated the possible anti-diabetic effects of probucol, an antioxidant widely used as an anti-hyperlipidemic agent, on preservation of β-cell function in diabetic C57BL/KsJ-db/db mice. Probucol-containing diet was given to mice from 6 to 16 weeks of age. Immunostaining for oxidative stress markers such as 4-hydroxy-2-nonenal (HNE)-modified proteins and heme oxygenase-1 revealed that probucol treatment decreased reactive oxygen species (ROS) in pancreatic islets of diabetic animals. Oxidative stress is known to enhance apoptosis of β-cells and to suppress insulin biosynthesis, but probucol treatment led to preservation of β-cell mass and the insulin content. According to intraperitoneal glucose tolerance tests, the probucol treatment preserved glucose-stimulated insulin secretion and improved glucose tolerance at 10 and 16 weeks: insulin, 280 ± 82 vs. 914 ± 238 pmol/l (120 min, at 16 weeks; $P < 0.05$); glucose, 44.6 ± 2.4 vs. 35.2 ± 2.6 mmol/l (120 min, at 16 weeks; $P < 0.05$). Thus, our present observations demonstrate the potential usefulness of probucol for treatment of type 2 diabetes. © 2002 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Probucol; Glucose toxicity; Glucotoxicity; Oxidative stress; Antioxidant

1. Introduction

Development of type 2 diabetes is associated with pancreatic β-cell dysfunction that is coupled to peripheral and hepatic insulin resistance. Normal β-cells can compensate for insulin resistance

* Corresponding author. Tel.: +81-6-6879-3633; fax: +81-6-6879-3639.

E-mail address: kajimoto@medone.med.osaka-u.ac.jp (Y. Kajimoto).

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by increasing insulin secretion, but insufficient compensation leads to the onset of glucose intol-erance. Once hyperglycemia becomes apparent, β-cell function progressively deteriorates: glucose-induced insulin secretion becomes further impaired and degranulation of β-cells becomes evident, often accompanied by a decrease in the number of β-cells [1–3]. To date, lines of evidence have demonstrated that hyperglycemia per se is a major factor involved in progressive impairment of β-cell function in type 2 diabetes [4–9]. Although this phenomenon has been known for years as a clinical concept, glucose toxicity, its underlying mechanism was poorly understood and no therapeutic approaches other than pursuing strict glycemic control have been tried to prevent glucose toxicity. However, strict glycemic control is not always achievable and sometimes impairs quality of life with frequent incidence of hypoglycemia. Thus there is a need for a new therapeutic approach based specifically on the mechanism of glucose toxicity.

Oxidative stress has recently been shown to be responsible, at least in part, for the β-cell dysfunction caused by glucose toxicity. Under hyperglycemia, production of various reducing sugars such as glucose-6-phosphate and fructose increases through glycolysis and the polyol pathway. All of these reducing sugars are known to promote protein glycation through the Maillard reaction, which in turn produces Schiff base, Amadori product and advanced glycosylation end products (AGE). During this process, reactive oxygen species (ROS) are produced and cause tissue damage [10–12]. Also, more recently, Brownlee and his colleagues have shown that oxidative stress is induced in the mitochondrial electron transport chain in hyperglycemia and this is involved in various tissue-damaging intracellular events such as activation of protein kinase C, polyol pathway, and glycation reaction [13]. Indeed, pancreatic β-cells kept under high glucose concentration contain AGE [14] and the level of 8-hydroxy-2′-deoxyguanosine (8-OHdG), a marker of oxidative stress, is increased in β-cells of diabetic Goto–Kakizaki (GK) rats [15]. Moreover, pancreatic islet cells are probably sensitive to oxidative stress because they express a relatively low amount of antioxidant enzyme genes compared with other tissues and organs [16]. As direct support for the significance of oxidative stress in progressive deterioration of β-cell function in type 2 diabetes, we and others have recently shown that administration of an antioxidant, *N*-acetylcysteine, either alone or in combination with antioxidative vitamins, to murine models of type 2 diabetes can preserve β-cell function [17,18].

As a step toward development of a mechanism-based therapeutic approach against glucose toxicity, we examined the possible usefulness of the potent antioxidant, probucol. It has been used for years as an anti-hyperlipidemic agent and exerted beneficial effects on preventing atherosclerosis or restenosis after coronary angioplasty in various clinical trials [19–21]. We have found in this study that the probucol treatment preserves β-cell mass and function and improves glucose tolerance in a mouse model of type 2 diabetes.

2. Material and methods

2.1. Experimental design

C57BL/KsJ-db/db female mice at 6 weeks of age were purchased from Clea Japan, Tokyo, Japan. The mice were allowed free access to food and water in a specific pathogen-free environment. At 6 weeks of age, the mice were divided into two groups, with one group kept on a regular diet and the other group started on a probucol-containing diet (1.0%). At 10 and 16 weeks of age, the mice were subjected to physiological or histological analyses. The animal studies were conducted in accordance with the 'Principles of laboratory animal care' given in NIH publication No. 85-23.

2.2. Glucose tolerance test

After an overnight fast, the mice were injected intraperitoneally with glucose at 1.0 g/kg body weight. Blood samples were taken at various time points (0–120 min) and used to measure plasma glucose level and plasma insulin level. Plasma glucose concentration was measured with a Beckman Glucose Analyzer II (Beckman Instruments,

Fullerton, CA), and plasma insulin concentration was determined using a Lebis® Radioimmunoassay kit (Shibayagi, Gunma, Japan) with mouse insulin as the standard.

2.3. Insulin tolerance test

After an overnight fast, the mice were intraperitoneally injected with 2.0 U/kg body weight human regular insulin. Blood samples were taken at various time points (0–90 min) and plasma glucose concentration was measured as described in Section 2.2.

2.4. Preparation of pancreas sections and immunohistochemical analyses

The mice were anesthetized using pentobarbital sodium. After a midline abdominal incision, pancreas was removed from the mice and fixed overnight in a solution of 4% paraformaldehyde. Fixed tissue was routinely processed for paraffin embedding and 5-μm sections were prepared and mounted on slides. Before each incubation with antibodies, the mounted sections were rinsed with PBS three times.

For detection of insulin, the avidin–biotin complex (ABC) method was performed using a Vectastain® ABC Kit (Vector Laboratories, Burlingame, CA). The mounted sections were incubated for 30 min with guinea pig polyclonal anti-insulin antibody (Dako, Glostrup, Denmark) diluted to 1:3000 in PBS containing 1% BSA. This was followed by incubation for 30 min with biotinylated *anti*-guinea pig IgG (Vector Laboratories), diluted 1:200, used as the secondary antibody. The sections were then incubated with ABC reagent for 30 min and positive reactions were visualized by incubation with peroxidase substrate solution containing 3,3′-diaminobenzidine tetrahydrochloride (DAB) (Zymed Laboratories, San Francisco, CA), and the nuclei were counterstained with hematoxylin.

Staining of 4-hydroxy-2-nonenal (HNE)-modified proteins or heme oxygenase-1 was done by a similar procedure, except for the use of anti-HNE-modified proteins antibody (JICA, Shizuoka, Japan) or anti-heme oxygenase-1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), respectively, as the primary antibody. Each antiserum was diluted 1:100.

2.5. Morphometry

The relative endocrine area was measured using an image analysis program (National Institute of Health Image 1.41). For each experimental group, four mice were killed and pancreases were isolated. The measurement and calculation were done with a total of 12 sections that had been prepared from the four pancreases (three sections per mouse). Whole area of each section was investigated and the percentage of islet cell area relative to the whole area was determined.

2.6. Measurement of islet insulin content

Four mice in each group (10 and 16 weeks of age) were used to measure the insulin content. They were made to fast, then pancreatic islets were isolated by collagenase digestion and hand-picking. The islet insulin content was measured using the supernatant of the acid–ethanol extraction from isolated islets and was determined using a Lebis® Radioimmunoassay kit (Shibayagi, Gunma, Japan) with mouse insulin as the standard. The data were normalized with respect to DNA content in isolated islets, which was measured fluorophotometrically.

2.7. Measurement of triglyceride (TG) content in islets

Four mice in each group (10 and 16 weeks of age) were used to measure the TG content. They were made to fast overnight, then pancreatic islets were isolated by collagenase digestion and hand-picking, and then immediately used for experiments. Fifty microlitres of 2 M NaCl/2 mM EDTA/50 mM sodium phosphate buffer, pH 7.4, was added to the islets, which were then sonicated for 1–2 min and centrifuged at 12 000 rpm for 5 min. The amounts of TG were measured using a 'TG-EN Kainos' kit (Kainos, Tokyo, Japan). The data were normalized with respect to DNA content in isolated islets, which was measured fluorophotometrically.

2.8. Statistical analyses

Statistical comparisons of means among individual groups used analysis of variance (ANOVA) followed by post hoc testing with the Fisher least significant difference test.

3. Results

3.1. Probucol treatment suppresses oxidative stress in islet cells of type 2 diabetes

To evaluate the potential of probucol as an antioxidant for the pancreas, we examined whether probucol treatment could reduce the expression of oxidative stress markers in islet cells of diabetic C57BL/KsJ-db/db mice. Immunostaining of HNE-modified proteins and heme oxygenase-1 (Fig. 1) were done to monitor lipid peroxidation and intracellular oxidative stress, respectively. In untreated mice, these oxidative stress markers were induced significantly in endocrine area, not much in exocrine area. This again shows that pancreatic islet cells are affected by oxidative stress. On the other hand, these markers were markedly reduced in probucol-treated mice and became close to the level of the non-diabetic control (Fig. 1), indicating that probucol treatment efficiently reduced the oxidative stress that had been induced in pancreatic islets of mice with type 2 diabetes.

3.2. Probucol treatment preserves both islet mass and insulin content in type 2 diabetes

The effects of probucol on β-cell morphology and function were investigated with type 2 diabetic animals. As shown in Fig. 2, insulin degranulation is far less evident in the islets of mice treated with probucol. Quantitatively, insulin content per islet cell was significantly higher in the probucol-treated group than in the untreated group (1.8- and 2.6-times higher at 10 and 16 weeks, respectively; Fig. 3A). Also, in the probucol-treated mice, total islet mass was significantly larger than that in the untreated mice (2.5-times at 16 weeks; Fig. 3B). These results indicated that probucol treatment can preserve both insulin content and islet mass in diabetic animals.

3.3. Probucol treatment improves glucose tolerance in type 2 diabetes

As shown in Table 1, the probucol treatment did not change food intake or body weight at 10 and 16 weeks of age. To determine whether this treatment could lead to improvement of glucose tolerance, intraperitoneal glucose tolerance tests were performed with diabetic C57BL/KsJ-db/db mice. As shown in Fig. 4, insulin secretion during the glucose tolerance tests was dramatically increased in probucol-treated mice both at 10 and 16 weeks of age (Fig. 4A and B): 280 ± 82 vs. 914 ± 238 pmol/l (120 min, at 16 weeks; $P < 0.05$; Fig. 4B). Because the insulin content was also increased in those mice despite the secretion of more insulin (Fig. 3A), the insulin biosynthesis should have been increased in those probucol-treated mice. In accordance with the increase in insulin secretion, the probucol-treated mice also displayed significantly better glucose tolerance both at 10 and 16 weeks (Fig. 4C and D): 44.6 ± 2.4 vs. 35.2 ± 2.6 mmol/l (120 min, at 16 weeks; $P < 0.05$; Fig. 4D). These results thus indicated that probucol treatment can preserve insulin secretion, probably supported by the preservation of islet mass and insulin biosynthesis, and thereby can improve glucose tolerance in diabetic C57BL/KsJ-db/db mice. In contrast to the positive effects of the probucol treatment in diabetic C57BL/KsJ-db/db mice, this treatment did not alter the glucose tolerance in non-diabetic C57BL/6 mice (Fig. 4E), agreeing with the idea that probucol exerts its effect by neutralizing the toxic effects of oxidative stress that are induced by prolonged hyperglycemia only in diabetic animals.

3.4. Probucol treatment does not change insulin sensitivity

To investigate the possible effects of probucol on insulin sensitivity, intraperitoneal insulin tolerance tests were performed. As shown in Fig. 5, both the probucol-treated and untreated groups of mice showed a similar reduction of blood glu-

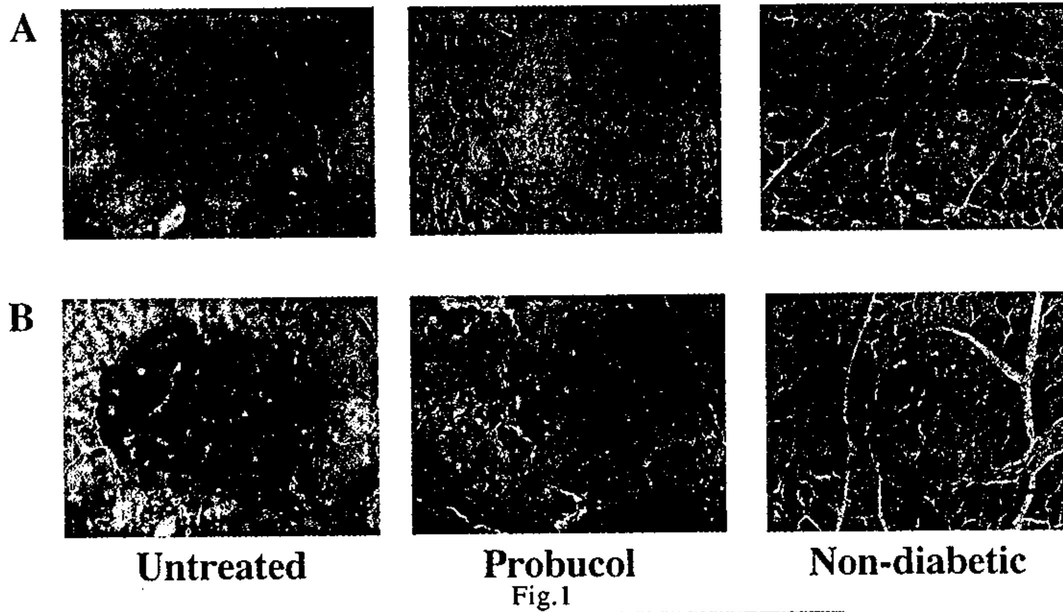

Fig.1

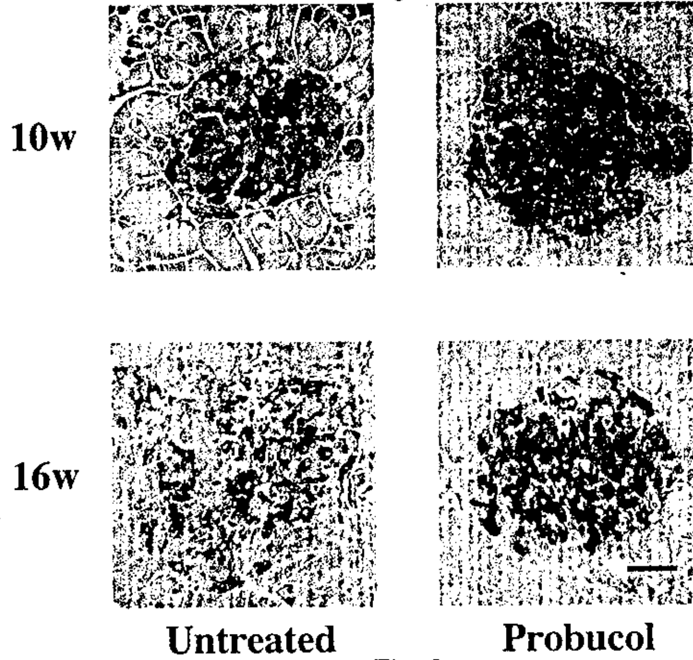

Fig. 2

Fig. 1. Effects of probucol on expression of oxidative stress markers. Pancreases isolated from 10 weeks old diabetic C57BL/KsJ-db/db mice that had been untreated (left) or treated with probucol (middle) and non-diabetic C57BL/6 mice (right) were immunostained for HNE-modified proteins (A) or heme oxygenase-1 (B). Probucol treatment suppressed oxidative stress markers to a level that is comparable to that of non-diabetes. The bar indicates 50 μm.

Fig. 2. Effects of probucol on insulin immunostaining. Pancreases isolated from 10 and 16 weeks old diabetic C57BL/KsJ-db/db mice that had been untreated (left) or treated with probucol (right) were immunostained with an anti-insulin antibody. Probucol treatment preserved the amount of insulin in the islet cells. The bar indicates 50 μm.

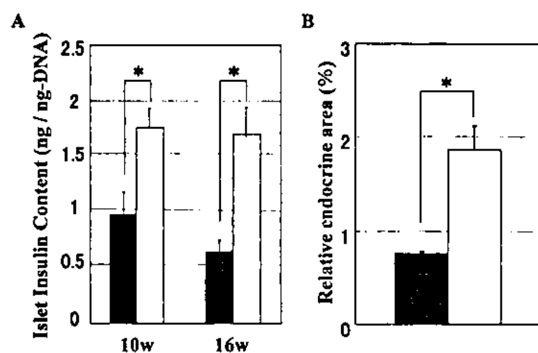

Fig. 3. Effects of probucol on insulin content and islet mass. (A) Insulin content was measured in isolated islet cells of 10 and 16 weeks old mice that had been untreated (■; $n = 4$ each) or probucol-treated (□; $n = 4$ each) diabetic C57BL/KsJ-db/db mice. Data were normalized with respect to DNA content and shown as means ± SE. Probucol treatment preserved islet insulin content both at 10 and 16 weeks (*$P < 0.05$). (B) Relative pancreatic endocrine area in 16 weeks old diabetic C57BL/KsJ-db/db mice that had been untreated (■; $n = 4$) or treated with probucol (□; $n = 4$) was determined as described in Section 2. Data were expressed as a percentage relative to the whole pancreas area (means ± SE). Probucol treatment significantly preserved islet mass (*$P < 0.05$).

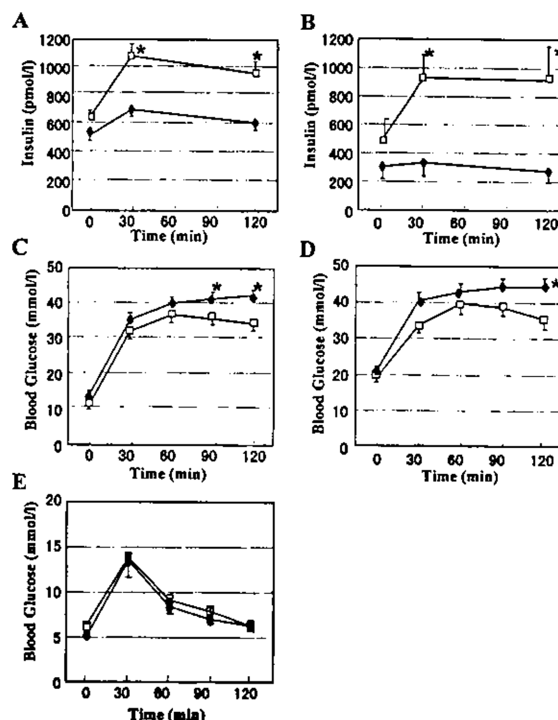

Fig. 4. Glucose tolerance in C57BL/KsJ-db/db mice treated with probucol. Intraperitoneal glucose tolerance tests were performed in diabetic C57BL/KsJ-db/db mice (panels A–D) or non-diabetic C57BL/6 mice (panel E) that had been untreated (●) or treated with probucol (□). After overnight fasting, glucose was injected intraperitoneally at a dose of 1.0 g/kg, and plasma insulin (A, B) and glucose (C–E) levels were measured. Both at the age of 10 (A, C) and 16 weeks (B, D), probucol significantly improved the glucose tolerance (C, D) with marked increase in glucose responsive insulin secretion (A, B), whereas it did not alter the glucose tolerance in non-diabetic control mice (E). Data are means ± SE (10 weeks, $n = 20$ each; 16 weeks, $n = 8$ each) (*$P < 0.05$).

cose levels in response to the injected insulin, suggesting that preservation of glucose tolerance in the probucol-treated group was not due to improvement of insulin sensitivity but to the preservation of insulin secretion.

3.5. Probucol treatment improves lipid metabolism in type 2 diabetes

As an anti-hyperlipidemic agent, probucol improved lipid metabolism in the diabetic mice: serum levels of total cholesterol, TG and free fatty acids (FFA) were significantly reduced by 69, 34, and 47%, respectively (Fig. 6A). In agreement with the reduction of serum lipid concentration in the probucol-treated mice, the TG content in islet cells was also reduced by more than 50% (Fig. 6B).

Table 1
Effects of probucol treatment on body weight and food intake

Weeks	Control (n)	Probucol (n)	
Body weight (g)			
6	25.9 ± 0.1 (20)	25.9 ± 0.2 (20)	n.s.[a]
10	37.0 ± 0.4 (20)	37.3 ± 0.4 (20)	n.s.
13	39.6 ± 0.6 (12)	37.2 ± 1.1 (12)	n.s.
16	37.9 ± 2.4 (8)	39.8 ± 1.3 (8)	n.s.
Food intake (g/day)			
6–10	5.2 ± 0.11 (20)	5.2 ± 0.09 (12)	n.s.
10–13	5.4 ± 0.03 (12)	5.3 ± 0.09 (12)	n.s.
13–16	5.3 ± 0.01 (8)	5.1 ± 0.11 (8)	n.s.

Data are shown as means ± SE.
[a] Not significant.

4. Discussion

Chronic hyperglycemia is not only a marker of poor glycemic control in diabetes but is itself a cause of impairment of both insulin secretion and

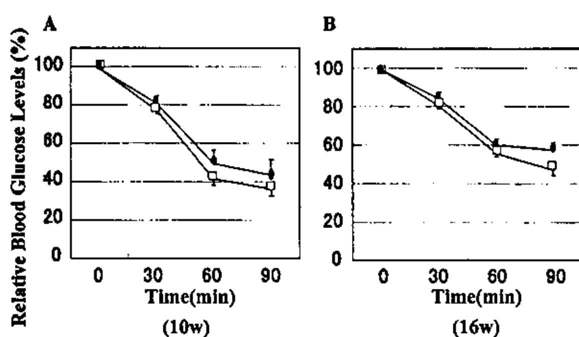

Fig. 5. Effects of probucol on insulin sensitivity in C57BL/KsJ-db/db mice. Intraperitoneal insulin tolerance tests were performed at 10 (A) and 16 weeks (B) of age in C57BL/KsJ-db/db mice that had been untreated (●; $n = 8$ each) or treated with probucol (□; $n = 8$ each). After overnight fasting, insulin was injected at a dose of 2.0 U/kg. Data were expressed as means ± SE. There was no difference in glucose lowering effects between the two groups.

biosynthesis: prolonged exposure of pancreatic β-cells to high glucose levels is known to cause β-cell dysfunction, called glucose toxicity [4–9]. Such damaged β-cells often display extensive degranulation when examined histologically, and are clinically associated with the development of diabetes in some model animals for type 2 diabetes

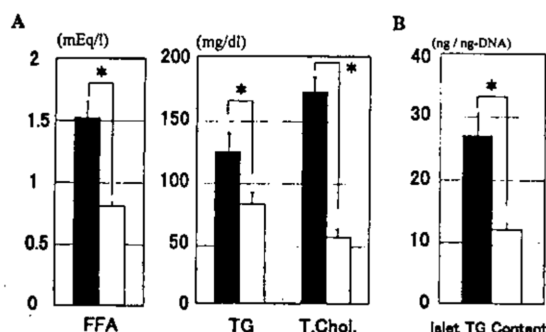

Fig. 6. Effects of probucol on lipid metabolism. (A) Serum levels of FFA, TG, and total cholesterol were measured for C57BL/KsJ-db/db mice (16 weeks) that had been untreated (■; $n = 8$) or treated with probucol (□; $n = 8$). Data are means ± SE. Probucol treatment significantly reduced serum levels of FFA, TG, and total cholesterol (* $P < 0.05$). (B) TG content in pancreatic islets was measured. The islets were isolated from C57BL/KsJ-db/db mice (16 weeks) that had been untreated (■; $n = 4$) or treated with probucol (□; $n = 4$). Data were normalized with respect to DNA content in isolated islets and expressed as means ± SE. Probucol treatment significantly reduced TG content in pancreatic islet cells (* $P < 0.05$).

[15,18]. Therefore, protection of β-cells against chronic hyperglycemia-induced damage is an important target for the treatment of type 2 diabetes.

To enable effective prevention and treatment of glucose toxicity to β-cells, it is essential to understand the biochemical aspects of the phenomenon. To date, lines of in vitro and in vivo studies have suggested the implication of oxidative stress in the progression of β-cell dysfunction in type 2 diabetes. Ihara et al. [15] examined oxidative stress markers in diabetic GK rats and found increased ROS in pancreatic islets. On the other hand, when we induced glycation and oxidative stress in pancreatic β-cell-derived HIT-T15 cells in vitro, the insulin gene promoter activity, insulin mRNA and insulin content were all suppressed in an ROS-dependent manner [12], suggesting that the insulin gene promoter is sensitive to oxidative stress and this in part explains the decrease of insulin biosynthesis in type 2 diabetes. Recently, as direct support for the implication of oxidative stress in β-cell glucose toxicity in vivo, Robertson's group and our group independently showed that suppression of oxidative stress by a potent antioxidant, *N*-acetylcysteine, increases β-cell mass, insulin content, and insulin secretion in rat and mouse models for type 2 diabetes and also improves glucose tolerance in them [17,18].

Because type 2 diabetes is a chronic disease, prevention of oxidative stress needs to be achieved not periodically but as long as the hyperglycemia persists. However, there are only a few antioxidants for which long-term safety has been established for clinical use. Among them are antioxidative vitamins, such as vitamins E and C; however, they do not display significant effects against the progression of diabetes in diabetic C57BL/KsJ-db/db mice [18]. Although speculative, this may be relevant to the fact that main function of vitamins E and C is to suppress lipid peroxidation. Whereas lipid peroxidation mainly takes place in the plasma membrane, the antioxidative vitamins may not be able to reduce intracellular ROS. Although, aminoguanidine may also reduce oxidative stress by inhibiting glycation reaction and the activity of inducible nitric oxide synthase (iNOS), it seems to have various toxic

effects including direct toxicity against pancreatic β-cells in vivo [22]. As an alternative, we decided to focus on another clinically available, widely used antioxidant, probucol.

Probucol, in agreement with its potent antioxidative effects that have been proven for many tissues and organs [23,24], reduced the expression of oxidative stress markers such as HNE-modified proteins and heme oxygenase-1 in the islets of C57BL/KsJ-db/db mice (Fig. 1). Indeed, probucol was previously shown to suppress intracellular oxidative stress in pancreatic islet cells [25] and to prevent in part alloxan-induced degranulation of the islet cells in rats [26] or islet cell destruction in NOD mice [25], suggesting that probucol may be useful for the prevention of type 1 diabetes. It should be noted that the latter study also used 1% probucol-containing diet in mice and plasma concentration of probucol in the treated mice was similar to that in humans given 750 mg/day probucol [25]. Therefore, it is likely that the clinical dose of probucol (500–1000 mg/day) also suppresses oxidative stress in pancreatic islets in humans.

As could be expected from the potent antioxidative effects of probucol in pancreatic β-cells, the β-cell mass, insulin content, and insulin secretion were significantly preserved in the probucol-treated C57BL/KsJ-db/db mice (Figs. 2–4). Because probucol did not alter the glucose tolerance when administered to non-diabetic control mice (Fig. 4E), it probably exerted its antidiabetic effects by neutralizing some types of cell toxicity that were induced in β-cells in association with prolonged hyperglycemia. Also, because the intraperitoneal insulin tolerance test revealed no significant difference in insulin sensitivity between the two groups (Fig. 5), the improvement of glucose tolerance is likely to depend mainly on the preservation of β-cell function in those mice.

It should be noted, however, that the degree of improvement in fasting or post-challenge plasma glucose levels was relatively small in spite of the large increase in insulin secretion (Fig. 4A–D). One reason for this could be the fact that these mice were very insulin-resistant and therefore relatively insensitive to the major increase in insulin secretion. Also, at least in mice, insulin signal seems not to be the single important factor that regulates glucose disposal by muscle tissue: according to the observations obtained with muscle-specific insulin receptor-knockout mice, the almost complete loss of insulin receptor in skeletal muscle led to abnormal lipid metabolism but did not cause impairment of glucose tolerance [27]. Because the glucose disposal after acute glucose challenge is known to take place predominantly in skeletal muscle, the physiological significance of insulin-independent glucose uptake in mouse muscle was suggested. Indeed, the insulin receptor-deficient muscle in those mice could react to physical exercise and almost normalize the glucose transport when assayed in vitro [28]. Although speculative, the modest improvement in glucose tolerance in the probucol-treated mice may be relevant to these observations.

In agreement with the results of previous studies in which probucol was given to various diabetic or non-diabetic animals [29,30], our present study revealed the TG-lowering effect of probucol in diabetic C57BL/KsJ-db/db mice (Fig. 6A). Whereas probucol does not activate lipoprotein lipase activity, the mechanism of its hypolipidemic action may be multifactorial in origin [31]. Although speculative, the improvement of insulin effects as results of preservation of β-cell function in the probucol-treated mice may also in part contribute to the reduction of TG: insulin deficiency is known to cause the elevation of serum TG due to low lipoprotein lipase activity and to insufficient suppression of hormone sensitive lipase [32]. The major decrease in serum FFA levels (approximately by 50%; Fig. 6A) also supports this possibility.

Possibly caused by the reduction of serum FFA levels (Fig. 6A), we also found that the islet TG content in those probucol-treated mice was reduced by approximately 50% (Fig. 6B). It has been suggested that deranged lipid metabolism in the pancreatic islet cells, most typically represented by accumulation of TG in the cells, causes β-cell damage through the induction of iNOS and excess nitric oxide (NO) generation in Zucker Diabetic Fatty (ZDF) rats [33]. Although we failed to detect iNOS expression in pancreatic islet cells even without the probucol treatment (unpub-

lished results), it is still possible that the improved lipid metabolism in islet cells of the probucol-treated mice may in part account for the preservation of β-cell function in them.

In conclusion, our present results demonstrated that probucol, a widely used anti-hyperlipidemic agent with potent antioxidative activity, can exert anti-diabetic effects by preserving β-cell function in an animal model of type 2 diabetes. Although, it needs to be investigated whether this can be observed also in other animal models of type 2 diabetes, probucol may be a suitable drug to use when a clinical trial for antioxidant therapy for type 2 diabetes is designed.

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PAX6 Mutation as a Genetic Factor Common to Aniridia and Glucose Intolerance

Tetsuyuki Yasuda,[1] Yoshitaka Kajimoto,[1] Yoshio Fujitani,[1] Hirotaka Watada,[1] Shuji Yamamoto,[2] Takao Watarai,[3] Yutaka Umayahara,[1] Munehide Matsuhisa,[1] Shin-ichi Gorogawa,[1] Yasuaki Kuwayama,[4] Yasuo Tano,[2] Yoshimitsu Yamasaki,[1] and Masatsugu Hori[1]

A paired homeodomain transcription factor, PAX6, is a well-known regulator of eye development, and its heterozygous mutations in humans cause congenital eye anomalies such as aniridia. Because it was recently shown that PAX6 also plays an indispensable role in islet cell development, a *PAX6* gene mutation in humans may lead to a defect of the endocrine pancreas. Whereas heterozygous mutations in islet-cell transcription factors such as IPF1/IDX-1/STF-1/PDX-1 and NEUROD1/BETA2 serve as a genetic cause of diabetes or glucose intolerance, we investigated the possibility of *PAX6* gene mutations being a genetic factor common to aniridia and diabetes. In five aniridia and one Peters' anomaly patients, all of the coding exons and their flanking exon-intron junctions of the *PAX6* gene were surveyed for mutations. The results of direct DNA sequencing revealed three different mutations in four aniridia patients: one previously reported type of mutation and two unreported types. In agreement with polypeptide truncation and a lack of the carboxyl-terminal transactivation domain in all of the mutated PAX6 proteins, no transcriptional activity was found in the reporter gene analyses. Oral glucose tolerance tests revealed that all of the patients with a *PAX6* gene mutation had glucose intolerance characterized by impaired insulin secretion. Although we did not detect a mutation within the characterized portion of the *PAX6* gene in one of the five aniridia patients, diabetes was cosegregated with aniridia in her family, and a single nucleotide polymorphism in intron 9 of the *PAX6* gene was correlated with the disorders, suggesting that a mutation, possibly located in an uncharacterized portion of the *PAX6* gene, can explain both diabetes and aniridia in this family. In contrast, the patient with Peters' anomaly, for which a *PAX6* gene mutation is a relatively rare cause, showed normal glucose tolerance (NGT) and did not show a *Pax6* gene mutation. Taken together, our present observations suggest that heterozygous mutations in the *PAX6* gene can induce eye anomaly and glucose intolerance in individuals harboring these mutations. *Diabetes* 51:224–230, 2002

From the [1]Department of Internal Medicine and Therapeutics, Osaka University Graduate School of Medicine, Suita, Japan; the [2]Department of Ophthalmology and Visual Science, Osaka University Graduate School of Medicine, Suita, Japan; the [3]Department of Internal Medicine, Osaka Kouseinenkin Hospital, Osaka, Japan; and the [4]Department of Ophthalmology, Osaka Kouseinenkin Hospital, Osaka, Japan.

Address correspondence and reprint requests to Yoshitaka Kajimoto, MD, Department of Internal Medicine and Therapeutics (A8), Osaka University Graduate School of Medicine, 2-2 Yamadaoka, Suita City, Osaka Pref. 565-0871, Japan. E-mail: kajimoto@medone.med.osaka-u.ac.jp.

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ADA, American Diabetes Association; EMSA, electrophoretic mobility shift assay; IFG, impaired fasting glucose; IGT, impaired glucose tolerance; IVTT, in vitro transcription and translation; MODY, maturity-onset diabetes of the young; NGT, normal glucose tolerance; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PISCES, pancreatic islet cell enhancer sequences; SNP, single nucleotide polymorphism.

The development and differentiation of organs such as the pancreas require the coordinated activation of a unique set of transcription factors. In rodents, a homozygous disruption of genes encoding the islet cell-related transcription factors causes severe abnormalities in pancreas development and early death due to diabetes. Diabetes, though in a milder form, can also be seen in human individuals with a heterozygous gene mutation that leads to a haploinsufficiency of those transcription factors. To date, heterozygous mutations of *IPF1* or *NEUROD1*, for example, are associated with maturity-onset diabetes of the young (MODY) or human type 2 diabetes (1,2), suggesting a gene-dosage effect for those transcription factors in humans.

A paired domain–containing transcription factor, Pax6, has recently emerged as a transcription factor regulating the differentiation of the endocrine pancreas. Disruption of the *Pax6* gene in mice caused marked reduction of all four types of endocrine cells in the pancreas (3). Whereas Pax6 binds to a common *cis* element called PISCES (pancreatic islet cell enhancer sequences) shared by the glucagon, insulin, and somatostatin gene promoters and activates their transcription, the amount of hormone production from the remaining cells is also substantially decreased in Pax6 mutant mice. Indeed, even in a mouse with a heterozygous mutation of *Pax6*, the mRNA and protein levels of insulin in the pancreatic islets were reduced by 40 and 25%, respectively (4). Although the physiological significance of the reductions was not evaluated in those mice, they may potentially contribute to the onset of diabetes if present in individuals genetically or environmentally predisposed to diabetes. Also, support for the gene-dosage effect of *Pax6* comes from the phenotype of a transgenic mouse overexpressing Pax6 in the pancreas; in the mouse, the increased Pax6 appears to induce islet neogenesis from hyperplastic epithelial cells of pancreatic ducts (5). Thus, it is likely that the *PAX6* gene mutations, if seen in humans, may affect the function of the endocrine pancreas and thereby contribute to the onset of diabetes.

TABLE 1
PAX6 mutaion in aniridia patients

Patient	Phenotype	Inheritance	Exon	Domain	Position	Mutation	Nucreotide change	Outcome
A	Aniridia	Familial	6	PD	590	7-bp Insertion (TAAACCG)	c.590ins7	TAA stop
B	Aniridia	Sporadic	7	LNK	790	4-bp Deletion (ATGA)	c.790del4	Frameshift→TAA stop
C	Aniridia	Familial	8	LNK	969	C→T	c.969C→T	R203X
D	Aniridia	Familial	8	LNK	969	C→T	c.969C→T	R203X
E	Aniridia	Familial				Not detected		
F	Peters' anomaly	Sporadic				Not detected		

ins, insertion; del, deletion.

Before its recent recognition as a pancreas factor, Pax6 had been known for years as a master regulator of eye development. Pax6 is expressed in the developing eye, nose, and central nervous system in addition to the pancreas, and mutations of the *Pax6* gene cause severe derangement in eye development in mammals as well as *Drosophila* (6–8). Although many of the islet cell–related transcription factors, such as *NeuroD1* and *Isl-1*, are also expressed and have functions in neural tissues, *Pax6* is a factor whose heterozygous mutation causes a developmental defect in the central nervous system of the affected animals. At least in some mammals, mutant alleles of *Pax6* are semidominant, because they cause various eye anomalies in individuals harboring the mutant allele in heterozygotes. In rodents, the heterozygous mutation causes a phenotype called "small eye" and, in humans, it typically causes aniridia, although there are cases of Peters' anomaly, keratitis, or isolated foveal hypoplasia (9–11).

Aniridia is a rare congenital eye anomaly characterized by the almost complete absence of the iris, often associated with cataracts, optic nerve hypoplasia, and glaucoma (12). Approximately one-third of aniridia cases are sporadic, but two-thirds are familial, with an autosomal dominant inheritance. In the familial cases, the penetrance is high, although the expressivity is variable. Importantly, *PAX6* gene mutations are the only cause identified to date (13). Peters' anomaly is an even rarer congenital eye anomaly characterized by dysgenesis of the ocular anterior segment and central corneal opacity. Although *PAX6* gene mutations were shown to be a cause of this disease in a few cases, the genetic cause is not known in most of the cases.

In this study, we tried to evaluate the possible significance of the *PAX6* gene mutation as a cause of glucose intolerance or diabetes. After screening for a *PAX6* gene mutation in patients with aniridia and Peters' anomaly, glucose tolerance was evaluated, and all of the aniridia patients studied were found to display glucose intolerance. In the family of one subject with both aniridia and diabetes, diabetes was very well cosegregated with aniridia. Although we were unable to identify a *PAX6* gene mutation in this patient, there was a correlation between a single nucleotide polymorphism (SNP) within the *PAX6* gene and the diseases in her family. Thus, our present observations indicate that a heterozygous *PAX6* gene mutation, which causes aniridia in humans, can also be a cause of glucose intolerance in affected individuals.

RESEARCH DESIGN AND METHODS

We recruited six unrelated Japanese patients diagnosed as having aniridia or Peters' anomaly by ophthalmologists. Four patients had familial aniridia, one had sporadic aniridia, and one had sporadic Peters' anomaly (Table 1). After obtaining written consent to participate in our study, we performed a general physical examination, blood sampling for DNA extraction and biochemical measurements, and an oral glucose tolerance test (OGTT). We also obtained written consent for participation from one family with familial aniridia. To diagnose diabetes, impaired fasting glucose (IFG), and impaired glucose tolerance (IGT), we used the American Diabetes Association (ADA) criteria. The study was approved by the ethical committee of Osaka University and was in accordance with the principles of the Helsinki Declaration.

Mutation screening. Genomic DNA was obtained from peripheral leukocytes using a QIAamp DNA Blood Mini Kit (Qiagen, Valencia, CA). The entire coding exons (exons 4–13), including the flanking exon-intron junctions of the *PAX6* gene, were amplified from the genomic DNA using previously reported polymerase chain reaction (PCR) primers and conditions (14). We purified the PCR products using centrifugal filter devices (Millipore, Tokyo) according to the manufacturer's instructions and sequenced them directly by the dideoxy-chain termination method using an ABI Prism 310 (PE Applied Biosystems, Osaka, Japan). To confirm the sequence of mutations, we subcloned each PCR product into the TA cloning vector pCR2.1 (Invitrogen, Carlsbad, CA) and sequenced it.

Plasmid construction. Full-length human *PAX6* cDNA (provided by Richard Maas) was cloned into an expression plasmid pcDNA3 to produce the *PAX6* expression vector pcDNA3-PAX6. To generate the mutated *PAX6* expression vectors, pcDNA3-c.590ins7, pcDNA3-c.790del4, and pcDNA3-c.969C→T, we used a Quick Change Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). All mutagenized constructs were checked by sequencing. The Pax-responsive firefly luciferase (Luc) reporter construct $(Pax)_6$TKLuc was described previously (15).

Electrophoretic mobility shift assays. Proteins were prepared by in vitro transcription and translation (IVTT) using a TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Electrophoretic mobility shift assays (EMSAs) were then performed using the G3 element of the glucagon gene promoter, a putative target sequence for PAX6. The sense strand of the sequence was as follows: GluG3;5'-GTAGTTTTTCACGCCTGACTGAGATTGA AGGGT-3'. The double-stranded oligonucleotide probe was end-labeled with [γ-^{32}P]ATP using T4 polynucleotide kinase. The binding reaction was performed as described previously using 2 μg each of proteins prepared by IVTT (15). The electrophoresis was performed on 6% nondenaturing polyacrylamide gels in 0.5 × TBE (44.5 mmol/l Tris, 44.5 mmol/l borate, and 1 mmol/l EDTA) for 90 min at 150V at 4°C.

Transient transfections and luciferase assays. An expression plasmid for wild-type PAX6 (pcDNA3-PAX6), mutant PAX6, or the mock vector (pcDNA3) (50 ng) was cotransfected into COS7 cells using LipofectAMINE reagent (Life Technologies, Rockville, MD) with the reporter plasmid $(Pax)_6$TKLuc (1 μg) and a Renilla luciferase expression vector, pRL-TK (10 ng). At 48 h after transfection, the cells were harvested and firefly and Renilla luciferase activity were measured following the manufacturer's protocol (Toyo Ink, Tokyo). The firefly luciferase data were normalized with respect to transfection efficiencies evaluated by the Renilla luciferase activity (Toyo Ink).

RESULTS

Detection of *PAX6* gene mutations in aniridia patients. Genomic DNA was isolated from the peripheral blood of five aniridia patients and one Peters' anomaly

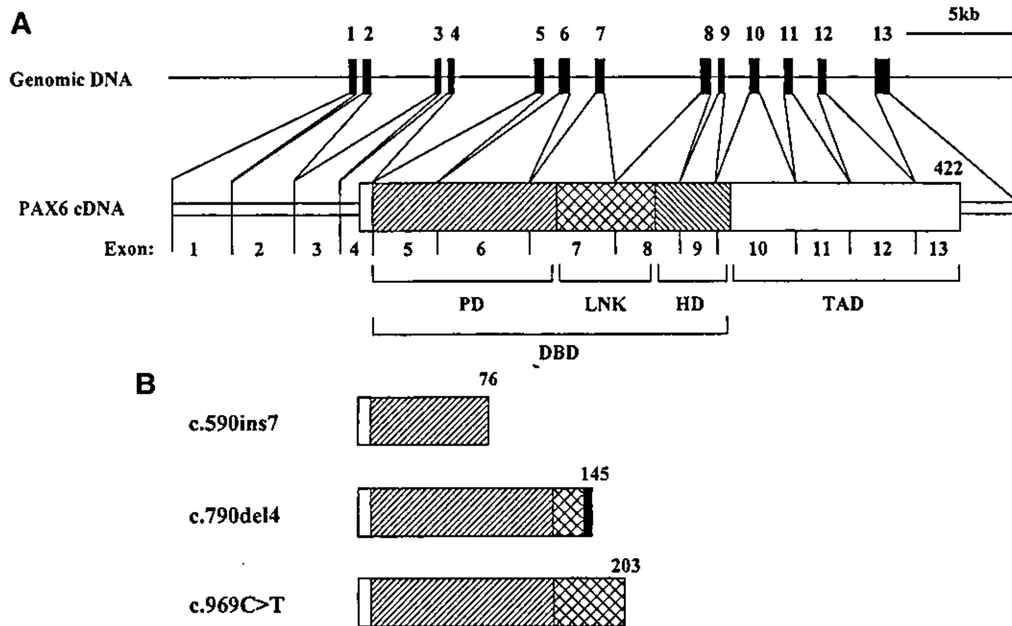

FIG. 1. Structures of human *PAX6* gene/cDNA and mutants. *A* (top): Diagram of the human *PAX6* gene. All exons (exons 1–13) are indicated. *A* (bottom): Diagram of the human *PAX6* cDNA. The coding regions are indicated by the wide bar and 5'- and 3'-untranslated regions by the thin bars. DBD, DNA-binding domain; PD, paired domain; LNK, linker region; HD, homeodomain; TAD, transactivation domain. *B*: Diagram of mutant *PAX6* cDNAs. The blackened area shows the altered open reading frame beyond the mutation site.

patient and screened for *PAX6* gene mutations. Using direct DNA sequencing, all of the coding exons and the flanking exon-intron junctions of the *PAX6* gene were examined, and three kinds of mutations were identified in four aniridia patients (Table 1). Figure 1 depicts the structures of the mutant *PAX6* cDNAs identified in aniridia patients. The human *PAX6* gene encodes a 422–amino acid protein comprised of multiple functional domains. The DNA-binding function is mediated by a paired domain and a paired-type homeodomain (residues 4–131 and 210–269, respectively) that are separated by a linker region (residues 132–209). Located in the COOH-terminal region of the protein is the transactivation domain (residues 270–422). The c.590ins7 mutation in patient A is a newly identified 7-bp insertion (TAAACCG) just after codon 76 of exon 6 (Fig. 1 and Table 1). This insertion contains a premature termination codon (TAA) and thereby generates a truncated protein that lacks part of the paired domain and all of the other domains. In patient B, the c.790del4 mutation was identified as a new type of *PAX6* gene mutation. The c.790del4 mutation is a deletion of four nucleotides (ATGA) at codon 143 of exon 7 that leads to a frame-shift of amino acid codons and creates a premature termination codon (TAA) within the linker region just two codons downstream of the deletion site (Fig. 1 and Table 1). The resulting truncated protein contains only the paired domain and some residues of the linker region and lacks the rest. The c.969C→T mutation seen in patient C and D was a C→T substitution at codon 203 that converts an Arg codon (CGA) to a termination codon (TGA). This also creates a truncated protein similar to that of c.790del4, with a slightly longer linker region (Fig. 1 and Table 1). Only the c.969C→T mutation was previously reported in several aniridia cases (16,17).

In one aniridia patient and one Peters' anomaly patient, we were not able to detect any mutation within the coding sequences or exon-intron junctions of *PAX6*. We thus surveyed the 346-bp DNA fragment of the promoter region and 5'-noncoding sequences of the *PAX6* gene for possible mutations. Moreover, to search for a chromosomal deletion or rearrangement within the region of 11p13 where PAX6 is located, we performed Southern blot and microsatellite polymorphism analyses. The Southern blot used a *PAX6* cDNA probe and gave exactly the same pattern of positive bands as obtained with normal subjects (data not shown). The microsatellite polymorphism analysis used three polymorphic markers located between the PAX6 and WT1 loci (PAX6, D11S929, and D11S914) (18) and detected no loss of heterozygosity in any of the markers (data not shown). Thus, no apparent deletion or rearrangement of the chromosomal locus for the *PAX6* gene could be found.

Evaluation of DNA-binding activity of mutant PAX6 proteins. To evaluate the function of the mutant PAX6 proteins identified in the aniridia patients, we first examined the DNA-binding capacity in gel-mobility shift analyses. A radiolabeled double-stranded oligonucleotide probe reproducing the G3 element of the glucagon gene was allowed to bind to either wild-type or mutant PAX6 proteins that had been produced by IVTT. All of the wild-type and mutant (truncated) PAX6 proteins could be produced properly according to the size of the radiolabeled proteins (data not shown).

As shown in Fig. 2, the wild-type PAX6 revealed a markedly retarded band that competed very well against the addition of cold competitors. Although they had different mobilities, two of the mutant PAX6 proteins, c.790del4 and c.969C→T, but not the other mutant, c.590ins7, revealed specifically formed protein-DNA com-

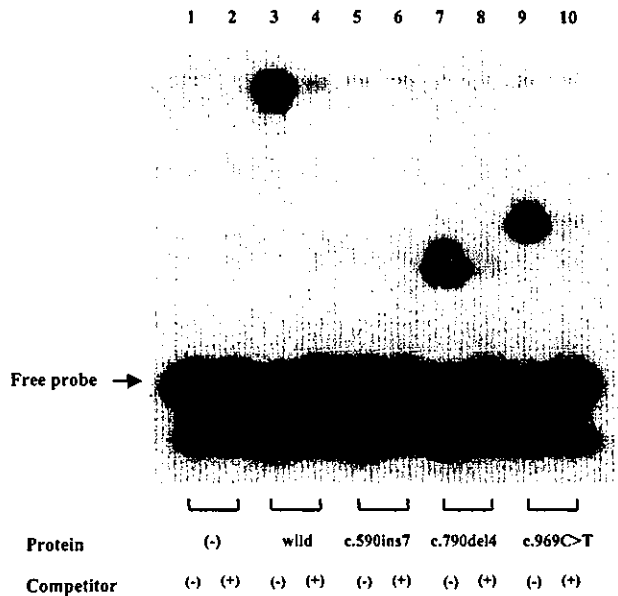

FIG. 2. DNA-binding capacity of mutant PAX6 proteins. EMSAs for the mutant PAX6 proteins are shown. An equal amount of protein yielded by IVTT was applied to each lane; wild-type PAX6 in *lanes 3* and *4*, mutant PAX6 (c.590ins7) in *lanes 5* and *6*, (c.790del4) in *lanes 7* and *8*, and (c.969C→T) in *lanes 9* and *10*. The glucagon G3 element (GluG3) is used as a probe for each lane. Unlabeled probes were applied in 50-fold excess to *lanes 2*, *4*, *6*, *8*, and *10* as specific competitors. Reticulocyte lysate (2 µl) was applied to *lanes 1* and *2* as negative controls. Identical results were obtained in three independent experiments.

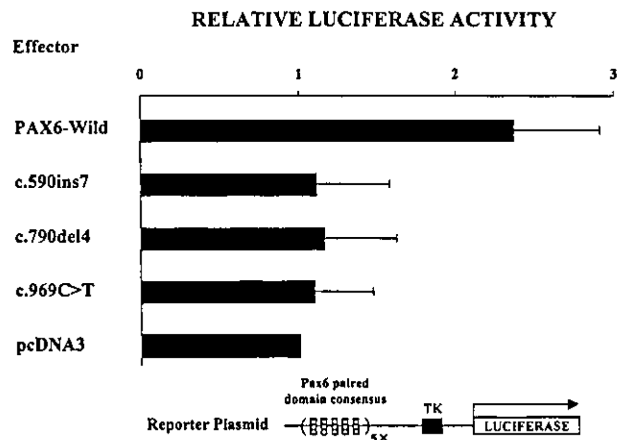

FIG. 3. Transcription properties of mutant PAX6 proteins. Wild-type or mutant PAX6 (50 ng) was transfected into COS7 cells with 1 µg reporter plasmid [(Pax)$_5$TKLuc] and 10 ng pRL-TK. At 48 h after transfection, the cells were harvested and firefly luciferase activity was measured. Transfection efficiencies were normalized with respect to Renilla luciferase activity. Each experiment was repeated four times, and data are expressed as the means ± SE.

plexes, suggesting that DNA-binding capacity is preserved in c.790del4 and c.969C→T mutants but not in the c.590ins7 mutant. This finding agrees with the observation that c.790del4 and c.969C→T mutants preserve an intact paired domain, whereas the c.590ins7 mutant lacks both the paired domain and the homeodomain required for DNA binding. Because of the lack of a transactivation domain, those two mutants with preserved DNA-binding capacity (c.790del4 and c.969C→T) may function in a dominant-negative manner in vivo.

Evaluation of the transactivation potential of mutant PAX6 proteins. Transcriptional activity of the mutant PAX6 proteins were evaluated by reporter gene analyses (Fig. 3). Plasmids expressing either the wild-type or a mutant *PAX6* were cotransfected into COS7 cells together with a luciferase reporter plasmid containing five copies of high-affinity Pax6 binding sites upstream of the minimal thymidine kinase promoter. As shown in Fig. 3, wild-type PAX6 protein activated the luciferase reporter by ~2.5-fold. In contrast, mutant PAX6 proteins did not exert any significant effects on the promoter activity of the reporter construct, in agreement with the observation that all of the mutant PAX6 proteins lacked the transactivation domain.

Evaluation of glucose tolerance in patients with aniridia. To determine whether the *PAX6* gene mutations causing aniridia can also cause glucose intolerance, we examined plasma glucose and insulin levels using OGTTs. One aniridia patient (patient E) was excluded because she had overt diabetes and was on insulin injection therapy.

As shown in Table 2, all of the nonovertly diabetic aniridia patients (patients A–D), who carried the *PAX6* gene mutations, displayed glucose intolerance (patient A: IFG; patients B, C, and D: IGT), whereas the patient with Peters' anomaly (patient F), in whom no apparent *PAX6* gene mutation was found, had NGT. None of the aniridia patients, including those with relatively high BMI values (patients C and D) (Table 2), were hyperinsulinemic. Instead, the insulinogenic index, which can be defined as the ratio of the increment of immunoreactive insulin (IRI) to the increment of plasma glucose (PG) 30 min after a glucose load ($\Delta IRI_{0-30\ min}/\Delta PG_{0-30\ min}$) (19), was relatively low or close to the lower limit of the normal range (>0.4) in the aniridia patients. According to previous reports with Japanese subjects, an insulinogenic index <0.4 manifests early-phase insulin deficiency and is a strong predictor of the development of type 2 diabetes (20–22). Thus, it was suggested that a β-cell defect, rather than insulin resistance, is the cause of the glucose intolerance in these patients.

Cosegregation of aniridia and diabetes in pedigree of patient E. Patient E was diagnosed as being diabetic at the age of 22 years. Her diabetes continued to progress, and she began requiring insulin when she was 31 years old. As is usually the case with poorly controlled diabetes, she had suffered from microangiopathy/triopathy for the 6 years before the study. She had neither elevated levels of GAD antibody nor a history of ketosis. Written consent was obtained from family members of this patient to conduct some genetic and laboratory analyses.

As shown in Fig. 4, the proband (patient E) and her father had aniridia and diabetes. Although one subject in this family (subject 3) revealed IGT, only those two who had aniridia revealed a diabetic pattern in OGTT (Fig. 4 and Table 3). This cosegregation of aniridia with diabetes in two of the family members suggests that a certain inherited genetic disorder may cause both aniridia and diabetes in this family.

Although we could not detect mutations within the surveyed portion of the *PAX6* gene, we found an SNP in intron 9 of the gene. This cannot be directly associated

TABLE 2
Clinical characteristics of aniridia patients

Patient	Age (years)	Sex	BMI (kg/m²)	Glucose levels (mg/dl) 0′	30′	120′	Insulin levels (μU/ml) 0′	30′	120′	Status	Insulinogenic index
A	54	F	23.1	118	149	121	7	17	17	IFG	0.32
B	29	M	16.8	93	145	142	2	24	34	IGT	0.38
C	37	F	25.5	102	173	147	9	39	47	IGT	0.42
D	28	F	28.7	115	203	154	9	39	44	IGT	0.34
*E	37	F	19.1	—	—	—	—	—	—	D	—
F	38	M	19.8	85	149	80	5	40	42	NGT	0.54

*Patient E has diabetes and is undergoing treatment with insulin. D, diabetic.

with the onset of aniridia or diabetes because individuals with the same genotype as the proband (GT) or her father (GG) did not necessarily develop the diseases. However, the pattern of inheritance of this marker (*G*) does not reject the hypothesis that a mutant gene allele of *PAX6* is responsible for the onset of aniridia and diabetes in this family (Fig. 4). Thus, it is still possible that the aniridia patients in this family have a mutation in the *PAX6* gene within a portion of the gene that has not been investigated.

DISCUSSION

In the present study, screening of five unrelated aniridia patients showed that they all had glucose intolerance or diabetes. In 1993, Sekikawa et al. (23) investigated the prevalence of the then "impaired glucose intolerance," which included both IGT and IFG according to current ADA/World Health Organization diagnostic criteria, and diabetes in 868 Japanese subjects who were >45 years old (BMI [mean ± SD] 23.4 ± 3.4 kg/m² for men and 24.2 ± 3.5 kg/m² for women) (Funagata Study). They found that 15.3% (11.9% in men and 16.6% in women) had either IGT or IFG, and 10.4% (8.8% in men and 14.0% in women) had diabetes according to the current diagnostic criteria. Considering that the average age of our aniridia subjects (37 years old) was much lower than that of the Funagata population (median age 55–64 years) (23), aniridia patients seem to have a high prevalence of glucose intolerance.

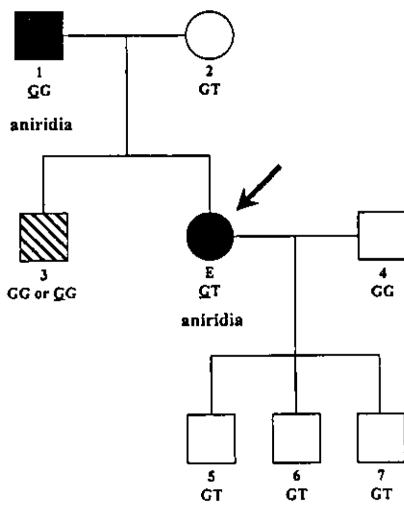

FIG. 4. Cosegregation of aniridia and diabetes in the pedigree of patient E. ■ And ●, male and female subjects, respectively, with type 2 diabetes; ▨, male subjects with IGT; ○ and □, female and male subjects, respectively, with NGT. The clinical characteristics for each subject are presented in Table 3. Numbers shown below the symbols match the subject's number in Table 3. Genotypes for the G/T SNP identified in *PAX6* intron 9 are also shown. The allele, which may carry a mutation responsible for the diseases, is underlined. An arrow indicates the proband.

The developmental processes of the ectoderm-derived neural tissues and the endoderm-derived pancreas share some key transcription factors, such as Isl-1, NeuroD1/Beta2, and HB9 (24). When those transcription factors are totally lost (e.g., as a result of homozygous gene disruption), there is a clear phenotype in both neural tissues and the pancreas (25–28). However, in the case of a heterozygous disruption, the effect varies. For example, the heterozygous gene mutation of *NEUROD1/BETA2* in humans causes early onset diabetes (MODY), but no neural phenotype has been reported to date, indicating that the gene-dosage effect is evident only in the pancreas and not in the brain. In terms of PAX6, the situation was different: heterozygous gene mutations of *PAX6* in humans have been known for years to be a cause of aniridia and some other eye anomalies, whereas no data were available for their effects on the pancreas. The OGTT data obtained with nonovert diabetic aniridia patients in this study indicated that they appear to develop glucose intolerance because of a defect in pancreatic β-cells rather than because of insulin resistance, suggesting that there is a gene-dosage effect of *PAX6* on the function of the endocrine pancreas as well as on eye development.

Among the five aniridia patients, four had mutations within the coding sequences for *PAX6*; however, we could not detect any mutations in one patient with diabetes. The possibility of a chromosomal deletion or rearrangement of

TABLE 3
Clinical characteristics of family members of patient E

Subject	Age (years)	Sex	BMI (kg/m²)	Status	Glucose levels (mg/dl) 0′	30′	120′
1	65	M	25.4	D	95	202	216
2	65	F	27.4	NGT	95	178	126
3	34	M	20.8	IGT	88	192	160
E	37	F	19.1	D	—	—	—
4	40	M	23.7	NGT	87	141	124
5	13	M	20.2	NGT	88	120	128
6	12	M	19.7	NGT	85	143	130
7	6	M	16.1	NGT	76	109	85

D, diabetic.

the *PAX6* gene locus, which in some cases causes a contiguous genetic syndrome, WAGR (Wilms' tumor, aniridia, genitourinary malformation, and mental retardation) syndrome, was also denied because the Southern blot analyses revealed exactly the normal band pattern, and microsatellite polymorphism analyses identified no loss of heterozygosity. Also, we found no mutations in the 346-bp fragment of the *PAX6* gene promoter by a direct sequencing analysis. However, this does not necessarily mean that aniridia is caused by some genetic factors other than the PAX6 gene mutations in the patient. According to the Human PAX6 Allelic Variant Database (found online at http://www.hgu.mrc.ac.uk/Softdata/PAX6/), *PAX6* mutations can be found in ~80% of aniridia patients. However, no other genetic cause that explains the aniridia in the remaining 20% has been identified (29). Thus, it is still possible that *PAX6* gene mutations are the only genetic cause for aniridia, although some mutations tend to be hidden in unscreened portions of the gene, such as 5'- and 3'-untranslated sequences, introns, or regulatory sequences further upstream. Because of the cosegregation of aniridia and diabetes in the family of patient E, we are tempted to consider that a mutation that causes both aniridia and diabetes in the patient and her father may be located in the uncharacterized portion of the *PAX6* gene (Fig. 4). Support for this comes from the inheritance of an SNP in the *PAX6* gene within the family, which does not dispute the hypothesis that a hidden mutation of *PAX6* gene is the cause of aniridia and diabetes in the family.

A patient with Peters' anomaly (patient F) who was also included in this study did not show glucose intolerance (Table 2). This clearly contrasts with the phenotype of the aniridia patients, all of whom display glucose intolerance, including diabetes. Although Hanson et al. (9) showed that Peters' anomaly can be caused by a missense mutation of the *PAX6* gene, the *PAX6* gene mutation does not seem to be a common cause of the disease. Calvas et al. (30) investigated the entire *PAX6* coding region in four patients with Peters' anomaly but were not able to find any mutations. Furthermore, Churchill et al. (31) investigated 15 individuals with Peters' anomaly and also found no mutations within the coding sequences of *PAX6*. Because we also failed to detect any mutations or gene rearrangement of the *PAX6* gene in our Peters' anomaly patient (patient F), the disease in the patient is not likely to be correlated with a *PAX6* gene mutation.

This study added two new types of mutations to the list of *PAX6* gene mutations and detected one previously identified mutation. All of the three mutant types give rise to truncated polypeptides lacking the carboxyl-terminal transactivation domain. However, the character of those mutants might differ because one of them (c.590ins7) has totally lost its DNA-binding capacity, whereas the other two preserve it. Although it needs to be defined whether the two mutants (c.790del4 and c.969C→T), which have an intact paired domain but lack a homeodomain, can indeed bind to DNA in vivo or not, there may be a dominant-negative effect in those mutants. However, we would like to note that, in agreement with previous studies that denied the correlation between the type of *PAX6* mutation and the severity of aniridia (32,33), there was no difference in the degree of iris defect among the subjects (T.Y., Y.K., S.Y., unpublished observations).

What remains to be elucidated is how a *PAX6* gene mutation can cause glucose intolerance represented by a decrease in early insulin secretion if a *PAX6* mutation is indeed a common cause of aniridia and glucose intolerance. According to an observation by Sander et al. (4), mice with a heterozygous mutation of *Pax6* had 40% lower insulin mRNA and 25% less insulin content in their β-cells. This is consistent with the fact that Pax6 is a binding factor for the PISCES element and is thereby involved in the production of endocrine hormones such as insulin in the pancreas. In general, humans have a much longer life span than rodents, and this may be important for amplifying the effects of relatively weak gene mutations, such as a heterozygous gene mutation, to a level that can lead to disease onset. Although a 25% decrease in insulin content is not likely to cause immediate glucose intolerance in mice, it may become pathophysiologically significant during aging in humans.

All of the aniridia patients with evident *PAX6* gene mutations (patients A–D) revealed glucose intolerance but did not develop diabetes until at least 28–54 years of age, suggesting that the *PAX6* gene mutations alone are not potent enough to induce diabetes in affected humans. In contrast, overt diabetes was observed in patient E, although the involvement of a *PAX6* gene mutation in this case has yet to be determined. This difference may depend on whether there were some genetic background for diabetes/glucose intolerance independent of *PAX6* gene mutations or aniridia in those patients. In the family of patient E, apart from the two patients with aniridia (patient E and her father [subject 1]), her brother (subject 3) was glucose intolerant, despite having no anomaly in eye development (Fig. 4). In contrast, although we were not allowed to obtain laboratory data from the family members of patients A–D, there was no family history of diabetes in their families, suggesting that a common genetic background for diabetes was not prevalent in their families. Thus, a *PAX6* gene mutation do not seem to cause diabetes by itself, but when combined with other diabetes-associated genes and environmental factors such as aging or obesity, it may contribute to the onset of diabetes.

In conclusion, we identified *PAX6* gene mutations as a possible cause common to aniridia and glucose intolerance in humans. Whereas various transcription factors are involved in the development of pancreas and neural tissue, *PAX6* may be the first gene whose heterozygous mutation provokes the defect of neural tissue and islets. To confirm our present observations and further elucidate the role of *PAX6* mutations in causing glucose intolerance, more aniridia patients and their families need to be recruited and studied.

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〔平成13年度日本医師会生涯教育講座〕

糖尿病性大血管症の病因と治療

大阪大学大学院病態情報内科学
山崎　義光

【キーワード】頚動脈エコー，IMT，早期動脈硬化，危険因子

緒　言

糖尿病はいうまでもなくIGT（Impaired Glucose Tolerance）でも，冠動脈疾患や脳血管性疾患による致死率が高くなることが知られている．軽症糖尿病あるいは耐糖能異常症例は，高血圧，異脂質血漿の合併頻度が高く，動脈硬化の危険因子として，高血糖以外のリスファクターの関与が想定されている．本稿では，検診時に発見しやすい，境界型糖尿病および軽症糖尿病の早期動脈硬化とその要因にかんして，著者らが現在までに検討してきたことについて述べたい．

方　法

超音波断層装置は，7.5MHz以上の中心周波数のリニア型パルスエコープローブを有するものを使用する．頭蓋外頚動脈は皮下浅層に存在するため，7.5MHz以上の周波数のものが使用可能で，高解像度（距離分解能0.1mm）を得ることができる．用いるプローベは長径が5～6cmぐらいが実用的であり，頚動脈専用プローブがない場合は，頚部，乳腺専用プローブが代用可能である．被験者を坐位の状態とし，頚動脈を斜め前から観察する前斜位，横からの側面，斜め後方からの後斜位の各縦断像と横断像をルーチンに観察する．検査のはじめに側面横断像にて総頚動脈から内外頚動脈分岐部，さらに末梢側にスキャンし，病変の位置，広がりを把握したうえで，縦断面を詳細に観察する．

血管壁は，血管内腔側の1層の低エコー輝度の部分と，その外の高エコー輝度の層の2層構造としてエコー像上解析される．Pignoliら[1]は，116例の病理標本との比較により前者が内膜中膜複合体（IMC：Intimal Plus Media Complex）に，後者が外膜と血管周囲の結合組織からなることを報告し，超音波的に測定したIMCの肥厚度が病理的に検索したIMCの肥厚度とよく相関することより，IMCが頚動脈の動脈硬化の優れた定量的指標に成りうることを示した．著者らも104例の健常例の観察より，IMCが10歳代より70歳代まで加齢とともに増加し，その肥厚度は1.1mmを越えないことを確認しており[2]，このIMCの健常人の上限は，各研究者の間でも1.0mmから1.2mmと意見が一致する[1,2]．

前斜位，側面の各縦断像で最大の内膜中膜肥厚度を示す部位を中心として中枢側1cmおよび遠位側1cmの計3ポイントの肥厚度の平均を求める．左右の頚動脈の2縦断面の皮膚に対する近位壁（near wall）および遠位壁（far wall）の合計8の壁肥厚度で最大肥厚度をIMT（avgIMT）とする．また，左右の肥厚度の平均をIMTとする研究者もいる．さらに，far wallの一定区画の平均肥厚度をmeanIMTとすることもある．

各縦断像で最大の内膜中膜肥厚度を示す部位は“プラーク”病変を含むことが多く，この部位の肥厚度をmaxIMTとする事もある．

測定の詳細は早期動脈硬化研究会のホームページを参照されたい．（http://www.imt-ca.com）

軽症糖尿病の動脈硬化の危険因子（表1）

早期動脈硬化の定量的指標である頚動脈の内膜中膜複合体肥厚度をNIDDM約500例，IDDM約100例で検索したとき，NIDDM，IDDMともに同年齢の健常人に比し，著しい動脈硬化の進展を認めた．また，IDDMでは糖尿病期間に一致して進展を認めたのに比し，NIDDMでは罹病期間の5年以下と短いものにもかなりの割合で高度の進展症例を認めた[2]．この結果

山崎義光（Yamasaki Yoshimitsu）
文献請求先：〒565-0871　大阪府吹田市山田丘2—2（A8）
大阪大学大学院病態情報内科学

表1　軽症糖尿病あるいは境界型糖尿病の動脈硬化の危険因子

1. 高血糖(食後高血糖)
   糖化の亢進→酸化の亢進→酸化LDL
2. インスリン抵抗性
   血管内皮細胞傷害→平滑筋細胞増殖，凝固能の亢進
3. 高インスリン血症
   血管平滑筋細胞増殖
4. 脂質異常
   レムナントリポ蛋白の増加，small dense LDLの増加
5. 凝固線溶系異常
   PAI-1活性の亢進(高インスリン血症，インスリン抵抗性の関与？)
   フィブリノーゲンの増加(高血糖によるmRNAの発現亢進)

—軽症糖尿病とは直接的に関連しないもの—

6. 高血圧
7. 喫煙
8. 閉経(女性ホルモン)
9. 遺伝素因
10. 炎症反応・感染症

は，軽症糖尿病でも有意に動脈硬化の進展がみられることを示している．動脈硬化は，高脂血症，高血圧，高血糖などの重積によって促進するとされている．軽症糖尿病や境界型糖尿病では，これら動脈硬化の危険因子が重積しやすいことより，以上の3つの危険因子に喫煙の危険因子を加味し，危険因子積算度別にNIDDMと境界型糖尿病のIMTを検索した．危険因子の積算度とともにIMTが肥厚することを認めたが，他の危険因子を認めず高血糖のみを有する症例にも，IMTの肥厚症例を認めた[3]．このことは，食後の高血糖のみでも動脈硬化の危険因子となりうる可能性を示している．

糖尿病の罹病期間を5年ごとに区切り，早期動脈硬化（IMT≧1.1mm）と網膜症（単純性網膜症以上の病変）の頻度を検索した．網膜症は罹病期間が5年以下ではほとんど認めなかったのに比べ，早期動脈硬化は5年以下でも50%以上と高率に認めた．また，食事療法治療群とインスリン治療群とを比較したとき食事療法群でも高率に早期動脈硬化を認めたことは，罹病期間が短く，あるいは“軽症”糖尿病でも動脈硬化が進展している可能性を示している．

軽症糖尿病の特徴的な病態は，食後高血糖と高インスリン血症である．食後高血糖の程度に比例して冠動脈疾患の致死率が高くなることが知られており，食後高血糖が大血管合併症の危険因子であることは明らかである[4]．食後高血糖の持続により，オキシラジカルの産生亢進あるいは分解低下により生体のオキシラジカルが増加し，LDLの酸化により催動脈硬化作用の強い酸化LDLが産生され，マクロファージの泡沫細胞化を促進する．また，AGE（Advanced Glycated Endproduct）の産生が亢進し，これもマクロファージに取り込まれ，泡沫細胞化を促進する．

軽症糖尿病あるいはIGTでは，中性脂肪にともむVLDLの産生が亢進し，レムナンドリポ蛋白や，small dense LDLなどが増加することが知られている[5]．食後のレムナントリポ蛋白増加の原因として蛋白リパーゼ活性低下によるレムナント代謝の停滞のほかにレムナント高値例にアポE23の頻度が高いことが知られており，肝臓レムナント受容体への結合損傷も考えられている．レムナントリポ蛋白は変性LDLと同様に動脈壁のマクロファージに容易に取り込まれることより，マクロファージの泡沫化をきたした動脈硬化促進性に作用する．

動脈硬化危険因子としての高インスリン血症の意義は未だ不明確であるが，潜在するインスリン抵抗性との関連性は明確である．空腹時血糖が未だ高くない軽症糖尿病は，IGTと同程度のインスリン抵抗性を示すとの報告もあり，境界型の時期に洗剤するインスリン抵抗性が動脈壁肥厚を来す可能性が考えられる．事実，耐糖能正常者でも，インスリン抵抗性と動脈壁肥厚度が相関するとの報告もある．インスリン抵抗性存在下では，インスリンによる血管内皮細胞からのNO産生が低下し，血管平滑筋の弛緩抑制，血小板凝集抑制作用低下のため，動脈硬化が進行しやすいことが想定される．

軽症糖尿病の危険因子に対する治療

軽症糖尿病にみられる大血管合併症の危険因子として高血糖の意義を検討するためには，高血糖を是正し，この大血管合併症に対する影響を検討することが望ましい．食後高血糖の選択的治療薬剤としてαグルコシデース阻害剤が臨床に用いられている．この薬剤により食後高血糖が特異的に改善し，さらに食後高インスリン血症の改善も報告されている．この食後高インスリン血症の改善はインスリン抵抗性の改善効果によるものかもしれない．本薬剤を糖尿病患者に投与し，small dense LDLが減少したとの報告もあり，特に食後高脂血症に対する効果も期待されている．著者らは，本薬剤をsulphonylurea剤投与糖尿病患者に併用投与し，動脈硬化進展に対する効果を検索している．SU剤単独投与時には明らかな頸動脈壁肥厚度の進展を認