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Increment of C-peptide after glucagon injection determines the progressive nature of Japanese type 2 diabetes: A long-term follow-up study

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Abstract. Type 2 diabetes (T2D) is characterized by a steady worsening of β-cell dysfunction as the disease progresses. The objective of this study was to estimate the decline of insulin secretion in Japanese type 2 diabetic patients (T2D-patients) by glucagon injection over an observation period of more than 10 years. Thirty-three T2D-patients were followed for 10.4±1.4 years. Fasting C-peptide immunoreactivity (FCPR), the 6 min value of CPR after glucagon injection (6MCPR), and the increment of CPR (ΔCPR) were measured at baseline and follow-up. FCPR, 6MCPR, ΔCPR were significantly lower at follow-up than at baseline (p<0.05, p<0.005, and p<0.0005, respectively). The annual change of ΔCPR was significantly (p<0.05) greater than the annual change of FCPR (-0.062±0.076 ng/mL/year and -0.025±0.067 ng/mL/year, respectively). In contrast, CPR-index (an index of β-cell function) and SUIT-index (secretory units of islets in transplantation) calculated based on fasting blood samples were unaltered. The annual changes of FCPR, 6MCPR, and ΔCPR were negatively correlated with the FCPR, 6MCPR, and ΔCPR values at baseline, respectively. Duration of diabetes, BMI, diabetic retinopathy, and secondary sulfonylurea failure at baseline were not correlated with the annual changes of FCPR, 6MCPR, and ΔCPR. In conclusion, our longitudinal observations suggest that β-cell function progressively declines in Japanese T2D-patients. The annual declines of ΔCPR were more prominent than the annual declines of FCPR. ΔCPR after glucagon injection may be more useful for estimating individual longitudinal insulin secretion than fasting blood samples.

Key words: Type 2 diabetes, Glucagon test, β-cell function

Type 2 diabetes (T2D) is a heterogeneous disease characterized by insulin resistance and defective insulin secretion [1]. Metabolic control deteriorates in most type 2 diabetic patients (T2D-patients) as the duration of the disease increases [2]. At the initial diagnosis, diet and lifestyle advice are generally provided to achieve glycemic control. Oral-anti-diabetic drugs (OADs) are later required in patients who fail to achieve glycemic control. Daily insulin injection is finally indicated if glycemic control becomes unachievable even with OADs. The results of the United Kingdom Prospective Diabetes Study (UKPDS) show that pancreatic β-cell function estimated by the Homeostasis model of assessment (HOMA) index in patients allocated to diet or OADs decreased by about 25% in 6 years [2]. Few studies, however, have reported the effect of a longer duration of diabetic exposure on β-cell function, including the stage when insulin injection is required. According to Meier, et al. [3], the CPR-to-glucose ratio after oral glucose ingestion appears to better predict the β-cell area in individual patients with diabetes than fasting measures such as the HOMA-β. This suggests that fasting measures may not well reflect β-cell function.

Given that the liver extracts insulin but not CPR, the serum CPR level reflects endogenous insulin secretion more directly than does serum insulin. CPR also serves as an index of β-cell function, even in patients on insulin therapy. The CPR response to 1 mg gluca-
gon injection (glucagon test) was introduced more than a decade ago as a measure of pancreatic responsiveness in type 1 diabetes [4, 5] and T2D [5-9]. The glucagon test has also been used in attempts to discriminate whether T2D-patients require insulin for hyperglycemic control [8]. Little is known, however, about the longitudinal course of β-cell function in T2D.

In this study we investigated individual longitudinal insulin secretion in Japanese T2D-patients by the glucagon test and measured the various factors influencing the declines of pancreatic β-cell function.

**Subjects and Methods**

We followed 33 consecutive T2D-patients who were admitted to our hospital from 1999 to 2003. Most of the patients were admitted because of poor glycemic control. Subjects positive for glutamic acid decarboxylase (GAD), insulin, or IA-2 antibody and subjects with renal failure (defined as a serum creatinine level of 1 mg/dL or more) were excluded, as renal insufficiency affects the CPR level. Subjects with progression of renal failure (defined as a serum creatinine level of 1 mg/dL or more) were excluded at follow-up. Patients with marked hyperglycemia, including diabetic ketoacidosis or ketosis, were also excluded.

Diabetic retinopathy was graded as simple, pre-proliferative, and proliferative retinopathy judged by ophthalmologists. Patients with retinopathy graded more severe than simple were defined as diabetic retinopathy in this study. Diabetic neuropathy was diagnosed by the presence of two or more components among clinical symptoms (bilateral spontaneous pain, hypoesthesia, or paresthesia of the legs), the absence of ankle tendon reflexes, and decreased vibration sensations in response to a C128 tuning fork. The criterion for microalbuminuria was urinary albumin excretion (UAE) of 30-300 mg/g creatinine. The patients with microalbuminuria were defined as diabetic nephropathy in this study. Diabetic complications were examined at baseline and at follow-up. Informed consent was obtained from all subjects. The study was approved by the ethics committee of the Showa University School of Medicine.

**Experimental designs**

Medical histories were established and physical examinations and laboratory evaluations were performed on the second or third day in the hospital from 1999 to 2003. The glucagon test was carried out in the morning (0800) after a 10-h fast. Fasting C-peptide immunoreactivity (FCPR) and the 6 min value of CPR after glucagon injection (6MCPR) were measured. The increment of CPR (ΔCPR) was obtained by subtracting FCPR from 6MCPR. The glucagon test was repeated for all patients from 2011 to 2012 at the outpatient clinic of Showa University Hospital.

The individual annual declines of FCPR, 6MCPR, and ΔCPR were estimated as follows. The individual changes of insulin secretion between baseline and follow-up were divided by follow-up intervals (years), as the follow-up intervals differed from patient to patient (8-14 years). The CPR index (CPI) and secretory unit of islet in transplantation (SUIT) were also measured. CPI was calculated by the formula \[100 \times \frac{FCPR \text{ (ng/mL)}}{\text{fasting plasma glucose (FPG) (mg/dL)} \times 10}\]. SUIT index was calculated by the formula \[\frac{FCPR \times 1500}{\text{FPG-61.7 (mg/dL)}}\].

All patients received the optimal dietary calorie intake calculated from their ideal body weight at admission. When fasting glucose and postprandial glucose were greater than about 140 mg/dL and 200 mg/dL, respectively, in patients taking more than 5 mg of glibenclamide (or 4 mg of glimepiride), the patients were switched from SU to insulin therapy and were defined as secondary SU failure in this study.

SU was discontinued while the patients were on insulin therapy, but insulin non-secretagogues (BG, TZD and αGI) were usually continued.

**Laboratory measurements**

Serum CPR was measured by immunoenzymometric assay (ST E test Tosoh II C-peptide; Tosoh, Tokyo, Japan). The value for HbA1c (%) in this report is estimated as an NGSP (National Glycohemoglobin Standardization Program) equivalent value (%) as calculated by the formula HbA1c (%) = HbA1c (Japan Diabetes Society [JDS]) (%) + 0.4% [12]. FPG was measured by the glucose oxidase method.

**Statistical analysis**

Data were expressed as means±standard deviations or as proportions (%). Statistical analysis was carried out by the paired t-test between clinical characteristics baseline and follow-up. Differences in the medication were analyzed by \(\chi^2\) analysis. Relationships between two variables were tested by Pearson’s correlation coefficient. \(P\) values of less than 0.05 were considered statistically significant.
Results

Clinical characteristics of the patients
The general patient profiles at baseline and follow-up are shown in Table 1. The average age of subjects at follow-up was 69.7±9.7 years. The duration of diabetes at follow-up was 18.9±6.9 years. The patients at follow-up showed significantly higher BMI (24.0±4.1 kg/m² vs 25.2±3.9 kg/m², p<0.05), lower fasting glucose levels (176±44 mg/dL vs 158±43 mg/dL, p<0.05), and HbA1c (10.1±2.1% vs 7.7±1.3%, p<0.001) compared with those at baseline.

A significantly lower proportion of patients at follow-up had used no medications or sulfonylurea derivatives compared with baseline (35.0% vs 10.0% and 61.0% vs 23.0%, p<0.05, p<0.005, respectively), and a significantly higher proportion of patients at follow-up had used metformin (6.0% vs 35.0%, p<0.05), α-GI (3.0% vs 20.0%, p<0.05), Dipeptidyl peptidase-4 (DPP-4) inhibitor (0% vs 16.0%, p<0.05), and insulin therapy (10.0% vs 61.0%, p<0.0001).

The incidence of diabetic nephropathy at follow-up was significantly higher than that at baseline (13.0% vs 50.0%, p<0.005). No significant difference was found between the incidences of diabetic retinopathy and neuropathy.

Change of β-cell function between baseline and follow-up (Fig. 1)
FCPR, 6MCPR, and ΔCPR at follow-up were significantly reduced compared with those at baseline (p<0.05, p<0.005, and p<0.0005, respectively). There were no significant differences in CPI and SUIT-index at follow-up compared with those at baseline.

Comparison of annual changes of FCPR and ΔCPR (Fig. 2)
The annual change of ΔCPR was significantly greater than the annual change of FCPR (-0.062±0.076 ng/mL/year vs -0.025±0.067 ng/mL/year, p<0.05).

Correlations FCPR, 6MCPR, and ΔCPR at baseline with the annual changes of FCPR, 6MCPR and ΔCPR (Figs. 3-5)
Fig. 3 to 5 show scattered plots of linear regression, simple regression coefficients, and formulas comparing FCPR, 6MCPR, and ΔCPR at baseline and the annual changes of FCPR, 6MCPR, and ΔCPR.

FCPR at baseline was negatively correlated with the annual changes of FCPR (r=-0.521), 6MCPR (r=-0.484) but showed no correlation with the annual changes of ΔCPR (r=0.261, r=-0.201) (Fig. 3a-c). 6MCPR at baseline was negatively correlated with the annual changes of FCPR (r=-0.565), 6MCPR (r=-0.613), and ΔCPR (r=-0.581) (Fig. 5a-c).

Correlations of the annual changes of FCPR, 6MCPR, and ΔCPR with clinical characteristics at baseline in simple regression analyses (Table 2)
Simple correlation coefficients of the annual changes of FCPR, 6MCPR, and ΔCPR with clinical characteristics (age, duration of diabetes, BMI, secondary SU failure, diabetic retinopathy, HbA1c) at baseline were calculated. The annual changes of FCPR, 6MCPR, and ΔCPR showed no correlations with any of the measured variables at baseline.

| Table 1 Clinical characteristics between baseline and follow-up |
|-----------------|-----------------|-----------------|
|                | baseline        | follow-up       | p       |
| N               | 33.0            | —               | —       |
| Male (%)        | 42.0            | —               | —       |
| Age (years)     | 59.2±10.0       | 69.7±9.7        | <0.0001 |
| Duration of diabetes (years) | 8.4±6.9       | 18.9±6.9        | <0.0001 |
| Family history of diabetes (%) | 58.0           | —               | —       |
| BMI (kg/m²)     | 24.0±4.1        | 25.2±3.9        | <0.05   |
| FPG (mg/dL)     | 176±44          | 158±43          | <0.05   |
| HbA1c (%)       | 10.1±2.1        | 7.7±1.3         | <0.0001 |
| Medication (%)  |                 |                 |         |
| no medication   | 35.0            | 10.0            | <0.05   |
| Sulfonylurea    | 61.0            | 23.0            | <0.005  |
| Glinide         | 0.0             | 3.0             | NS      |
| Biguanide       | 6.0             | 35.0            | <0.05   |
| TZD             | 3.0             | 10.0            | NS      |
| α-GI            | 3.0             | 20.0            | <0.05   |
| Insulin Treatment | 10.0          | 61.0            | <0.0001 |
| GLP-1 analog    | 0.0             | 10.0            | NS      |
| DPP-4 inh       | 0.0             | 16.0            | <0.05   |
| Complications (%) |               |                 |         |
| Diabetic retinopathy | 29.0           | 29.0            | NS      |
| Diabetic nephropathy | 13.0           | 50.0            | <0.005  |
| Diabetic neuropathy | 42.0           | 60.0            | NS      |
FPG, fasting plasma glucose; TZD, thiazolinedione; α-GI, α-glucosidase inhibitor; GLP-1, Glucagon-Like Peptide-1; DPP-4 inh, Dipeptidyl peptidase-4 inhibitor
Fig. 1  Change of β-cell function between baseline and follow-up
FCPR, Fasting C-peptide immunoreactivity; 6MCPR, 6 min value of CPR after glucagon injection; ΔCPR, increment of CPR; CPI, CPR index; SUIT, Secretory units of islets in transplantation

Fig. 2  Comparison of annual change of ΔCPR and FCPR
FCPR, Fasting C-peptide immunoreactivity; ΔCPR, increment of CPR
Fig. 3  The relationship between FCPR at baseline and annual change of FCPR (a), 6MCPR (b) and ΔCPR (c)
FCPR, Fasting C-peptide immunoreactivity; 6MCPR, 6 min value of CPR after glucagon injection; ΔCPR, increment of CPR

Fig. 4  The relationship between 6MCPR at baseline and annual change of FCPR (a), 6MCPR (b) and ΔCPR (c)
FCPR, Fasting C-peptide immunoreactivity; 6MCPR, 6 min value of CPR after glucagon injection; ΔCPR, increment of CPR

Fig. 5  The relationship between ΔCPR at baseline and annual change of FCPR (a), 6MCPR (b) and ΔCPR (c)
FCPR, Fasting C-peptide immunoreactivity; 6MCPR, 6 min value of CPR after glucagon injection; ΔCPR, increment of CPR
Clinic characteristics at baseline, change of β-cell function between baseline and follow-up, and the annual changes of FCPR, 6MCPR, and ΔCPR in the insulin-treated group and non-insulin-treated group (Table 3)

The diabetic subjects were classified into two groups, an insulin-treated group (insulin or insulin plus OADs without SU) and a non-insulin-treated group (diet/exercise or OADs). The insulin-treated group received insulin during the observation period, and 92% of them received insulin four times daily in a basal-bolus manner. The non-insulin-treated group was managed by diet/exercise or OADs during the observation period. There was no significant difference in age or BMI at baseline. The duration of diabetes at baseline was significantly longer in the insulin-treated group than in the non-insulin-treated group. There was no significant difference in the change of β-cell function between baseline and follow-up or in the annual change of FCPR, 6MCPR and ΔCPR in either group.

Changes of FPG, FCPR, CPI, and SUIT-index between baseline and follow-up in the insulin-treated group (including basal insulin) and non-insulin-treated group (Fig. 6 a-d)

In the insulin-treated group (n=20), FPG and FCPR were significantly lower at follow-up than at baseline (both \( p<0.05 \)). In the non-insulin-treated group (n=13, diet/exercise or OADs), there was no significant difference in FPG or FCPR between follow-up and baseline. There was no significant difference in CPI or SUIT-index between follow-up and baseline in either group.
Fig. 6  Changes of FPG (a), FCPR (b), CPI (c), and SUIT-index (d) between baseline and follow-up in the insulin-treated group (including basal insulin) and non-insulin-treated group.

FCPR, Fasting C-peptide immunoreactivity; FPG, Fasting Plasma Glucose; CPI, CPR index; SUIT, Secretory units of islets in transplantation.
Discussion

Decreases in both β-cell function and number can contribute to insulin deficiency in T2D. Progressive loss of pancreatic β-cell function in T2D-patients may be caused, at least in part, by a defect in β-cell mass [13, 14].

This study shows progressive declines in β-cell function in Japanese T2D-patients. The annual declines of ΔCPR were more prominent than the annual declines of FCPR measured by the glucagon test over an observation period of more than 10 years. However, CPI and SUIT-index calculated based on fasting blood samples were unaltered during the 10-year follow-up.

Although FCPR was significantly lower at follow-up than at baseline, there was no significant difference in CPI or SUIT-index between follow-up and baseline in the present study. One possible explanation for this could be the reduction of FPG conferred by the enhanced diabetes treatment during follow-up, mainly the basal insulin. We also investigated how the effects of the basal insulin on FPG related to changes in CPI and SUIT-index at baseline and at follow-up. We surmised, from the evidence we could gather, that the existence of basal insulin had no effect on CPI or SUIT-index at baseline or at follow-up in the present study.

Acute insulin secretion in response to glucose, a parameter widely assessed by insulinogenic index, is an important determinant of the oral glucose tolerance test (OGTT) and plays an important role in the pathogenesis of T2D when it declines [15].

However, the OGTT takes longer to perform and has lower reproducibility than the glucagon test because of the variability of gastric emptying [16]. The glucagon test is used mainly to evaluate residual insulin secretion and may help the physician in selecting the most appropriate choice of treatment for given individuals within the T2D population [8, 17]. The insulin assays cross-react with the exogenous insulin in patients under insulin therapy. The incidence of insulin therapy was significantly higher at follow-up than at baseline in the present study. We therefore measured CPR after glucagon injection to evaluate residual insulin secretion.

Evidence from earlier studies, mainly in vitro, have implicated glucolipotoxicity, cytokines secreted by adipocytes, immune response, and medication, especially SU, as the causes of the deterioration of pancreatic β-cell function during diabetic exposure [18-20]. β-cell mass declines progressively during the course of diabetes [3], and various antidiabetic treatment regimens have been postulated to modulate β-cell mass. To the best of our knowledge, no earlier studies have comprehensively examined the influence of clinical characteristics on the annual decline of β-cell function. The present study has revealed that clinical characteristics (age, duration of diabetes, BMI, secondary SU failure, diabetic retinopathy, HbA1c) at baseline are not correlated with the annual changes of FCPR, 6MCPR, and ΔCPR.

Our findings are not consistent with those of Funakoshi et al. [10], who proposed that the duration of diabetes exposure and BMI are major factors in β-cell function in Japanese T2D-patients. Based on evidence from a cross-sectional study, they reported that the time-dependent decline of CPR was steeper in obese subjects than in lean subjects [10]. Although FCPR, 6MCPR, and ΔCPR at baseline were negatively correlated with duration of diabetes in the present study (data not shown), BMI and the duration of diabetes exposure were not correlated with the annual changes of FCPR, 6MCPR, and ΔCPR. The difference between Funakoshi’s study and ours may be due to the study design (cross-sectional vs. longitudinal) and sample size.

In the present study, HbA1c at baseline, a marker of shorter-term diabetes exposure, was not correlated with the annual change of FCPR, 6MCPR, and ΔCPR. Surprisingly, the mean HbA1c of sequential follow-ups, a marker of longer-term diabetes exposure, was also uncorrelated with the annual changes of FCPR, 6MCPR, and ΔCPR (data not shown). Chronic elevation of the blood glucose concentration impairs β-cell function in animal models [21, 22] and in human [23, 24]. Butler et al. [13], on the other hand, reported that patients with IFG, a group at high risk of developing T2D, had a 40% deficit in relative β-cell volume compared with control subjects. This observation implies that the deficit in β-cell volume is mainly involved with an early process in the development of T2D and is likely to be of primary importance rather than simply secondary to chronic hyperglycemia.

SU binds the SU receptor of the β-cell and stimulates insulin secretion. This results in desensitization of insulin secretion, a condition generally referred to as “secondary SU failure” [25], in some patients receiving SU treatment. This has led to speculation that the mechanisms of the secondary β-cell failure may include long-time over-stimulation of the β-cell with SU [26, 27] and glucose toxicity [28]. Unexpectedly,
secondary SU failure at baseline was not correlated with the annual changes of FCPR, 6MCPR, and ΔCPR in the present study. The United Kingdom Prospective Diabetes Study (UKPDS) has shown, however, that a loss of insulin secretory function occurs at similar rates among diet-treated, SU-treated, and metformin-treated patients [2]. This may imply that progressive β-cell deterioration occurs independently of the model of therapy. Though the correlation between SU secondary failure and β-cell function must be clarified in further detail with larger sample populations, this finding indicates that secondary SU failure may not be predictive of further deterioration of β-cell function.

This study has several limitations and issues to be considered. First, this study offers no suggestions or prospects for a suitable medication to clinically prevent this progressive deterioration of β-cell function. Recent studies have demonstrated that the early introduction of insulin protects β-cell function [29, 30]. However, no longitudinal data are currently available to support the beneficial effect of insulin treatment on progressive declines in β-cell function. Meanwhile, insulin treatment during the observation period in the present study failed to prevent this progressive deterioration of β-cell function. This may have been attributable to the timing of the insulin treatment, which was started after secondary SU failure in the course of long-duration diabetes. A prospective clinical study will be required to determine whether prolonged insulin treatment after early insulin introduction prevents the progressive deterioration of β-cell function in T2D.

Second, the annual changes of FCPR, 6MCPR, and ΔCPR in the present study were negatively correlated with FCPR, 6MCPR, and ΔCPR at baseline, respectively (Figs. 3-5). The decreases of these parameters were larger in the subjects with higher residual β-cell function at baseline. We were unable to clarify the reason, however, as this study was retrospective. Our study also would have benefited from a calculation of the speed of β-cell function decline. However, since the observation period varied from 8 to 14 years, we were unable to calculate the rate of β-cell function decline, as this rate was presumably larger in cases observed over the longer period. To the best of our knowledge, however, no earlier reports have discussed the change of residual β-cell function in a single patient for ten years. Based on our results, residual β-cell function can predict reduction of future β-cell function. Our findings are therefore important clinical data. To research the secular change of residual β-cell function correctly, we believe it will be necessary to perform a prospective study on patients undergoing the glucagon load test every year.

In conclusion, our longitudinal observations suggest that β-cell function progressively declines in Japanese T2D-patients. The annual declines of ΔCPR were more prominent than the annual declines of FCPR. ΔCPR may be more useful for estimating individual longitudinal insulin secretion than fasting blood samples.

**Disclosure Summary**

All of the authors declare no conflict of interest.

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**References**