Pseudo-hyperglucagonemia was observed in pancreatectomized patients when measured by glucagon sandwich enzyme-linked immunosorbent assay

Masaki Kobayashi1, Hironori Waki2, Hitomi Nakayama3, Atsushi Miyachi4, Eri Mieno4, Hitoshi Hamajima4, Moritaka Goto4, Kentaro Yamada3, Takashi Kadowaki2,5, Tadahiro Kitamura1*

1Metabolic Signal Research Center, Institute for Molecular and Cellular Regulation, Gunma University, Gunma, Japan, 2Department of Diabetes and Metabolic Diseases, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan, 3Division of Endocrinology and Metabolism, Department of Medicine, Kurume University School of Medicine, Kurume, Fukuoka, Japan, 4Pharmaceutical Research Laboratories, Sanwa Kagaku Kenkyusho Co., Ltd, Inabe, Japan, and 5Department of Metabolism and Nutrition, Teikyo University Mizonokuchi Hospital, Kanagawa, Japan

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*Correspondence
Tadahiro Kitamura
Tel.: +81-27-220-8845
Fax: +81-27-220-8849
E-mail address: kitamura@gunma-u.ac.jp

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INTRODUCTION
Glucagon is a 29-amino-acid peptide that is associated with diabetes pathophysiology1. Although it is predominantly produced by and secreted from pancreatic \( \alpha \)-cells, glucagon is also produced in the gastrointestinal tract2. Plasma glucagon has been detected in patients even after total pancreatectomy3, and reports of post-pancreatectomy changes in plasma glucagon levels are controversial. Bajorunas et al. reported that total pancreatectomy greatly reduced plasma glucagon levels, as measured by radioimmunoassay with gel chromatography4. However, conventional radioimmunoassay lacked sufficient sensitivity and specificity for measuring glucagon in plasma5, and thus more accurate sandwich enzyme-linked immunosorbent assay (ELISA) kits were developed6. Using sandwich ELISA, Lund et al.7 showed much higher plasma glucagon levels after glucose loading in total pancreatectomy patients compared with healthy individuals. More recently, we have developed a new glucagon measurement system using liquid chromatography–high-resolution mass spectrometry (LC-HRMS)8, which enables more accurate quantification of glucagon values in plasma by excluding the cross-reactivity with other molecules that might affect immunoassay results, including sandwich ELISA measurements. Here, we report the evaluation of plasma glucagon levels using LC-HRMS, revealing hypoglucagonemia in four pancreatectomized patients, with values that clearly differed from the measurements obtained using sandwich ELISA.

CASE REPORT
We measured plasma glucagon levels in four pancreatectomized patients who gave their consent. Table S1 and Data S1 provide information regarding the patients and study methods. This

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ABSTRACT
Glucagon is detected in plasma even after total pancreatectomy, and it is debated whether this glucagon is derived from the gastrointestinal tract. Here, we applied sandwich enzyme-linked immunosorbent assay (ELISA) and liquid chromatography–high-resolution mass spectrometry to measure plasma glucagon levels in one patient after partial pancreatectomy (one-seventh of the pancreas remaining) and three patients after total pancreatectomy. Sandwich ELISA detected higher glucagon levels in pancreatectomy patients than in healthy individuals. In contrast, liquid chromatography–high-resolution mass spectrometry showed that plasma glucagon levels in pancreatectomy patients were below the lower limit of quantification. Plasma glucagon measured by sandwich ELISA showed a striking correlation with plasma glicentin, suggesting cross-reaction with this gastrointestinal glucagon-related peptide. These results indicated that pancreatectomized patients falsely showed pseudo-hyperglucagonemia when measured by glucagon sandwich ELISA.
study was carried out under approval by the ethical review committees of Gunma University (#2017-170), University of Tokyo Hospital (#11857e) and Kurume University School of Medicine (#20010).

Patient 1 was partially pancreatectomized, with one-seventh of the pancreas remaining, and was subjected to glucose and meal tolerance tests. All plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification, except for at 30 min after meal loading. In contrast, sandwich ELISA results showed that plasma glucagon levels dramatically increased after both glucose and meal loading (Table 1). Notably, the peak plasma glucagon level after glucose loading in patient 1 was approximately 15-fold higher than in healthy individuals (Figure S1). The results showed similar dramatic increases in plasma glicentin and glucagon-like peptide-1 levels in patient 1 (Table 1; Figure S1). Plasma gastric inhibitory polypeptide level also increased after glucose loading in patient 1, but this increase was also observed in the healthy individuals, suggesting that the gastric inhibitory polypeptide response after glucose loading was not affected by pancreatectomy (Figure S1).

Patients 2–4 had undergone total pancreatectomy, and their plasma glucagon levels were measured before and after breakfast. As observed in patient 1, plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification, whereas the levels measured by sandwich ELISA were comparable to those in healthy individuals (Table 2). Even though the plasma glucagon levels measured by LC-HRMS were below the lower limit of quantification value in all four pancreatectomized patients, the sandwich ELISA results showed incorrectly high glucagon values in these patients. One possible reason for the incorrect measurement was that sandwich ELISA might have been affected by cross-reaction with glucagon-related peptides, including glicentin⁹. We found no significant correlation between the plasma concentrations of glucagon and glicentin measured by sandwich ELISA in healthy individuals; however, we detected a highly positive correlation between these two hormones in pancreatectomized patients (Figure S2). This suggested that pancreatectomy increased glicentin secretion, whereas markedly reduced glucagon secretion, which might have been reflected as a good correlation between glicentin and glucagon measured by sandwich ELISA. Another possible explanation was that the sandwich ELISA system per se is generally affected by pancreatectomy. However, plasma gastric inhibitory polypeptide levels measured by sandwich ELISA and LC-triple quadrupole mass spectrometry showed a

| Table 1 | Blood glucose and various plasma hormone levels during glucose and meal tolerance tests in patient 1 (partially pancreatectomized) |
|-----------------|-----------------|-----------------|-----------------|
| **Time (min)**  | 0               | 30              | 60              | 120             |
| **Oral glucose tolerance test** | | | | |
| Glucagon (pmol/L) | LC-HRMS  | <LLOQ | <LLOQ | <LLOQ |
| Sandwich ELISA | 5.6          | 65.0 | 56.1 | 280.0 |
| Glucose (mmol/L) | 4.89 | 125  | 164  | 11.1 |
| Insulin (pmol/L) | 6.09 | 54.8 | 97.4 | 365.0 |
| C-peptide (pmol/L) | 0.1 | 0.3   | 0.6   | 0.7 |
| Glicentin (pmol/L) | 29.4 | 441  | 513  | 251  |
| Total GLP-1 (pmol/L) | 29.3 | 62.4 | 39.8 | 225.0 |
| Total GIP (pmol/L) | LC-TQMS  | 25.1 | 288  | 196  | 85.4 |
| Sandwich ELISA | 24.8 | 238  | 221  | 61.0 |
| Active GIP (pmol/L) | LC-TQMS  | 6.09 | 572  | 36.5 | 7.85 |
| Sandwich ELISA | 4.56 | 67.4 | 48.8 | 4.41 |
| **Meal tolerance test** | | | | |
| Glucagon (pmol/L) | LC-HRMS  | <LLOQ | 0.7 | <LLOQ |
| Sandwich ELISA | 4.6 | 15.7 | 30.7 | 264.0 |
| Glucose (mmol/L) | 4.94 | 104  | 129  | 872.0 |
| Insulin (pmol/L) | 6.09 | 79.2 | 146  | 85.3 |
| C-peptide (pmol/L) | 0.1 | 0.4   | 1.0   | 1.2 |
| Glicentin (pmol/L) | 44.5 | 237  | 276  | 257  |
| Total GLP-1 (pmol/L) | 42.5 | 21.7 | 25.5 | 16.4 |
| Total GIP (pmol/L) | LC-TQMS  | 17.7 | 265  | 313  | 219  |
| Sandwich ELISA | 32.5 | 357  | 390  | 214.0 |
| Active GIP (pmol/L) | LC-TQMS  | 5.61 | 80.2 | 61.7 | 24.6 |
| Sandwich ELISA | 4.53 | 110  | 88.2 | 30.4 |

ELISA, enzyme-linked immunosorbent assay; GIP, gastric inhibitory polypeptide; GLP-1, glucagon-like peptide-1; LC-HRMS, liquid chromatography–high-resolution mass spectrometry; LC-TQMS, liquid chromatography–triple quadrupole mass spectrometry; LLOQ, lower limit of quantification in glucagon measurement (0.5 pmol/L with liquid chromatography–high-resolution mass spectrometry).
In the present report, LC-HRMS showed that plasma glucagon levels were much lower in pancreatectomized patients compared with those in healthy individuals. In contrast, sandwich ELISA results showed incorrectly high plasma glucagon levels in pancreatectomized patients, most likely due to cross-reaction with glicentin.

Consistent with the present results, Lund et al.\(^7\) used sandwich ELISA, and reported that oxyntomodulin, glucagon-like peptide-1 and glucagon levels after glucose loading were higher in total pancreatectomy patients compared with those in healthy individuals. They concluded that this hyperglucagonemia could result from extrapancreatic glucagon secretion\(^7\). They also carried out more precise analysis using gel chromatography and mass spectrometry, which showed glucagon presence in the plasma even after total pancreatectomy, but they did not compare these measurements between pancreatectomized patients and healthy individuals. Although glucagon sandwich ELISA shows higher specificity than conventional glucagon radioimmunoassays, there can still be cross-reactivity with glucagon-related peptides, such as glicentin and oxyntomodulin\(^9\). Therefore, as the plasma glicentin level is extremely elevated after pancreatectomy, sandwich ELISA might show incorrect glucagon values as a result of cross-reaction with glicentin. Similar observations have been reported in the patients after bariatric surgery\(^10\). It will be of importance to elucidate how glicentin secretion is enhanced by pancreatectomy or bariatric surgery.

Among the four pancreatectomized patients, only patient 1 had a partial pancreatectomy and therefore pancreatic exocrine function, as well as endogenous insulin secretion, remained, whereas patients 2–4 had total pancreatectomy and thereafter received daily insulin injections (Table S1), which might have affected the incretin secretion and accounted for the different correlation coefficient of glucagon and glicentin between patient 1 and patients 2–4. Furthermore, because patient 1 had a nephrectomy, and patient 2 had a resection of the small
intestine, impaired renal function and intestinal function might have affected the stability and secretion of incretin in these patients (Table S1). These are the limitations of this clinical report.

In conclusion, LC-HRMS measurement showed that glucagon secreted from the gastrointestinal tract was very scarce in the pancreatectomized patients; nevertheless, sandwich ELISA falsely showed pseudo-hyperglucagonemia as a result of cross-reaction with gastrointestinal glucagon-related peptides.

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DISCLOSURE
The authors declare no conflict of interest.

REFERENCES
1. Unger RH, Orci L. The essential role of glucagon in the pathogenesis of diabetes mellitus. Lancet 1975; 1: 14–16.
2. Sasaki H, Rubalcava B, Baetens D, et al. Identification of glucagon in the gastrointestinal tract. J Clin Invest 1975; 56: 135–145.
3. Holst JJ, Pedersen JH, Baldissera F, et al. Circulating glucagon after total pancreatectomy in man. Diabetologia 1983; 25: 396–399.
4. Bajorunas DR, Fortner JG, Jaspan JB. Glucagon Immunoreactivity and Chromatographic Profiles in Pancreatectomized Humans: Paradoxical Response to Oral Glucose. Diabetes 1986; 35: 886–893.
5. Holst JJ, Christensen M, Lund A, et al. Regulation of glucagon secretion by incretins. Diabetes Obes Metab 2011; 13(Suppl 1): 89–94.
6. Wewer Albrechtsen NJ, Hartmann B, Veedfald S, et al. Hyperglucagonaemia analysed by glucagon sandwich ELISA: nonspecific interference or truly elevated levels? Diabetologia 2014; 57: 1919–1926.
7. Lund A, Bagger JI, Wewer Albrechtsen NJ, et al. Evidence of Extrapancreatic Glucagon Secretion in Man. Diabetes 2016; 65: 585–597.
8. Miyachi A, Kobayashi M, Mieno E, et al. Accurate analytical method for human plasma glucagon levels using liquid chromatography-high resolution mass spectrometry: comparison with commercially available immunoassays. Anal Bioanal Chem 2017; 409: 5911–5918.
9. Matsuo T, Miyagawa J, Kusunoki Y, et al. Postabsorptive hyperglucagonemia in patients with type 2 diabetes mellitus analyzed with a novel enzyme-linked immunosorbent assay. J Diabetes Investig 2016; 7: 324–331.
10. Roberts GP, Kay RG, Howard J, et al. Gastrectomy with Roux-en-Y reconstruction as a lean model of bariatric surgery. Surg Obes Relat Dis 2018; 14: 562–568.

SUPPORTING INFORMATION
Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 | Comparisons of blood glucose and various plasma hormone levels during glucose tolerance testing between patient 1 and healthy subjects. For comparison, data from healthy subjects are presented, some of which were published in Reference 8 and others were unpublished. We used the criteria for healthy as those who are not taking regular medication and whose fasting blood glucose <6.1 mmol/L and 2 h blood glucose <7.8 mmol/L during OGTT according to the WHO guideline. Data from healthy subjects are shown as mean ± SD. The number of healthy subjects is indicated on each panel. Red horizontal line indicates the lower limit of quantitation (0.5 pM).

Figure S2 | Correlations between the plasma glicentin levels and glucagon levels measured by sandwich ELISA in the pancreatectomized patients. For comparison, we present data from healthy subjects—some published in Reference 8 and others unpublished (n = 85).

Figure S3 | Correlations between liquid chromatography–triple quadrupole mass spectrometry and sandwich enzyme-linked immunosorbent assay results in terms of measured plasma levels of total gastric inhibitory polypeptide (left) and active gastric inhibitory polypeptide (right) in pancreatectomized patients.

Table S1 | Clinical characteristics of the pancreatectomized patients.

Data S1 | Supplementary methods.