Insulin Wakayama: familial mutant insulin syndrome in Japan

K. Nanjo¹, M. Miyano¹, M. Kondo¹, T. Sanke¹, S. Nishimura¹, K. Miyamura¹, K. Inouye², B. D. Given³, S. J. Chan³, K. S. Polonsky³, H. S. Tager³, D. F. Steiner³ and A. H. Rubenstein³

¹Department of Medicine, Wakayama University of Medical Science, Wakayama, and ²Shionogi Research Laboratories, Osaka, Japan;

³Department of Medicine, Biochemistry and Molecular Biology, University of Chicago, Chicago, Illinois, USA

Summary. We describe a family from Japan displaying the mutant insulin syndrome with hyperinsulinaemia and an increased insulin:C-peptide molar ratio. Serum insulin isolated from several family members showed reduced in vitro biological activity, and analysis by high performance liquid chromatography revealed a peak co-eluting with human insulin and a second species of increased hydrophobicity co-migrating with the previously reported Insulin Wakayama. The insulin genes from the proband were cloned and sequenced, revealing one normal allele; the second allele, encoding a leucine for valine amino acid substitution at position 3 of the insulin A

chain, was similar to that previously described for Insulin Wakayama. Synthesized [Leu^{A3}] insulin showed 0.14% of receptor binding activity on rat adipocytes and a 10-fold prolonged half-life in a somatostatin-infused dog compared with human insulin. The finding of the same mutant gene in two unrelated Japanese families suggests that Insulin Wakayama may be discovered in additional Japanese families with hyperinsulinaemia and/or diabetes.

Key words: Insulin, mutant, familial, gene, high performance liquid chromatography.

Diabetes associated with the synthesis and secretion of a structurally abnormal insulin has been identified in three families with three distinct defects characterized at the genomic level [1–3]. The relationship of the mutation in the insulin gene to the frequently co-existing glucose intolerance is incompletely understood. Additionally, the frequency with which these mutant genes occur in the general population and, therefore, the overall incidence of diabetes associated with abnormal insulins is unknown.

We have identified a fourth family with the mutant insulin syndrome. Characterization of the mutant insulin by high performance liquid chromatography (HPLC) and DNA analysis has confirmed that this family demonstrates the identical point mutation leading to the expression of [Leu^{A3}] insulin previously described for Insulin Wakayama [3], and raises the possibility that this genetically transmitted defect may be present in additional Japanese diabetic patients. Additionally, this insulin analog has been synthesized, allowing us to characterize its in vivo and in vitro characteristics.

Subjects and methods

Subject

The subject was a 57-year-old, non-obese Japanese man from Arida, Japan, who presented with glucosuria on routine examination. Three

years later, he showed increased glucosuria accompanied by thirst and general fatigue. He had no family history of diabetes. Physical examination revealed eczematoid dermatitis on his arms, but he was otherwise normal. Laboratory tests were normal except for marked fasting hyperinsulinaemia (0.83 pmol/ml; nl < 0.13). Possible causes of hyperinsulinaemia were evaluated. Counterregulatory hormone excess was ruled out (fasting growth hormone 5.8 ng/ml, cortisol 12.6 µg/dl, glucagon 97 pg/ml). Anti-insulin and insulin antireceptor antibodies [4, 5] and islet cell surface antibodies [6] were absent, and binding of ¹²⁵I-insulin to the patient's erythrocyte insulin receptors [7] was normal.

Clinical studies

An oral glucose tolerance test (OGTT, 50 g) and an insulin tolerance test (ITT, 0.1 U/kg intravenous pure pork insulin) were performed on the subject after an overnight fast. A second OGTT was performed 18 months later, at which time samples were collected for the HPLC study (see below).

To assess whether the defect was hereditary, fasting glucose, immunoreactive insulin (IRI) and C-peptide (CPR) levels were measured in other family members.

Characterization of purified serum insulin

Insulin isolated by Sep-Pak [8] from the serum of the subject and selected family members was subjected to gel-filtration on Bio-Gel P30 (Bio-Rad, Richmond, Calif, USA) and separation on reverse-phase HPLC as previously described [9]. Serum samples collected from the subject during the second OGTT at baseline and 30, 60, 90 and 120 min after 50 g of glucose were also subjected to HPLC analysis.

Fig. 1. Changes with time in plasma glucose, insulin immunoreactivity (IRI) (●—●), C-peptide immunoreactivity (CPR) (○—○), and IRI/CPR molar ratios after the oral administration of a 50-g oral glucose tolerance test to the subject. The bottom panel displays the response of plasma glucose to an intravenous bolus injection of 0.1 U/kg of pork insulin. The test was terminated due to hypoglycaemia at 30 min. The shaded area in the third panel represents mean $\pm$ 2 SEM for IRI/CPR molar ratios in 30 normal subjects

Insulin-gene characterization

To determine the coding sequence of the insulin gene, DNA was isolated from peripheral leukocytes obtained from the subject [1]. Both insulin alleles were cloned [10, 11] and sequenced [12] according to our published methods [1, 2, 11, 13].

Semisynthetic [Leu^{A3}] insulin

[Leu^{A3}] insulin was produced by semi-synthetic methods [14]. The identity of the analog was confirmed by sequence analysis and by comparison with Insulin Wakayama on HPLC (data not shown). Binding of this analogue to insulin receptors was assessed using rat adipocytes [15]. In vivo clearance of the [Leu^{A3}] insulin analog and normal human insulin were measured in the dog according to previously described methods in which the disappearance of insulin from serum is calculated following the abrupt discontinuation of a steady state infusion [16].

Results

Clinical studies

The initial OGTT revealed impaired glucose tolerance with a normal fasting plasma glucose of 5 mol/l which peaked at 14.4 mol/l at 60 min (Fig. 1). This hypergly-

Fig. 2. Pedigree and clinical data for the subject and studied family members. Numbered subjects were available for screening. P=subject, †=deceased. Diagonal lines signify fasting hyperinsulinaemia; the asterisk signifies impaired glucose tolerance. Insulin immunoreactivity (IRI) is expressed as pmol/ml with μ U/ml in parentheses. CPR=C-peptide immunoreactivity

caemia occurred despite pronounced hyperinsulinaemia. In contrast, the plasma C-peptide levels were relatively normal. Resultant IRI/CPR molar ratios were thus markedly elevated (Fig. 1).

To assess insulin sensitivity *in vivo*, exogenous insulin was administered intravenously. Despite the patient's marked endogenous hyperinsulinaemia, he responded normally to exogenous insulin. The test was terminated due to symptomatic hypoglycaemia at 30 min (Fig. 1).

To determine whether the hyperinsulinaemia was familial, screening of other family members was arranged. Five family members from three generations were available for study and were willing to cooperate. As shown in Figure 2, four of the five were hyperinsulinaemic with abnormally elevated IRI/CPR molar ratios. None were diabetic, as assessed by fasting plasma glucose levels and the absence of post-prandial glucosuria. They did not agree to have oral glucose tolerance tests.

Characterization of circulating insulin

Since proinsulin and its intermediates crossreact in the insulin assay and result in elevated IRI levels [17], sera from the subject and his four family members were subjected to size exclusion gel chromatography. The peak co-eluting with ¹²⁵I-insulin accounted for >90% of serum IRI in all subjects (data not shown).

Insulin was purified from the serum of the subject and the other four available family members and subjected to reverse phase HPLC analysis (Fig. 3). All four subjects with hyperinsulinaemia demonstrated the presence of a major peak of increased hydrophobicity reacting in the insulin assay. A normal insulin peak was also

detected on two of the chromatograms, but was not detected at these low concentrations in subjects 1 and 2. The unaffected son (subject 5) displayed only normal insulin on HPLC analysis.

Because the HPLC pattern closely resembled that of Insulin Wakayama [3, 9], insulins purified from the sera of the index cases from both of the families were chromatographed separately and in combination. The patterns were indistinguishable from each other (data not

Fig. 3. HPLC analysis of insulin purified from sera obtained from the father (1), brother (2), subject (3), daughter (4) and son (5). Chromatography of a mixture of bovine (a), human (b) and porcine (c) insulins is shown in the upper panel (S). The peak corresponding with [Leu^{A3}] insulin is designated (d)

shown), a result strongly suggesting that a second family expressing Insulin Wakayama had been identified.

Having observed glucose intolerance during the first OGTT despite the presence of marked hyperinsulinemia, a second OGTT was performed with samples collected and extracted by Sep-Pak for HPLC analysis. The normal and abnormal insulin peaks were separately quantitated and expressed as a ratio of total HPLC insulin immunoreactivity. By then multiplying this ratio by the total serum IRI level, the actual concentrations of each insulin species at various time points could be calculated (Table 1).

Insulin gene characterization

To identify the mutation resulting in the expression of [Leu^{A3}] insulin in this family, the allelic insulin genes from the subject were cloned and sequenced using recombinant DNA techniques (Fig. 4). One allele contained the normal coding sequence consistent with the presence of normal insulin in the circulation. However, the second allele contained a thymine for guanine substitution in the first position of the triplet normally coding for valine at position 3 in the insulin A chain. This is the identical point mutation previously identified in the insulin gene coding for Insulin Wakayama [3, 11], and results in a leucine for valine substitution. Thus, both families synthesize and secrete [Leu^{A3}] insulin as well as normal human insulin.

In vitro and in vivo characterization of semi-synthetic [Leu^{A3}] insulin

Binding of normal human and semisynthetic [Leu^{A3}] insulin to rat adipocytes was compared. As shown in Table 2, there was a greater than 700-fold reduction in the ability of the analog to displace 50% of the pork insulin tracer compared to normal human insulin standards. In vivo clearance was assessed in a somatostatin

Table 1. Serum and plasma samples during a 50 g OGTT in the subject were collected and assayed for glucose, insulin and C-peptide. Insulin was then extracted from serum and separated by HPLC, and normal human insulin (normal) and [Leu^{A3}] insulin (abnormal) were quantitated by insulin radioimmunoassay and expressed as a ratio of the total of both peaks. Multiplying this ratio by the serum insulin level yields the circulating concentration of the two insulin species displayed

Time (min)	Oral glucose tolerance test (50 g)				
	0	30	60	90	120
Plasma glucose (mol/l)	4.1	9.3	10.1	7.3	6.9
Insulin Total (pmol/ml)	0.58 (84)*	1.73 (247)	4.72 (674)	2.78 (398)	3.58 (513)
Normal (pmol/ml)	0.08 (12)	0.23 (33)	0.52 (74)	0.14 (20)	0.27 (39)
Abnormal (pmol/ml)	0.50 (72)	1.50 (214)	4.20 (600)	2.64 (378)	3.31 (474)
C-peptide (pmol/ml)	0.77	3.30	3.90	6.17	7.0

* Values in μU per ml are in parenthesis

Fig. 4. Autoradiograph of portions of the sequencing gel showing the partial nucleotide sequence of both normal and mutant alleles in the area of the point mutation. The single base transversion is indicated by an asterisk (*)

Fig. 5. Serum insulin concentrations in a somatostatin infused dog studied on two occasions. On the first, human insulin (●—●) was infused at a concentration of $1.44 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 120 min, at which point the infusion was abruptly discontinued and frequent insulin levels drawn. In a separate experiment, semisynthetic [Leu^{A3}] insulin (○—○) was infused at $0.382 \text{ mU} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ for 120 min and abruptly discontinued. The insert shows the change in insulin level expressed as a percent of the steady state level following discontinuation of the infusion

infused dog by measuring the half-life following abrupt discontinuation of a steady state infusion of the analog (Fig. 5). A similar experiment employing normal human insulin is included for comparison. A 10-fold difference in half-life was observed (35.0 vs 3.6 min, Fig. 5, Table 2).

Discussion

With the development of the insulin radioimmunoassay and the subsequent finding that many Type 2 (non-insulin-dependent) diabetic subjects possessed normal or even elevated plasma insulin levels, the speculation

Table 2. Binding potency of human and semisynthetic [Leu^{A3}] insulins on rat adipocytes expressed in percent (in terms of 50% displacement of a ¹²⁵I-pork insulin tracer). Human insulin was assigned a value of 100% for comparison with the analog. Also shown is the clearance half-life ($t_{1/2}$) following the abrupt discontinuation of human insulin or analog in the somatostatin-infused dog (see also Fig. 5)

	Adipocyte binding (%)	$t_{1/2}$ (min)
Human insulin	100	3.6
[Leu ^{A3}] insulin	0.14	35.0

arose that the insulin molecules in some of these patients might be genetic mutations resulting in biologically less active insulins which still retained immunoreactivity [18]. Since 1978, three such cases have been reported and characterized at the genomic level [1–3, 19, 20]. Other potential mutant species have been identified, but these species await gene confirmation [21, 22].

Studies of affected families have led to the description of the “mutant insulin syndrome” consisting of hyperinsulinaemia, lack of counterregulatory hormone excess or circulating antibodies to insulin or its receptor, demonstration of a normal response to exogenous insulin, increased insulin:C-peptide molar ratio and reduced biological activity of endogenous insulin [2, 3, 20].

This report describes the fourth such patient characterized at the level of the insulin gene (Fig. 4). As in the other examples [9, 16], the predominant circulating insulin form in affected family members is the mutant species (Fig. 3). In two of the family members studied (subjects 1 and 2), normal insulin was not detected in the circulation due to the small quantity of serum analyzed. This predominance of the mutant form in serum has been ascribed to delayed plasma clearance based on two lines of evidence. First, when insulin was extracted from the pancreas of the first patient identified with the mutant insulin syndrome, approximately 50% was authentic human insulin [9]. Second, when the *in vivo* clearance of semi-synthetic [Ser^{B24}] insulin (Insulin Los Angeles) was compared to normal human insulin in the dog, its half-life was markedly prolonged [16]. Equimolar infusions of [Ser^{B24}] insulin and normal human insulin resulted in the marked accumulation of the abnormal form. Our present findings are in agreement with these results. [Leu^{A3}] insulin showed a 10-fold increase in half-life compared to normal human insulin in the dog (Fig. 5). Since the clearance of human insulin in dog and man are similar [23, 24], this experiment probably mimics the *in vivo* situation closely. Moreover, since insulin clearance is closely related to receptor binding, the reduced clearance is presumably due to reduced receptor binding, as suggested by the *in vitro* rat adipocyte data (Table 2), which showed that [Leu^{A3}] insulin displaced ¹²⁵I pork insulin tracer only 0.14% as well as the human insulin standard. That a neutral for neutral amino acid substitution results in a marked change in hydrophobicity as demonstrated by the HPLC analysis

suggests an underlying alteration in the tertiary structure of this mutant insulin molecule. In addition, the A3 site may be directly involved in the receptor binding surface of the insulin molecule. Three dimensional X-ray crystallographic analysis of the mutant insulin will be required to definitively answer this issue.

Perhaps the most perplexing question concerning the mutant insulin syndrome is the inconsistent finding of glucose intolerance or overt diabetes in affected family members. In an attempt to clarify this issue, we separated the serum insulin components circulating during the second OGTT in the subject. The results indicated the presence of normal concentrations of authentic human insulin similar to those observed in healthy subjects [25]. This is in keeping with his normal glucose tolerance at the time. Unfortunately, insufficient serum was available from the initial OGTT to perform the same analyses at a time when he was glucose intolerant. The patient's normal response to exogenous insulin at this time (Fig. 1) indicates that insulin resistance was an unlikely contributor to his glucose intolerance. However, it should be noted that the total serum IRI and C-peptide levels increased approximately eight times above basal during the second OGTT, which was normal, while in the first test the increase was only 3- to 4-fold. In the interim the patient had been treated with diet and exercise, and his glucose tolerance had improved. The implication is that, assuming the ratio of the abnormal to normal insulin was similar in the two tests, the concentration of the normal insulin would have been relatively low and thus inadequate during the initial OGTT, resulting in a diabetic curve (Fig. 1, Table 2).

With the opportunity to study four families secreting mutant insulins, it has become apparent that the expression of a single allele coding for a structurally abnormal insulin is not always associated with diabetes. However, it does appear to increase the risk for the occurrence of this disorder; furthermore, when it coexists with an inherited or acquired defect in pancreatic secretion or peripheral insulin resistance, glucose intolerance is more likely to occur than in subjects with two normal insulin genes. Thus, the expression of diabetes may vary between and within families expressing the same defect depending on the presence or absence of other diabetogenic factors, either genetic or acquired.

We have now had the opportunity to compare two families with the same mutant insulin species. Diabetes was present in 3 of 5 members of the first family [3], while in the family reported in this manuscript only transient glucose intolerance in the index case was noted. When four members of the first family were given oral glucose tolerance tests, the degree of hyperglycaemia correlated inversely with the magnitude of their insulin secretory response as judged by circulating C-peptide levels. The patient reported in this study showed an initial insulin secretory response which was similar to that seen in the previously described diabetic

subjects. His second OGTT was normal, and his insulin response during this test compared favourably to normoglycaemic members of both families. It seems reasonable to conclude that maintenance of significant pancreatic insulin secretory reserve can mitigate the effects of the reduced biological activity of the mutant insulin and permit the maintenance of euglycaemia.

Finally, while the mutant insulin syndrome may not generally be a numerically important contributor to the overall incidence of diabetes, in isolated, relatively inbred populations an abnormal insulin gene could have an appreciable impact on the prevalence of Type 2 diabetes. The significance of Insulin Wakayama may thus lie in its gene frequency in the Japanese population. The finding of the same point mutation in two unrelated families living over 100 km apart and with no common relatives for at least 4 generations is adequate justification for careful consideration of this etiological possibility in diabetic patients, especially those with a strong family history of diabetes. Screening efforts aimed at determining the frequency of this gene in various Japanese population groups using an elevated IRI:C-peptide ratio as a potential marker for the condition are currently underway.

Acknowledgements. The authors wish to thank K. Watanabe, J. Iimura, W. Pugh, A.H.J. Huen and N. Ditekowsky for valuable technical assistance, and V. Kuvakos and M. Puciata for preparing the manuscript. This study was supported by the Wakayama Nokyō Association (Japan) and grants AM13914, AM13941 and AM20595 (The Diabetes Research and Training Center) from the National Institutes of Health.

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Received: 13 October 1986
and in revised form: 16 December 1986

Dr. A. H. Rubenstein
Department of Medicine
University of Chicago
5841 S. Maryland Ave.
Chicago, IL 60637
USA