Immunoreactive insulin in diabetes mellitus patient sera detected by ultrasensitive ELISA with thio-NAD cycling

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To minimize patient suffering, the smallest possible volume of blood should be collected for diagnosis and disease monitoring. When estimating insulin secretion capacity and resistance to insulin in diabetes mellitus (DM), increasing insulin assay immunosensitivity would reduce the blood sample volume required for testing. Here we present an ultrasensitive ELISA coupled with thio-NAD cycling to measure immunoreactive insulin in blood serum. Only 5 μ L of serum was required for testing, with a limit of detection (LOD) for the assay of 10^{-16} moles/assay. Additional recovery tests confirmed this method can detect insulin in sera. Comparisons between a commercially available immunoreactive insulin kit and our ultrasensitive ELISA using the same commercially available reference demonstrated good data correlation, providing further evidence of assay accuracy. Together, these results demonstrate our ultrasensitive ELISA could be a powerful tool in the diagnosis and treatment of not only DM but also many other diseases in the future.

Insulin is a peptide hormone secreted from β cells in pancreatic islets that serves as a signal of the fed state (1). It is central to regulating carbohydrate and fat metabolism in the body (2). Normally, insulin is secreted in a constant proportion to remove excess glucose from the blood (3); however, this is not the case in patients with the metabolic disorder diabetes mellitus (DM) or metabolic syndrome. When control of insulin levels fails, DM can result (www.diabetes.niddk.nih.gov/dm/pubs/type1and2/index.aspx). DM occurs in two major forms: insulin-dependent

(Type 1) and non-insulin-dependent (Type 2). Patients with Type 2 DM are often insulin-resistant and therefore may suffer from a relative insulin deficiency. In both Type 1 and Type 2 DM, the blood insulin concentration (i.e., immunoreactive insulin) should be carefully measured to estimate insulin secretory capacity and insulin resistance (4–6).

Because DM patients, as well as those with various other conditions, must undergo frequent blood sampling, efforts should be made to reduce the volume of blood per sample required for testing (7–10). One way to accomplish this is to increase

the measurement sensitivity for particular target proteins. In particular, reduction of blood sample volume is very important in the case of infants with Type 1 DM. Blood collection from infants is very difficult, and the amount collected per sample is very small (11). Thus, an ultrasensitive assay for insulin has been strongly desired for progression-monitoring of DM in infants.

The sandwich enzyme-linked immunosorbent assay (ELISA) is a suitable method for precisely quantifying trace amounts of proteins (12). The ELISA for insulin was established in the 1980s and 1990s. The limits of detection (LODs) at

METHOD SUMMARY

The combination of a sandwich ELISA with a thio-NAD cycling method enables the ultrasensitive detection of immunoreactive insulin in patient sera by measuring the quantity of thio-NADH generated. This approach allows for the detection of trace amounts of immunoreactive insulin just by the application of thio-NAD cycling reagents to the standard ELISA system.

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Table 1. Protocol for additional recovery test using recombinant human insulin and human serum.

Serum	Blank (only including insulin in serum)		Recombinant human insulin (2.8 μ IU/mL) (Ray Biotech) (i.e., endogenous insulin in serum + 2.8 μ IU/mL insulin)	
DM-patient serum 0	no dilution	Solution 1	no dilution	Solution 3
	1:10 (2.5 μ L/25 μ L)	Solution 2	1:10 (2.5 μ L/25 μ L)	Solution 4
	1:20 (1.25 μ L/25 μ L)		1:20 (1.25 μ L/25 μ L)	
DM-patient serum 120	1:10 (2.5 μ L/25 μ L)		1:10 (2.5 μ L/25 μ L)	
	1:50 (0.5 μ L/25 μ L)		1:50 (0.5 μ L/25 μ L)	
	1:100 (0.25 μ L/25 μ L)		1:100 (0.25 μ L/25 μ L)	
Control serum low	1:10 (2.5 μ L/25 μ L)		1:10 (2.5 μ L/25 μ L)	
	1:20 (1.25 μ L/25 μ L)		1:20 (1.25 μ L/25 μ L)	
Control serum high	1:10 (2.5 μ L/25 μ L)		1:10 (2.5 μ L/25 μ L)	
	1:50 (0.5 μ L/25 μ L)		1:50 (0.5 μ L/25 μ L)	
	1:100 (0.25 μ L/25 μ L)		1:100 (0.25 μ L/25 μ L)	

that time were on the order of μ IU/mL, which corresponded to tens of femtomoles per milliliter. The molecular weight of human insulin is 5807, and for this conversion, 1 IU was estimated as $\sim 43 \mu$ g (www.nibsc.org/documents/ifu/66-304.pdf). For example, Bürgi et al. demonstrated that their sandwich ELISA detected insulin at 3.7 mIU/L, which corresponded to $\sim 2.8 \times 10^{-14}$ moles/mL (13). Storch et al. could detect insulin at 2 μ IU/mL, which was $\sim 1.5 \times 10^{-14}$ moles/mL (14). Mecklenburg et al. could detect it at 0.025 μ g/mL, corresponding to 4.3×10^{-12} moles/mL (15). The measurements obtained by Andersen et al. were very sensitive: 5 picomoles/L (5×10^{-15} moles/mL) (16). Recently, assays for insulin have moved to lab-on-a-chip methods. Using these methods, Park et al. showed that the detection level of insulin was 0.4 ng/mL, or 6.9×10^{-14} moles/mL (17), and Oyama et al. detected 17 ng/mL, or 2.9×10^{-12} moles/mL (18). Thus, these newer methods have never exceeded the limitation of the ELISA methods, and the overall LOD for insulin reported so far is on the order of 10^{-14} moles/mL.

More recently, Watabe et al. proposed a novel ultrasensitive ELISA in which a sandwich ELISA is combined with enzyme cycling (19). Enzyme cycling assays measure trace amounts of substrate using amplification techniques (20,21). Thus, a detectable signal is amplified in the a quadratic function-like response, resulting in an LOD for human insulin of 10^{-19} moles/assay using recombinant human insulin

(19). Watabe et al. showed that their ultra-sensitive determination was at least 4 orders of magnitude more sensitive than other highly sensitive methods reported in the literature (19).

Here we describe an ultrasensitive ELISA for immunoreactive insulin that we tested using human sera obtained from DM patients, using World Health Organization (WHO) international standard insulin or its equivalent products, widely used at clinical sites, as references. Our results confirm that the sensitivity of this technique was sufficiently high and reliable enough to determine immunoreactive insulin in DM-patient sera.

Materials and methods

Chemicals

Insulin as an antigen for ELISA was purchased from the following companies: The WHO international standard distributed by the National Institute for Biological Standards and Control (NIBSC), NIBSC code (66/304), was purchased from the Health Care Technology Foundation (Tokyo, Japan). The reference insulin included in the TOSOH II (IRI) kit (Tosoh, Tokyo, Japan) was also used in this study. Three kinds of recombinant human insulin were purchased from Kamiya Biomedical (Seattle, WA), Ray Biotech (Norcross, GA) and BioVision (Milpitas, CA). Intact proinsulin distributed by the NIBSC, NIBSC code (09/296), was purchased from the Health Care Technology Foundation. C-peptide was purchased from Anaspec

(Fremont, CA). The primary and secondary antibodies for anti-human insulin were: monoclonal mouse 7F8 antibody (Cat. # 211; HyTest, Turku, Finland) and monoclonal mouse D4B8 antibody (Cat. # 211; HyTest), respectively. The secondary antibody, which was linked to alkaline phosphatase (AP) (EC. 3.1.3.1), digested to F(ab')₂ by pepsin, and reduced to Fab' by 2-mercaptoethylamine. A maleimide terminal was then introduced by reacting AP with N-(6-maleimidocaproyloxy (sulfo-EMCS) purchased from Dojindo, Kumamoto, Japan. Finally, an SH group on Fab' and a maleimide terminal on AP were joined. Thionicotinamide-adenine dinucleotide (thio-NAD) and NADH were purchased from Roche (Mannheim, Germany). 3 α -hydroxysteroid dehydrogenase (3 α -HSD; derived from *Comamonas testosteroni* and recombined by *E. coli*) was purchased from Kikkoman Biochemifa (Tokyo, Japan) and purified by BL Co., Ltd. (Izunokuni, Japan). 5 β -androsterone was purchased from Steraloids (Newport, RI). 17 β -Methoxy-5 β -androstane-3 α -ol 3-phosphate was synthesized as described previously (19). Absorption was measured at 405 nm with a Corona Electric MTP-500 microplate reader (Hitachinaka, Japan) thermostated at 37°C.

Human sera

Blood was taken from DM patients at Kagawa University Hospital, and the sera were separated by centrifugation. Our method requires only 5 μ L serum per sample (i.e., a blood sample of ~10 μ L). In contrast, a commercially available method, for example the TOSOH II (IRI) kit, which is used at Kagawa University Hospital, requires at least 200 μ L, including the dead volume; thus, the blood sample must be at least 500 μ L. The serum samples were cryopreserved at -80°C. We avoided repeated freeze/thaw cycles beyond the minimum necessary (22). The experiments using human blood were performed with the permission of the Ethics Committees of Tokushima Bunri University and Kagawa University. Control sera were purchased from Eiken (Tochigi, Japan).

Ultrasensitive ELISA coupled with thio-NAD cycling

The primary antibody solution, which was adjusted to 20 μ g/mL in a 50 mM Na₂CO₃ solution (pH 9.6), was added

to 96-well microplates in aliquots of 50 μ L per well and incubated for 1 h at room temperature. The microplates were washed with Tris-buffered saline (TBS) including 0.05% Tween 20 and incubated with 20% Blocking One-P (Nacalai Tesque, Kyoto, Japan) for 45 min at room temperature. The microplates were again washed 3 times with TBS including 0.05% Tween 20. Then, an antigen or a serum was added at 50 μ L per well. The sera were diluted 10 $\times$ with TBS including 5% Blocking One-P. Note that this dilution was taken into account when approximating the sensitivity. The microplates were shaken for 60 min at room temperature and then washed with TBS including 0.05% Tween 20. The solution containing the secondary antibody (linked to AP) was adjusted to 5×10^{-15} moles/mL in TBS including 0.05% Tween 20 and 5% Blocking One-P before being added to the plate at 50 μ L per well. The microplates were kept overnight at 4°C and washed 9 times with TBS including 0.05% Tween 20. To amplify the ELISA signal, 50 μ L thio-NAD cycling solution was added to each well. This solution contains 1.0 mM NADH, 1.5 mM thio-NAD, 0.25 mM 17 β -methoxy-5 β -androstane-3 α -ol 3-phosphate, and 5 U/mL 3 α -HSD in 100 mM Tris-HCl (pH 9.0). Absorbance at 405 nm was measured with a microplate reader every 5 min for

60 min at 37°C. A detailed protocol can be found in the Supplementary Material.

Additional recovery test

The experimental procedures for this test were the same as for the ultrasensitive ELISA experiments without the antigen solution. We prepared four types of solutions (Table 1) containing:

Solution 1: 50 μ L TBS including 5% Blocking-One P.

Solution 2: 25 μ L 5% Blocking-One P, 50 mM NaCl, 10 mM PIPES-HCl, and 25 μ L human control serum, which was diluted 10–1000 $\times$ with TBS including 5% Blocking-One P (see Table 1).

Solution 3: 25 μ L recombinant human insulin (Ray Biotech) at a concentration of 0.14 μ IU/25 μ L and 25 μ L TBS including 5% Blocking-One P.

Solution 4: 25 μ L recombinant human insulin (Ray Biotech) at a concentration of 0.14 μ IU/25 μ L and 25 μ L human serum, which was diluted 10–1000 $\times$ with TBS including 5% Blocking-One P (see Table 1).

The additional recovery ratio was calculated as: [(the absorbance of Solution 4 - that of Solution 1) - (the absorbance of Solution 2 - that of Solution 1)] / [the absorbance of Solution 3 - that of Solution 1].

Figure 1. Ultrasensitive detection of insulin in serum by ELISA coupled with thio-NAD cycling using alkaline phosphatase, androsterone derivatives, 3 α -hydroxysteroid dehydrogenase, and its co-enzymes. During the cycling reaction, thio-NADH accumulates in a quadratic function-like fashion with time. Accumulated thio-NADH can be measured directly by absorbance at 400 nm (i.e., 405 nm with a commercially available microplate reader) without any interference from other cofactors.

Figure 2. Linear calibration curves for ultrasensitive ELISA coupled with thio-NAD cycling using commercially available insulin references. (A) Ultrasensitive ELISA calibration curve for the WHO international standard insulin reference. The absorbance of thio-NADH was measured at 405 nm after a cycling reaction time of 60 min. The linear regression equation is for the curve is $y = 0.045x + 0.11$, $R^2 = 1.00$. The blank values (i.e., the absorbances of the 0 $\mu\text{IU/mL}$ insulin samples) were not subtracted from the absorbances of the other concentrations of insulin because this linear curve was needed to calculate the limit of detection and the limit of determination. (B) Ultrasensitive ELISA calibration curves for other insulin references. The blank values (i.e., the absorbances of the 0 $\mu\text{IU/mL}$ insulin samples) were subtracted from the absorbances of the other concentrations of insulin. That is, this figure directly expresses the values corresponding to the concentrations of insulin. The equation for the linear calibration curve using the reference in the TOSOH II (IRI) kit was $y = 0.067x - 0.015$, $R^2 = 0.99$; that for the recombinant human insulin from Kamiya Biomedical was $y = 0.056x - 0.023$, $R^2 = 1.00$; that for the recombinant human insulin from Ray Biotech was $y = 0.038x - 0.025$, $R^2 = 0.95$; and that for the recombinant human insulin from BioVision was $y = 0.025x - 0.005$, $R^2 = 0.93$.

Limit of detection, limit of determination, and coefficient of variation

The experimental data were obtained by subtracting the mean value of the blank signals from each of the corresponding measurements. The LOD was estimated from the mean of the blank, the standard deviation of the blank, and a confidence factor of 3. The limit of determination was estimated by the same method as used for the LOD, but with a confidence factor of 10. The coefficient of variation calculated from 3 data points was obtained for 8.0 $\mu\text{IU/mL}$ of the WHO international standard insulin reference.

Results and discussion

The substrate-cycling reaction uses a single dehydrogenase (i.e., $3\alpha\text{-HSD}$) as follows (23–29): $3\alpha\text{-HSD}$ catalyzes substrate cycling between $3\alpha\text{-hydroxysteroid}$ and its corresponding 3-ketosteroid in the presence of an excess of NADH and thio-NAD because $3\alpha\text{-HSD}$ utilizes both NADH and thio-NAD as cofactors (30). During each cycle, 1 molecule of thio-NAD is reduced to thio-NADH, which can be measured directly as an increase in the absorbance at 400 nm ($11900 \text{ M}^{-1} \text{ cm}^{-1}$) (i.e., 405 nm with a commercially available

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microplate reader) without any interference from other cofactors such as thio-NAD, NAD, and NADH, the absorbance maxima of which are all <340 nm. These features make it possible to determine the amount of 3 α -hydroxysteroid with high sensitivity by measuring cumulative amounts of thio-NADH. This detectable signal changes linearly with time.

Our ultrasensitive ELISA coupled with thio-NAD cycling involves (Figure 1) a sandwich ELISA using a primary antibody against insulin and a secondary antibody for detection. An androstereone derivative, 17 β -methoxy-5 β -androstan-3 α -ol, is produced by the hydrolysis of 17 β -methoxy-5 β -androstan-3 α -ol 3-phosphate catalyzed by AP linked to the secondary antibody. This 17 β -methoxy-5 β -androstan-3 α -ol is oxidized to 17 β -methoxy-5 β -androstan-3-one by 3 α -HSD with the cofactor thio-NAD. In the opposite reaction, 17 β -methoxy-5 β -androstan-3-one is reduced to 17 β -methoxy-5 β -androstan-3 α -ol by 3 α -HSD with the cofactor NADH. During this cycling reaction, thio-NADH accumulates in a quadratic function-like fashion over time.

When using the WHO international standard insulin reference, an anti-human insulin 7F8 antibody (Hytest), and an AP-linked anti-human insulin D4B8 antibody (Hytest), the ultrasensitive ELISA coupled with thio-NAD cycling in TBS yielded a linear calibration curve ($y = 0.045x + 0.11$; $R^2 = 1.00$) in the range of 0–8 μ U/mL (Figure 2A). This curve was obtained from the absorbance of accumulated thio-NADH at 60 min. The LOD for the WHO international standard insulin reference was 19 nIU/assay, which corresponded to $\sim 1.4 \times 10^{-16}$ moles/assay. Because washing steps are included in the ELISA protocol, the absolute number of moles (on the order of subfemtomoles) that can be detected should be noted, rather than the concentration. If we convert this

value to the concentration (per mL), which is generally how ELISA data are expressed, 19 nIU/assay is equivalent to 0.38 μ U/mL, or $\sim 2.8 \times 10^{-15}$ moles/mL for a 50 μ L assay volume. These results show that the LOD of our method is less than one-tenth of the LODs reported for conventional ELISA and lab-on-a-chip methods, as discussed above. The minimum limit of determination for the WHO international standard insulin reference was 1.0 μ U/mL. The coefficient of variation was 9% for the 8 μ U/mL WHO international standard insulin reference.

Many manufacturers claim their insulin references are equivalent to the WHO international standard insulin reference. We compared linear calibration curves using our ultrasensitive ELISA for insulin references offered by different manufacturers. A linear calibration curve using the reference included in the TOSOH II insulin assay (IRI) kit was $y = 0.067x - 0.015$, $R^2 = 0.99$; for recombinant human insulin from Kamiya Biomedical: $y = 0.056x - 0.023$, $R^2 = 1.00$; for recombinant human insulin from Ray Biotech: $y = 0.038x - 0.025$, $R^2 = 0.95$; and for recombinant human insulin from BioVision: $y = 0.025x - 0.005$, $R^2 = 0.93$ in the range of 0.5–4.0 μ U/mL (Figure 2B).

It is clear that the slopes of these linear calibration curves are different, despite claims from the manufacturers that these references are equivalent to the WHO international standard insulin reference. The maximum variation for these results was 35% for 405 nm absorbance at 1 μ U/mL insulin. For example, when we used the WHO international standard insulin reference as the antigen, the linear calibration curve was $y = 0.045x + 0.011$, $R^2 = 1.00$ (Figure 2A). These results suggest that we cannot compare the data of immunoreactive insulin among different commercially available references.

For additional recovery tests, we examined 4 kinds of sera with 280 μ U/mL of recombinant human insulin from

Ray Biotech: (i) DM-patient serum 0 (i.e., serum obtained from a fasting DM patient), (ii) DM-patient serum 120 (i.e., serum obtained from the same DM patient 120 min after a meal), (iii) Control serum low (i.e., including 8.7 μ U/mL insulin; QC-RE Eiken Type B Low), and (iv) Control serum high (i.e., including 78.1 μ U/mL insulin; QC-RE Eiken Type B High). The data are shown in Table 2. In summary, the additional recovery ratio was $100.4 \pm 13.9\%$ (mean $\pm$ SD, $n = 9$). These additional recovery tests clearly showed that our ultrasensitive ELISA can detect immunoreactive insulin in serum.

In the previous section, we confirmed that our ultrasensitive ELISA can be used to detect immunoreactive insulin in serum, so we next measured levels of immunoreactive insulin in DM-patient sera with our ELISA. First, blood samples were collected from DM patients for testing using the TOSOH II (IRI) kit to measure the serum levels of immunoreactive insulin. Next, the same samples were measured by our ultrasensitive ELISA. As shown in Figure 3A, to compare these values for DM patients, we plotted the concentration of immunoreactive insulin measured by the TOSOH II (IRI) kit on the x-axis and the concentration of immunoreactive insulin measured by our ultrasensitive ELISA on the y-axis. Here, the calibration curve used for our ultrasensitive ELISA was drawn using the same data as used for the reference insulin from the TOSOH II (IRI) kit as shown in Figure 2B. The regression line between these two data sets was expressed as $y = 1.07x + 13.56$, $R^2 = 0.84$ (Figure 3A). Because the slope of this line was ~ 1 , the data from the TOSOH II (IRI) kit and from our ultrasensitive ELISA were in good agreement.

We also compared the data from the TOSOH II (IRI) kit and the data from our ultrasensitive ELISA using insulin references provided by other manufacturers (Figure 3B). When using recombinant human insulin from Kamiya Biomedical, the linear correlation curve was expressed as $y = 1.23x + 17.75$, $R^2 = 0.84$. For the WHO international standard insulin reference, $y = 1.48x + 20.86$, $R^2 = 0.84$; for the recombinant human insulin from Ray Biotech, $y = 1.93x + 26.94$, $R^2 = 0.84$; and for the recombinant human insulin from BioVision, $y = 2.94x + 33.09$, $R^2 = 0.84$ (Figure 3B). The variation in the slopes of these regression lines was very large. These results demonstrated that although our ultrasensitive ELISA can be

Table 2. Additional recovery test for human insulin in an ultrasensitive ELISA.

Serum	Dilution ratio	Recovery ratio (%)
DM-patient serum 0	1:10	90
	1:20	90
DM-patient serum 120	1:10	88
	1:50	112
	1:100	88
Control serum low	1:10	102
	1:20	117
Control serum high	1:50	124
	1:100	93

Figure 3. Comparison between the concentration of immunoreactive insulin in diabetes mellitus (DM) patients measured by a commercially available kit and that measured by the ultrasensitive ELISA coupled with thio-NAD cycling. (A) The regression line between the data from DM patients measured by Kagawa University Hospital using a TOSOH II (IRI) kit and the data measured by an ultrasensitive ELISA using the same reference is expressed as $y = 1.07x + 13.56$, $R^2 = 0.84$. (B) Regression lines between the data measured by Kagawa University Hospital using a TOSOH II (IRI) kit and the data measured by our ultrasensitive ELISA using other insulin references that were provided by other manufacturers. For the recombinant human insulin from Kamiya Biomedical, the linear correlation curve was expressed as $y = 1.23x + 17.75$, $R^2 = 0.84$; for the WHO international standard insulin reference, $y = 1.48x + 20.86$, $R^2 = 0.84$; for the recombinant human insulin from Ray Biotech, $y = 1.93x + 26.94$, $R^2 = 0.84$; and for the recombinant human insulin from BioVision, $y = 2.94x + 33.09$, $R^2 = 0.84$.

used for any kind of reference insulin, the values obtained using different references cannot be directly compared.

The present study showed that the LOD for the WHO international standard insulin reference was 10^{-16} moles/assay using our ultrasensitive ELISA. This is more sensitive than the LODs reported for other insulin

ELISA assays. This sensitivity is comparable to that for a commercially available chemiluminescent enzyme immunoassay (Lumipulse Insulin-N; Fujirebio, Tokyo, Japan), which is not colorimetric and is therefore expensive and requires a specialized instrument. By contrast, our colorimetric ultrasensitive ELISA provides

several major advantages in terms of cost and simplicity, without requiring a specialized instrument. However, this sensitivity of 10^{-16} moles/assay does not surpass that for recombinant human insulin (10^{-19} moles/assay) (19) or that for HIV-1 p24 (10^{-18} moles/assay) (31), which have also been obtained by our ultrasensitive ELISA. This issue of sensitivity thus seems to depend on the antigen and antibodies used.

Previously it has been reported that data from different insulin immunoassays are not easily compared (32). One important issue here is the quality of the insulin reference; thus, use of recombinant human insulin has been suggested for calibration traceability (33). Indeed, when the recombinant insulin reference was used in our previous study, our ultrasensitive ELISA resulted in subattomolar detection (19). In the present study, however, we used the WHO international standard insulin reference, which was purified from human pancreatic insulin, because hospitals and diagnostic companies still use assay kits including this WHO international standard insulin reference or equivalent products. As can easily be seen, the data obtained using the different references from different assay kits were highly variable, even in our ultrasensitive ELISA. Miller et al. have suggested that all of the commercially available kits should be standardized using a pure insulin primary reference material at the manufacturer (34). We suggest that each clinical test laboratory choose one method and stick to that in order to ensure proper patient follow-up.

An ELISA for insulin generally detects intact proinsulin as well. It has been argued that an immunoassay for insulin may overestimate the true insulin levels in Type 2 DM, and subjects with impaired glucose tolerance, due to cross-reaction with intact proinsulin, C-peptide, and split proinsulin (35,36). However, this is not an issue specific to our ultrasensitive ELISA, rather is an issue of the antibodies used. In any case, we examined cross-reactivity of intact proinsulin and C-peptide with the anti-insulin antibodies used in the present study. We applied 50 pg/mL, 100 pg/mL, and 200 pg/mL of insulin, intact proinsulin, and C-peptide to our ultrasensitive ELISA and measured the absorbance of thio-NADH at 405 nm.

In our ultrasensitive ELISA, when we increased intact proinsulin concentrations,

the absorbance of thio-NADH increased. That is, the antibodies used in the present study do detect intact proinsulin. For example, we found that the absorbance of 50 pg/mL intact proinsulin was 0.038 and that of the same volume of insulin was 0.054. Considering that the molecular masses of intact proinsulin and insulin are 9000 Da and 5807 Da, respectively, the antibodies used cross-react with intact proinsulin at a ratio of 45%. We believe that this issue originates from the antibodies used and not from our ultrasensitive ELISA method. Furthermore, the probable amount of intact proinsulin in serum is about one tenth (molar ratio) that of insulin (37–39). Because the cross-reaction ratio with intact proinsulin for the antibodies used is about 50%, intact proinsulin may exert an influence upon our ultrasensitive ELISA for insulin of ~5%. If higher specificity to insulin is required, then more specific antibodies should be used.

For C-peptide, the antibodies used do not cross-react at all. Even when we increased the concentration of C-peptide, the absorbance of thio-NADH in our ultrasensitive ELISA was not altered.

It should be noted that we did not try to examine the cross-reaction with split proinsulin by the antibodies used in the present study. The split proinsulin consists of only two amino acids, so any antibodies can cross-react with these amino acids. That is, the antibodies used in the present study may cross-react with split proinsulin completely or may not cross-react at all. However, the probable amount of split proinsulin in serum is about one tenth (mole ratio) that of insulin (37,40,41). Therefore, split proinsulin may influence our ultrasensitive ELISA for insulin at less than 10%. This is not an issue particular to our ultrasensitive ELISA method, but an issue of the antibodies used.

In the present study, our ultrasensitive ELISA for insulin needs only 5 μ L serum, which is equivalent to a blood sample of about 10 μ L. Thus a finger stick or earlobe prick method for blood collection can be used in conjunction with our assay. Collecting blood from an earlobe prick instead of a vein greatly reduces patient suffering. It is also possible for DM patients to collect their own blood using a minimally invasive lancet technique for our ultrasensitive ELISA. On the other hand, a commercially available method, such as the TOSOH II (IRI) kit, which is used at Kagawa University Hospital, requires at least 200 μ L of blood, including the dead volume. Thus, the blood sample must be at least 500 μ L.

The reduction in sample volume achieved when using our ELISA assay is an important advantage.

Furthermore, because our ultrasensitive ELISA is colorimetric, there is no requirement for special tools or chemicals such as radioisotopes or fluorescent probes. Thus, it is hoped that our ultrasensitive ELISA will pave the way for the development of a simple insulin measurement apparatus that can be easily used by general practitioners or DM patients themselves. Such an apparatus will enhance the measurement of the homeostasis model assessment ratio (HOMA-R) for resistance to insulin so that general practitioners will be able to further counsel DM patients accurately and rapidly. As such, our ultrasensitive ELISA for insulin measurement is a first step toward better treatment for DM.

In conclusion, we demonstrated that our ultrasensitive ELISA coupled with thio-NAD cycling is useful for the detection of insulin at subfemtomoles/assay in DM-patient sera, and leads to a reduction of blood sample volume required, allowing for less invasive blood sampling techniques. Recombinant human insulin, but not the WHO international standard insulin reference, should be used as a reference even in our ultrasensitive ELISA. Furthermore, as long as two suitable antibodies for a sandwich ELISA can be provided for the target protein, our ultrasensitive ELISA can be widely applied to detect trace amounts of various proteins in human sera with ultrasensitivity merely by combining thio-NAD cycling reagents with the conventional ELISA system.

Author contributions

E.I. and S.W. conceived and designed the experiments. M.K., H.K., M.M., M.T., K.A., S.H., S.T., N.K., H.I., and K.M. performed the experiments. E.I., M.K., H.K., S.W., K.N., M.Y., T.Y., and T.M. analyzed the data. E.I., S.W., K.N., S.H., K.M., M.Y., T.Y., and T.M. wrote the paper.

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Competing interests

E.I., M.K., H.K., M.T., K.A., S.H., S.T., N.K., H.I., K.M., M.Y., T.Y., and T.M. declare that they have no competing interests. M.M. and K.N. are employees of TAUNS Laboratories, Inc., and S.W. is an employee of BL Co., Ltd.

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