Analytical evaluation of a high-molecular-weight (HMW) adiponectin chemiluminescent enzyme immunoassay

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A B S T R A C T

Background: The measurement of high-molecular-weight (HMW) adiponectin concentration provides valuable clinical information. However, the conventional ELISA method requires complicated and lengthy assay procedures to obtain assay results.

Methods: We prepared new assay reagents based on chemiluminescent enzyme immunoassay (CLEIA) on a fully-automated analyzer system using the same IH7 monoclonal antibody as for ELISA as solid phase and detection antibodies (CLEIA/cartridge-type and CLEIA/bottle-type).

Results: The assay range of both CLEIA reagents were from 0.20 to 15.00 μg/ml, and lower limit of detection and quantification were lower than 0.0928 and 0.1346 μg/ml in CLEIA/cartridge-type and in CLEIA/bottle-type reagents, respectively. A good correlation was observed between both reagents (y = 1.000x + 0.120). The imprecision test as % of coefficient variation in both reagents were less than 3.3% and recovery test showed the range from 100% to 109%. No or little interference of blood components was observed in both reagents. HMW adiponectin concentration measured by CLEIA reagents was approximately half that measured by the previous ELISA because of reevaluation using freshly and highly purified HMW adiponectin standard.

Conclusion: The newly-prepared CLEIA reagents are robust and adequate and can be used for the measurement of HMW adiponectin in the clinical laboratory.

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1. Introduction

Adiponectin, an adipocyte-specific secretory protein carrying 244 amino acids with 18 signal residues, was originally isolated from human materials as GBP28 [1] and apM1 [2], and from murine cell lines as Acrp30 [3] and AdipoQ [4]. Adiponectin exists in human blood as multiple isoforms such as trimeric low-molecular-weight (LMW), hexameric middle-molecular-weight (MMW) and high-molecular-weight (HMW) forms, and oligomer formation of adiponectin critically depends on disulfide bond formation mediated by cys-39 [5,6].

Several reports have indicated that adiponectin regulates insulin sensitivity and lipid metabolism, and has protective activities on the vasculature [7,8]. Adiponectin levels have also been found to be negatively correlated with the degree of hyperinsulinemia, insulin resistance, obesity, diabetes and coronary artery diseases [9–25]. Additionally, increased levels of adiponectin concentration have been observed in patients treated with pioglitazone [18–21] and losartan (angiotensin II receptor blocker) [22]. Since the biological activity of adiponectin was carried by HMW form [19,23], the absolute amount of HMW adiponectin and/or the ratio of HMW adiponectin against total adiponectin rather than absolute total adiponectin concentration correlated better with the degree of improvement in insulin sensitivity by thiazolidinedione treatment [18–21], glucose tolerance [24], severity of vascular complications associated with obesity associated diseases [15–17], metabolic syndrome [25] and weight reduction [23].

Total adiponectin was measured using an enzyme-linked immunosorbent assay (ELISA) after treatment with sodium dodecyl sulfate (SDS) and heating [9]. In this ELISA, recombinant adiponectin was used as standard/calibrator; namely, a 693-bp adiponectin cDNA (nt 69–761) encoding a protein without a leader peptide was subcloned into the pET3c expression vector and transformed to host E. coli, BL21(DE3)pLyS S. To measure HMW adiponectin and/or percentage of HMW adiponectin in total adiponectin, previous studies have combined with gel-chromatography method and immunoblotting method [26], but this type of assay is cumbersome and cannot easily be adapted to high though-put analyses of multiple samples.

In a recent study, ELISA kits were used for the detection of various forms of human adiponectin using a combination of monoclonal antibodies and proteases [26,27]. However, sample pretreatment with proteases is required for the measurement of HMW adiponectin.
We previously reported on an ELISA for the measurement of HMW adiponectin using IH7 monoclonal antibody [28]. Since IH7 monoclonal antibody specifically reacts with the C-terminal globular domain of one trimer, and HMW adiponectin contains 4 and/or 6 trimers, the combination of IH-7 monoclonal antibodies as solid phase and enzyme-labeled antibodies can detect HMW but not LMW adiponectin.

Recently, 2 kinds of new assay reagents based on the chemiluminescent enzyme immunoassay (CLEIA) in combination with fully automatic analyzer systems were developed using IH7 monoclonal antibody. The value of HMW adiponectin concentration of the standard/calibrator was reevaluated based on the study using highly purified HMW adiponectin fraction in combination with a protein concentration assay by Lowry’s method and a determination of the extinction coefficient of adiponectin [29]. Therefore, the HMW adiponectin concentration of each sample by these new assay reagents is different from that of the ELISA kit, although purified HMW adiponectin is used as the standard/calibrator in both reagents.

2. Materials and methods

2.1. Human serum, plasma and reagents

Serum samples for the evaluation of CLEIA reagents were obtained from 299 individuals with their informed consent. Paired serum and plasma samples were purchased from ProMedDx (Norton, MA). Reagents for interference tests were purchased as follows: Interference Check A Plus containing conjugated-form bilirubin (Bilirubin-C), free-form bilirubin (Bilirubin-F), hemoglobin and chyle from Sysmex (Kobe, Japan), rheumatoid factor from TRINA (Nanikon, Switzerland), and human anti-mouse antibody (HAMA)-positive samples from ProMedDx. Reagents for interference tests were purchased as follows: Interference Check A Plus containing conjugated-form bilirubin (Bilirubin-C), free-form bilirubin (Bilirubin-F), hemoglobin and chyle from Sysmex (Kobe, Japan), rheumatoid factor from TRINA (Nanikon, Switzerland), and human anti-mouse antibody (HAMA)-positive samples from ProMedDx.

2.2. Assay principle of CLEIA

Two kinds of CLEIA reagents were used in combination with the fully-automated analyzer systems (Lumipulse System, Fujirebio Inc., Tokyo, Japan). Namely, a cartridge-type CLEIA reagent (CLEIA/cartridge-type) was used in combination with Lumipulse f and Lumipulse G-1200, and a bottle-type CLEIA reagent (CLEIA/bottle-type) was combined with Lumipulse Presto II. Ferrite particles coupled with IH7 monoclonal antibody as solid phase, alkaline phosphatase-conjugated IH7 monoclonal antibody, and AMPPD [3′-(16/60)-3″-(3′″-phosphoryloxy)phenyl-1,2-dioxetane disodium salt] (Applied Biosystems, Bedford, MA) as substrate were used for both types of CLEIA reagents.

2.2.1. Assay procedure of cartridge-type CLEIA reagent (CLEIA/cartridge-type)

Ten microliters of serum and/or plasma sample was automatically diluted with 180 μl of sample dilution buffer solution and incubated with ferrite microparticles previously coupled with IH7 monoclonal antibody in an assay cartridge. After 10 min incubation at 37 °C and washing, further incubation was done for 10 min at 37 °C with alkaline phosphatase-conjugated IH7 monoclonal antibody. After washing, 200 μl of substrate solution was added to the test cartridge and further incubated for 5 min at 37 °C, and relative chemiluminescent intensity was measured. HMW adiponectin concentration was calculated by calibrator curve written using the standards of 5 concentrations or the calibrators of 3 concentrations, and was expressed as μg/ml. In this study, the concentrations of the standards were 0, 0.49, 1.53, 5.31 and 17.14 μg/ml, and those of the calibrators were 0, 1.53 and 17.14 μg/ml, respectively.

2.2.2. Assay procedure of bottle-type CLEIA reagent (CLEIA/bottle-type)

Automatically diluted samples obtained by mixing 10 μl of serum and/or plasma with 190 μl of sample dilution buffer solution were incubated for 8 min at 37 °C with IH7 monoclonal antibody-coupled ferrite microparticle suspension. After washing, further incubation was done for 8 min at 37 °C with alkaline phosphatase-conjugated IH7 monoclonal antibody. After washing, 50 μl of substrate solution was added and further incubation was done for 4 min at 37 °C. HMW adiponectin concentration was calculated using 3 kinds of calibrators and HMW adiponectin concentration was expressed as μg/ml. The concentrations of the calibrators were 0, 1.56 and 16.95 μg/ml in this study.

2.3. Standard/calibrator of HMW adiponectin for CLEIA reagents

A standard/calibrator of HMW adiponectin was prepared and its concentration was determined as described previously [29]. Briefly, HMW adiponectin was purified from pooled human serum by the combination of the precipitation by 30% ammonium sulfate, chromatography with Superdex 200 (26/60), DEAE-Sepharose chromatography by stepwise elution method with 50 mmol/l Tris–HCl (pH8.0) and 100 mmol/l, 300 mmol/l, 500 mmol/l and 1 mol/l sodium chloride (NaCl), and finally column chromatography with Superdex 200 (16/60). The purity of the final fraction was checked by HPLC G3000SWXL column analysis and SDS-PAGE–Western blot analysis. The extinction coefficient of adiponectin was calculated by the molar absorptivity values for tryptophan and tyrosine residues by the combination of the amino acid sequence of The National Center for Biotechnology Information (NCBI) database and DC Protein Assay kit (BioRad, Tokyo, Japan) using bovine serum albumin and bovine IgG as standards.

2.4. ELISA

Both CLEIA reagents were compared using 297 serum samples using a commercially available ELISA kit for the measurement of HMW adiponectin (Fujirebio Inc., Tokyo, Japan) established based on the previous report [28].

2.5. Evaluation of CLEIA reagents

The performance of CLEIA/cartridge-type and CLEIA/bottle-type reagents was evaluated in terms of lower limit of detection (LOD) and lower limit of quantification (LOQ), imprecision test, reproducibility test, interference of blood components, comparison of serum and plasma samples, and correlation of assay values between both reagents. We also tested the stability of HMW adiponectin in serum samples by stock condition at −20 °C and/or 10 °C and by repeat freezing–thawing.

3. Results

3.1. Calibration curve, lower limit of detection (LOD) and lower limit of quantification (LOQ)

The assay range of both reagents was the same from 0.20 to 15.00 μg/ml. The LOD and LOQ of the CLEIA reagents were determined by the NCCLS document EP17-A [30]. The LOD of 2 different lots of each reagent was 0.0828 and 0.0928 pg/ml in CLEIA/cartridge-type and 0.1046 and 0.1346 μg/ml in CLEIA/bottle-type, and the LOQ was the same as the LOD.

3.2. Imprecision

The imprecision test was also performed in duplicate on the basis of 2 runs per day, 20 days within 1 month (80 assays per control panel in total). The CV% of within-run, between-day and between-run assays ranged from 0.7% to 2.2% in CLEIA/cartridge-type and from 1.2% to 2.7% in CLEIA/bottle-type (Table 1) [31]. Additionally, the intra-assay was performed through 6 repeated measurements of 3 control
panels during the same day, and the inter-assay was done by the measurement of 3 control panels on 6 different days. The intra-assay CV were 2.2% (range, 1.0–2.2%) for the CLEIA/cartridge-type and 3.1% (range, 1.4–3.1%) for the CLEIA/bottle-type; the inter-assay of the CLEIA/cartridge-type and CLEIA/bottle-type was 3.3% (range, 1.7–3.3%) and 3.3% (range, 2.8–3.3%), respectively (data not shown).

We also confirmed reproducibility using 297 samples by duplicate assay, and good data was observed: $y = 1.000x - 0.010$, $r = 0.999$ in CLEIA/cartridge-type and $y = 1.000x + 0.000$, $r = 0.997$ in CLEIA/bottle-type.

3.3. Linearity

Linearity was tested using 2 samples by serial dilutions with sample buffer solution, and HMW adiponectin concentrations were measured. Good linearity results were observed for both CLEIA reagents, as shown in Fig. 1A and B.

3.4. Recovery

The recovery test was performed by adding one-tenth volume of various concentrations of HMW adiponectin standard/calibrator to 5 serum samples. The percentage of recovery was calculated by assessing the HMW adiponectin concentration of mixed samples against the added HMW adiponectin standard/calibrator concentrations. Results show that recovery was from 101% to 106% in CLEIA/cartridge-type and from 100% to 109% in CLEIA/bottle-type (Table 2).

3.5. Interference of blood components

The interference of blood components to CLEIA reagents was analyzed by adding bilirubin-C, bilirubin-F, hemoglobin and chyle to 3 serum samples at various concentrations, and HMW concentrations were measured by both CLEIA reagents. The alteration of HMW adiponectin values was within 5% by the addition of 19.7 mg/dl of bilirubin-C, 18.1 mg/dl of bilirubin-F, 456 mg/dl of hemoglobin, and 1540 formazin turbidity of chyle (data not shown).

We also tested the influence of the rheumatoid factor and HAMA-positive samples, and observed an alteration of assay value of $<$11% by the addition of maximum 1000 IU/ml of rheumatoid factor and 291.3 ng/ml of HAMA (data not shown).

3.6. Comparison of serum and plasma samples

The differences between serum and plasma were studied using plasma obtained by treatment with heparin sodium and/or heparin lithium from the same individuals. As shown in Fig. 2A and B, the correlation between serum and plasma was $y = 0.978x - 0.057$, $r = 0.996$ (n = 104) in CLEIA/cartridge-type and $y = 0.984x - 0.099$, $r = 0.996$ (n = 106) in CLEIA/bottle-type.

### Table 1

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Control panels</th>
<th>Average (μg/ml)</th>
<th>Within-run (CV%)</th>
<th>Between-day (CV%)</th>
<th>Between-run (CV%)</th>
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<td>2.7</td>
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</table>

The imprecision test was performed by duplicate on the basis of two runs per day, 20-times assay within one month (80 assays per control panel in total).

### Table 2

<table>
<thead>
<tr>
<th>Reagents</th>
<th>Serum samples</th>
<th>Standard/calibrator added (μg/ml)</th>
<th>HMW adiponectin conc. (μg/ml) in samples</th>
<th>Measured HMW adiponectin conc. (μg/ml)</th>
<th>Recovery (%)</th>
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<td>2.16</td>
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</table>
3.8. Correlation of HMW adiponectin value between CLEIA reagents and ELISA

When CLEIA/cartridge-type and CLEIA/bottle-type were compared with ELISA using 297 serum samples, a good correlation was observed: $r = 0.984$ in CLEIA/cartridge-type and $r = 0.981$ in CLEIA/bottle-type; however, the slope of these reagents against ELISA was $y = 0.576x + 0.318$ and $y = 0.577x + 0.445$, respectively (Fig. 4A and B). These data suggest that the correlation between CLEIA reagents and ELISA is good and that the HMW adiponectin concentration value in CLEIA reagents was approximately half that by the ELISA kit.

3.9. Stability of HMW adiponectin in samples

Serum samples were stored at $-20\,^\circ\mathrm{C}$ and/or $10\,^\circ\mathrm{C}$ for 14 days, and the alteration of HMW adiponectin value was examined. No alteration or just a slight alteration of less than 8.2% in CLEIA/cartridge-type and less than 6.3% in CLEIA/bottle-type was observed.

Next, 3 control panels each were treated by 5 times freezing–thawing, and the alteration of HMW adiponectin concentration was measured. An alteration of less than 3.3% and 3.5% was observed in CLEIA/cartridge-type and CLEIA/bottle-type (data not shown).

4. Discussion

Adiponectin plays several important physiological roles in glucose and lipid metabolism, and the simple measurement of HMW adiponectin constitutes a good tool for the prediction of insulin resistance and metabolic syndrome [15–26]. According to LMW, MMW and HMW adiponectin concentrations, no significant difference was observed between males and females with regard to the levels of LMW adiponectin, whereas the total, HMW and MMW adiponectin levels in females were significantly higher than in males [27]. Further, the total adiponectin levels depended mostly on the HMW adiponectin levels in both males and females [27].

For the measurement of HMW adiponectin, several methods were reported [26–30]. One such method is based on the treatment of the sample with proteinase K and monoclonal antibodies for detection, where total (untreated with proteinase K) and HMW (resistant to proteinase K treatment) adiponectin are measured separately [26]. In this HMW adiponectin assay, pooled human serum, which calibrated by FPLC analysis was used as a standard/calibrator.

Another method is based on an assay principle that combines monoclonal antibodies with 2 kinds of proteases for the detection of various forms of human adiponectin such as HMW, MMW or LMW [27]. In this assay kit, purified HMW adiponectin was used as a standard/calibrator after conversion to the dimeric form with SDS sample buffer [100 mmol/l sodium citrate (pH 3.0) containing 2% SDS]; adiponectin protein content was quantified by Lowry’s method using bovine serum albumin as the standard.

Another assay was reported [32], namely, HMW adiponectin ELISA using monoclonal antibody (ANOC9121) against HMW adiponectin and pooled human serum calibrated by purified human HMW adiponectin as the standard/calibrator. However, the details of the standard/calibrator and monoclonal antibody are unclear.

Although several assay methods have been reported, an international standard/calibrator of adiponectin, especially HMW adiponectin, has not yet been established. It is therefore difficult to compare the assay
CLEIA assay kits were prepared using the newly expressed numerically by the protein concentration assay by Lowry’s method. Recently, new to the value obtained by the amino acid sequence of adiponectin and highly purified calibration standard/calibrator, and the extinction coefficient of HMW adiponectin was determined as 1.34 at 1.0 mg/ml (A280nm: 0.1%) [29]. The extinction coefficient value of 1.34 is similar to the value obtained by the amino acid sequence of adiponectin and by the protein concentration assay by Lowry’s method. Recently, new CLEIA assay kits were prepared using the newly expressed numerically standard/calibrator and IH7 monoclonal antibody based on fully automatic CLEIA analyzer systems. Since the expressed value of HMW adiponectin concentration in standard/calibrator of CLEIA reagents was different from that of the ELISA kit [28,29], the HMW adiponectin concentration value in CLEIA reagents was approximately half of that by ELISA kit; thus, the correlation of HMW adiponectin values of 297 serum samples in CLEIA reagents against ELISA was \( y = 0.576x + 0.318 \) in CLEIA/cartridge-type and \( y = 0.577x + 0.445 \) in CLEIA/bottle-type.

In this study, we evaluated the performance of CLEIA reagents in combination with automatic analyzer systems in which assay results were out-putted automatically within 30 min, and obtained satisfactory results with respect to LOD and LOQ, precision test, linearity, and recovery test. No or little interference was observed by the addition of blood components such as Bilirubin-C, Bilirubin-F, hemoglobin, chyle, rheumatoid factor and HAMA to serum samples. In addition, a good correlation was observed between CLEIA/cartridge-type and CLEIA/bottle-type reagents (\( y = 1.000x + 0.120 \), \( r = 0.997 \)) and between serum and plasma (\( r = 0.996 \) in both CLEIA/cartridge-type and CLEIA/bottle-type). These results clearly establish that these CLEIA reagents are sufficiently robust to be implemented in the clinical laboratory for the measurement of HMW adiponectin, and that they constitute a useful tool to investigate the physiological roles of HMW adiponectin.

Acknowledgement

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References


Fig. 4. Correlation between CLEIA and ELISA, HMW adiponectin concentrations (µg/ml) in 297 serum samples were measured by CLEIA/cartridge-type (A), CLEIA/bottle-type (B) and commercially available HMW adiponectin assay ELISA kit \( y = 0.576x + 0.318 \) in CLEIA/cartridge-type and \( y = 0.577x + 0.445 \) in CLEIA/bottle-type.


