Title
A new enzyme-linked immunosorbent assay system for human hepatic triglyceride lipase.

Permalink
https://escholarship.org/uc/item/65x7k4cc

Journal
Clinica chimica acta; international journal of clinical chemistry, 424

ISSN
0009-8981

Authors
Miyashita, Kazuya
Kobayashi, Junji
Imamura, Shigeyuki
et al.

Publication Date
2013-09-01

DOI
10.1016/j.cca.2013.06.016

Peer reviewed
A new enzyme-linked immunosorbent assay system for human hepatic triglyceride lipase

Kazuya Miyashita a, Junji Kobayashi b,⁎, Shigeyuki Imamura c, Noriaki Kinoshita d, Kimber L Stanhope e, Peter J Havel e, Katsuyuki Nakajima f, Tetsuo Machida f, Hiroyuki Sumino f, Makoto Nara f, Masami Murakami f

Articles info

Article history:
Received 18 March 2013
Received in revised form 12 June 2013
Accepted 13 June 2013
Available online 27 June 2013

Keywords:
Hepatic triglyceride lipase
Endothelial lipase
ELISA

Background: The objective of this study was to establish a new sandwich based enzyme linked immunosorbent assay (ELISA) for measuring the protein mass of human hepatic triacylglyceride lipase (HTGL).

Method: Two mouse monoclonal antibodies raised against human HTGL were used for the sandwich ELISA. The post-heparin plasma (PHP) samples obtained at a heparin dose of 50 unit/kg from 124 normolipidemic subjects were used for this ELISA.

Results: The dynamic assay range of the developed ELISA for the HTGL was from 0.47 to 30 ng/ml. The CV was <7% in both intra- and inter-assays, and it did not cross-react with lipoprotein lipase or endothelial lipase (EL).

The HTGL concentration in PHP showed a strong correlation with HTGL activity \[n = 121, r = 0.778, p < 0.001\]. There was a weak relation of HTGL concentration against high-density lipoprotein cholesterol (HDL-C) \[n = 123, r = -0.229, p = 0.011\] but no relations against total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), triglycerides (TG), small dense LDL, remnant like particles cholesterol (RLP-C) and RLP-TG were confirmed. Interestingly, a weak but positive correlation between HTGL concentration and EL concentration was shown \[n = 122, p = 0.013, r = 0.224\].

Conclusion: These results indicate that this new sandwich ELISA for measuring HTGL concentration in PHP can be applied in a daily clinical practice.

© 2013 Elsevier B.V. All rights reserved.

1. Introduction

Hepatic triglyceride lipase (HTGL), a lipolytic enzyme, is a secreted glycoprotein, and is synthesized by hepatocytes and bound to heparin sulfate proteoglycans at the surface of liver sinusoidal capillaries. HTGL plays a major role in lipoprotein metabolism as a lipolytic enzyme that hydrolyzes triglycerides (TGs), phospholipids in chylomicron remnants, intermediate-density lipoproteins (IDLs) and high-density lipoproteins (HDLs) \[1,2\]. Patients with HTGL deficiency present with hypercholesterolemia or hypertriglyceridemia, and accumulate very-low-density lipoproteins (VLDLs), chylomicron remnants, IDLs, TG-rich low-density lipoproteins (LDLs) and HDLs \[3–8\].

Although there was a commercially available sandwich ELISA kit that was developed in Japan \[9\] for measuring HTGL concentration, it used 2 distinct monoclonal antibodies raised against human HTGL purified from post-heparin plasma (PHP) as a starting material, it was withdrawn from the market with unknown reasons almost a decade ago. Since no high sensitive assay system for measuring the protein concentration in plasma was available, we reported an easy and quick PHP–HTGL activity assay system several years ago \[10,11\]. But we believe that it is still important to determine quantity of HTGL concentration in order to understand the clinical significance of this enzyme in lipoprotein metabolism, especially in the case of detecting dysfunctional types of HTGL. We have now established a new sandwich ELISA method using 2 distinct monoclonal antibodies which are raised against purified human HTGL from a concentrated conditioned medium of human HTGL-471 transfected Chinese hamster ovary (CHO) cells.

⁎ Corresponding author at: Department of General Medicine Kanazawa Medical University, 1-1 Daigaku, Uchinada Kahoku District Ishikawa Prefecture 920-0293, Ishikawa, Japan.
E-mail address: mary@kanazawa-med.ac.jp (J. Kobayashi).
2. Materials and methods

2.1. Preparation of purified HTGL

The purified human HTGL was prepared as previously reported [12]. We generated recombinant human HTGL as follows. A full-length of human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using primers in which a FLAG-epitope tag was added to the 3' end of cDNA before sub-cloning. To generate the plasmid encoding hHTGL-Full, the cDNA was inserted into pcDNA3.1(+) expression vector (Invitrogen). However, human HTGL is exhibited on cell surface through the binding of 5' carboxyl-terminal residues (KRKIR) [12]. Therefore, we also prepared a truncated human HTGL mutant (hHTGL-471) this time by using deleted 5' carboxyl terminal residues in order to promote its secretion in culture supernatant. Then, we used antisense PCR primers for generating the plasmids encoding hHTGL-Full and hHTGL-471, respectively. Each PCR product was inserted into pcDNA3.1(+) expression vector after a FLAG-epitope tag was added to the 3' end. CHO cells were transfected with the hHTGL-Full plasmid or the hHTGL-471 plasmid and selected with 500 \( \mu \)g/ml of G418 for establishing of stable transfectants. Next, we screened the transfectants by detecting the expression of hHTGL-Full or hHTGL-471 expression with anti-c-FLAG rabbit IgG (Immuno-Biological Laboratories) and selected the high-expression clones as a human HTGL-Full-Flag/CHO named as 8A4 or human HTGL-471-Flag/CHO named as 3B1, respectively. We purified HTGL protein from the concentrated conditioned medium of human HTGL-471-Flag/CHO 3B1 using an anti-FLAG M2 affinity gel (Sigma-Aldrich). We estimated the purity of the recombinant human HTGL protein using the Multi Gauge densitometry and determined the concentration of the protein by comparison with bovine serum albumin (BSA) as an indicator (Fig. 1).

2.2. Preparation of monoclonal antibody (MoAb)

The monoclonal antibody for human HTGL was prepared as reported previously [10]. In brief, we used the purified HTGL protein which was emulsified with Freund complete adjuvant for first immunization and then immunized it with Freund incomplete adjuvant after 2 weeks. It was immunized into BALB/c mice (Charles River) 2 times with Freund incomplete adjuvant every week. After the immunizations, we removed the spleen from the mice and hybridized it with mouse myeloma cells X63-Ag 8.653 as a fusion partner. We generated monoclonal antibodies up to 36 clones, and finally selected the best 2 monoclonal antibodies named 26A1 MoAb and 31A1 MoAb

### Table 1

<table>
<thead>
<tr>
<th>Clinical profile of the study subjects.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
</tr>
<tr>
<td>Men/women</td>
</tr>
<tr>
<td>Total cholesterol, mg/dl</td>
</tr>
<tr>
<td>Triglycerides, mg/dl</td>
</tr>
<tr>
<td>HDL-cholesterol, mg/dl</td>
</tr>
<tr>
<td>LDL-cholesterol, mg/dl</td>
</tr>
<tr>
<td>sdLDL, mg/dl</td>
</tr>
<tr>
<td>RLP-cholesterol, mg/dl</td>
</tr>
<tr>
<td>RLP-triglycerides, mg/dl</td>
</tr>
</tbody>
</table>

Fig. 1. Quantification of purified human HTGL protein and immunoblotting analysis. We quantified the purified Flag tagged HTGL-471 protein from the concentrated conditioned medium of human HTGL-471/CHO 3B1 using an anti-FLAG M2 affinity gel and sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE, right side) followed by staining with Coomassie Brilliant Blue (left side). The purity of the recombinant human HTGL protein was determined at the concentration of 17 \( \mu \)g/ml by densitometry using a Multi gauge and the concentration of the protein was determined by comparison with BSA as an indicator.

Fig. 2. Demonstration of the specificities of anti-human HTGL mouse monoclonal IgGs by western blotting. For immunoblotting, 3 \( \mu \)l conditioned medium of human HTGL Full-Flag/CHO 8A4 was used for 12.5% SDS–PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with 2 \( \mu \)g of 26A1 MoAb or 31A1 MoAb followed by incubation with secondary antibody conjugated with horseradish peroxidase.
which specifically and highly reacted with human HTGL in plasma. For testing the cross-reactivity of the ELISA with other lipase members including lipoprotein lipase (LPL) and endothelial lipase (EL), we obtained recombinant human LPL protein from BioVend and generated recombinant EL as we previously reported [12].

2.3. HTGL sandwich ELISA

Each well of microtiter plates (96 wells) was coated with 100 μl of 100 mmol/l concentrated carbonate buffer (pH 9.5) containing 0.25 μg purified 31A1 mouse monoclonal IgG followed by incubation overnight at 4 °C. The plates were then washed with PBS-T and blocked with 200 μl of 1% (wt/vol) BSA in PBS containing 0.05% NaN3/well overnight at 4 °C. After washing 2 times with PBS-T, 100 μl of both the serially diluted standard of recombinant human HTGL-471 and the 8-fold plasma samples with 1% BSA in PBS-T was added into each well of the coated microtiter plates in duplicate and incubated overnight at 4 °C. After washing 4 times with PBS-T, 100 μl of HRP-conjugated 26A1 mouse IgG Fab' was added into each well and the samples were incubated for 30 min at 4 °C. The wells were washed 8 times with PBS-T and 100 μl of tetramethyl benzidine solution (Kem-En-Tec) was added into each well as a substrate followed by incubation in the dark for 30 min at room temperature. The reaction was stopped by adding 100 μl of 0.5 mol/l H2SO4. We measured absorbance of the solution at 450 nm by means of an ELISA reader (E-Max; Molecular Devices). For assessing the intra- and inter-assay precisions of the ELISA, we established 3 QC samples covering the high, middle and low ranges of the calibration curves. We determined intra-assay precision by 24 repeated measurements of each QC sample in a plate and inter-assay precision was determined by assessing each QC sample across 6 different plates with quintuple wells. Additionally, for assessing the recovery rate in blood samples, different concentrations of recombinant human HTGL-471 added into samples were measured and the recovery rate was validated as the difference between the measured concentration and the theoretical concentration. The analytical limit of quantification of this system was determined on the basis of the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI) standard EP17-A evaluation protocols.

The ELISA assay system was finally designed as an ELISA kit (Immuno-Biological Laboratories).

2.4. Immunoblotting and immunoprecipitation–immunoblotting

We analyzed the supernatant obtained from human HTGL Full-Flag/CHO 8A4 Integra cells by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by staining it with Coomassie Brilliant Blue. For immunoblotting, 5 μl conditioned medium of human HTGL Full-Flag/CHO 8A4 was used for 12% SDS–PAGE and transferred to nitrocellulose membrane (Bio-Rad). The membrane was incubated with 2 μg of the 26A1 MoAb or 31A1 MoAb followed by incubation with secondary anti-mouse IgG antibody conjugated with horseradish peroxidase (HRP) (1:4000, Immuno-Biological Laboratories).

We also assessed the reactivity of the antibodies by immunoprecipitation–immunoblotting. The supernatant obtained from either the human HTGL Full-Flag/CHO 8A4 Integra cells, pre-heparin or post-heparin plasma pretreated with 0.1% Tween20-PBS was incubated with 2 μg of either 26A1 MoAb, 31A1 MoAb or mouse IgG (as negative control) and then it was added into Protein-G Sepharose (GE Healthcare Japan). After further incubation, the supernatant was centrifuged and the resulting pellet was washed 3 times with 500 μl of TNE buffer (10 mmol/l Tris–HCl, pH 7.8, 150 mmol/l NaCl, 1 mmol/l EDTA, 1% Nonidet P-40). The pellet was subsequently lysed and we performed immunoblot analysis using biotinylated

<table>
<thead>
<tr>
<th>STD (ng/mL)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>30.</td>
<td>2.600</td>
</tr>
<tr>
<td>15.</td>
<td>1.165</td>
</tr>
<tr>
<td>7.5</td>
<td>0.554</td>
</tr>
<tr>
<td>3.75</td>
<td>0.266</td>
</tr>
<tr>
<td>1.88</td>
<td>0.130</td>
</tr>
<tr>
<td>0.94</td>
<td>0.067</td>
</tr>
<tr>
<td>0.47</td>
<td>0.035</td>
</tr>
<tr>
<td>0</td>
<td>0.007</td>
</tr>
</tbody>
</table>

Fig. 4. Typical standard curves of HTGL concentration measured by the sandwich ELISA. Recombinant HTGL-471 as a standard was assayed at the concentrations of 30, 15, 7.5, 3.75, 1.88, 0.94 and 0.47 ng/ml in the ELISA.
26A1 MoAb followed by HRP-conjugated streptavidin system. We visualized the HTGL signal using an ECL reagent (GE Healthcare, Piscataway, NJ).

2.5. Other method

LPL and HTGL activities were measured using the method we previously reported [10,11]. Briefly, lipase activities were assayed using dioleylglycerol solubilized with detergent and measuring the increase in absorbance at 546 nm (subwave length; 660 nm) due to the production of quinonediimine dye. Automated assay of lipase activity was visualized the HTGL signal using an ECL reagent (GE Healthcare, Piscataway, NJ).

2.6. Preparation of blood samples

The investigation conformed to the principles outlined in the Declaration of Helsinki. The UC Davis Institutional Review Board approved the experimental protocol and subjects provided informed consent to participate in the study. We collected PHP samples from overweight and obese participants in a nutritional research study conducted at the University of California, Davis Clinical and Translational Science Center’s Clinical Research Center as described previously [13]. The clinical profiles of the subjects in this study are shown in Table 1. The activity of HTGL in the plasma samples was measured as described and previously reported [11]. PHP was obtained at 10 min after heparin (50 units/kg) was injected. The sera and plasma were immediately separated and kept frozen at −80 °C until assay.

Plasma concentrations of HDL-C, LDL-C and TG were measured enzymatically using kit from Sekisui (Tokyo, Japan).

2.7. Statistical analysis

The data was analyzed with Dr. SPSS II (SPSS, Chicago, IL). Quantitative variables are reported as mean ± standard deviation values. The statistical significance of difference was determined by the Mann–Whitney U test. Pearson’s correlation coefficients (r-value) were determined and single linear regression analysis was performed to detect associations between variables. A p < 0.05 was considered statistically significant.

3. Results

3.1. Identification of recombinant human HTGL and characterization of antibodies against human HTGL

We generated a pair of monoclonal antibodies named 26A1 and 31A1 that exhibited a highly specific reactivity with the HTGL protein derived from human HTGL-471/CHO 3B1 stable transfectant. Immunoblotting revealed a strong signal against 65-kDa mature HTGL protein (Fig. 2). To confirm whether the antibodies had the ability to react with native HTGL in aqueous conditions, we performed an immunoprecipitation–immunoblotting analysis. Both 26A1 MoAb and 31A1 MoAb were able to immunoprecipitate HTGL originated from the conditioned medium of human HTGL-471/CHO 3B1 stable transfectant (Fig. 3).

3.2. Specificity, recovery and imprecision of the ELISA

Both 26A1 MoAb and 31A1 MoAb showed highly specific reactivity with HTGL protein. Thus, we decided to use them for developing the new sandwich ELISA system. The standard dose–response curve for the HTGL ELISA system exhibited a linear shape when it was plotted on a log/log scale over a range from 0.47 to 30.0 ng/ml (Fig. 4). Although HTGL has a 53% and 41% amino acid sequence homology with LPL and EL [14], the cross-reactivity of this ELISA against human LPL and EL was less than 0.1% (The data is not shown.). Imprecision was determined with 3 supplemented QC controls (high, middle and low). The CVs of intra-assay imprecision were 5.1% in the high, 4.6% in the middle and 7.0% in the low controls (Table 2). Additionally, the inter-assay results for the CVs were 6.8% in the high, 5.0% in the middle and 6.1% in the low controls (Table 2). Imprecision was considered statistically significant.

Table 2

<table>
<thead>
<tr>
<th>Validation results.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay value (ng/ml)</td>
</tr>
<tr>
<td>1. Intra-assay reproducibility</td>
</tr>
<tr>
<td>13.8</td>
</tr>
<tr>
<td>3.4</td>
</tr>
<tr>
<td>1.2</td>
</tr>
<tr>
<td>2. Inter-assay reproducibility</td>
</tr>
<tr>
<td>14.1</td>
</tr>
<tr>
<td>3.4</td>
</tr>
<tr>
<td>1.2</td>
</tr>
<tr>
<td>3. Specificity test</td>
</tr>
<tr>
<td>Human HTGL</td>
</tr>
<tr>
<td>Human LPL</td>
</tr>
<tr>
<td>Human EL</td>
</tr>
</tbody>
</table>

Fig. 5. Dilution linearity of sample materials for the assay. Doubling dilution of original solution with 15 ng/ml of standard added samples were defined as dilution rate 1. The mixture of condition medium (A) or post-heparin plasma (B) and standard HTGL (original concentration 15 ng/ml) was diluted as indicated in x-axis in the figure.
3.3. Dilution linearity of several materials for the assay

As shown in Fig. 5, a good linearity was shown with culture medium of recombinant HTGL (15 ng/ml of standard added) and doubled with diluted medium. In contrast, a poor linearity was shown with PHP (15 ng/ml of standard added) when it was diluted less than 16 fold, which has different matrix from culture medium, and this indicates that those specimens need to be diluted at least 16 fold to obtain reliable results.

We also investigated dilution effect of PHP without standard HTGL being added on measured HTGL concentration (Table 3). This data also shows that PHP need to be diluted at least 16 fold to obtain reliable results in clinical use.

3.4. Correlation of HTGL concentration to lipids and lipoproteins

HTGL concentration in PHP from 124 human subjects was 172 ± 147 ng/ml with a range of 42 to 1200 ng/ml. No patients had an HTGL concentration below the limit of quantification.

HTGL concentration had a strong correlation with HTGL activity measured by the method we previously reported (Fig. 6). An inverse correlation was indicated in HTGL concentration with serum HDL-C (n = 123, p = 0.011, r = −0.23; Fig. 6) while having no correlation with LDL-C, sdLDL, TG, RLP-C or RLP-TG (Fig. 6). Positive correlation was observed between HTGL and EL concentration (n = 122, p = 0.013, r = −0.22; Fig. 6).

4. Discussion

We generated specific HTGL monoclonal antibodies, 26A1 MoAb and 31A1 MoAb against recombinant human HTGL and we used 2 of them for establishing a sandwich ELISA system for quantification of human HTGL protein mass in PHP. The immunoblot and immunoprecipitation–immunoblot analysis were demonstrated and the obtained monoclonal antibodies specifically detected human HTGL.
Previously, Nishimura et al. [9] reported a sandwich ELISA system for measuring human HTGL in PHP using 2 distinct monoclonal antibodies established for specifically detecting purified HTGL in PHP. The average HTGL concentration in PHP obtained from 64 healthy subjects measured by their ELISA system was reported at around 2000 ng/mL. Compared with their results, our average HTGL concentration in PHP using the 2 distinct monoclonal antibodies, 26A1 MoAb and 31A1 MoAb, specifically detecting purified HTGL obtained from cultured media of CHO cells transfected with HTGL cDNA, showed as 172 ng/mL, which is less than 1 out of 10 values previously reported by Nishimura et al. In relation to this fact, the detection range of their method was 40–800 ng/mL of HTGL protein, whereas that of the present method was 0.47–30 ng/mL. We are not certain of the exact reason why HTGL concentration measured by our method was much lower than the measurement previously reported by Nishimura et al. One possibility is that the materials for immunizing mice for generating the monoclonal antibodies in our method could be much purer than the method previously reported by Nishimura et al. since the cultured media for HTGL cDNA stable transfectant may contain much larger amounts of HTGL protein than PHP.

An inverse correlation of HTGL concentration against HDL-C was observed in this study. It is consistent with previous reports in that it is showing the polymorphism of the HTGL gene causing lowered observed in this study. It is consistent with previous reports in that amounts of HTGL protein than PHP.

The monoclonal antibodies in our method could be much purer than the measurement previously reported by Nishimura et al. [9] reported a sandwich ELISA system for measuring HTGL concentration in PHP using the 2 distinct monoclonal antibodies, 26A1 MoAb and 31A1 MoAb, specifically detecting purified HTGL obtained from cultured media of CHO cells transfected with HTGL cDNA, showed as 172 ng/mL, which is less than 1 out of 10 values previously reported by Nishimura et al. In relation to this fact, the detection range of their method was 40–800 ng/mL of HTGL protein, whereas that of the present method was 0.47–30 ng/mL. We are not certain of the exact reason why HTGL concentration measured by our method was much lower than the measurement previously reported by Nishimura et al. One possibility is that the materials for immunizing mice for generating the monoclonal antibodies in our method could be much purer than the method previously reported by Nishimura et al. since the cultured media for HTGL cDNA stable transfectant may contain much larger amounts of HTGL protein than PHP.

Acknowledgment

We thank Keiko Koyanagi and Ayano Enya, Immuno-Biological Laboratories Co., Ltd. for their assistance in reading and discussions of this manuscript.

References