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

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ABSTRACT

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.

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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 assay system using 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 on 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.

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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 µg/ml by densitometry using a Multi gauge and the concentration of the protein was determined by comparison with BSA as an indicator.

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 µg/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 after electrophoresis (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
Clinical profile of the study subjects.

Age, y	26.4 ± 6.1
Body mass index, kg/m ²	25.0 ± 3.7
Men/women	67/57
Total cholesterol, mg/dl	163 ± 30
Triglycerides, mg/dl	57.2 ± 37.3
HDL-cholesterol, mg/dl	48.5 ± 11.8
LDL-cholesterol, mg/dl	93.3 ± 26.4
sdLDL, mg/dl	26.7 ± 12.7
RPL-cholesterol, mg/dl	4.1 ± 2.1
RPL-triglycerides, mg/dl	7.1 ± 10.6

Fig. 2. Demonstration of the specificities of anti-human HTGL mouse monoclonal IgGs by western blotting. For immunoblotting, 5 µl 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 µg of 26A1 MoAb or 31A1 MoAb followed by incubation with secondary antibody conjugated with horseradish peroxidase.

Fig. 3. Demonstration of the specificities of anti-human HTGL mouse monoclonal IgGs by immunoprecipitation–immunoblotting analysis. The supernatant obtained from the human HTGL Full-Flag/CHO 8A4 Integra cells (Lanes 1–3) and pre-heparin (Lanes 4–6) or post-heparin plasma (Lanes 7–9) pretreated with 0.1% Tween20–PBS were used in this demonstration. The immunoprecipitation is shown with 26A1 (Lanes 2, 5, 8) or 31A1 (Lanes 3, 6, 9) or mouse IgG (Lanes 1, 4, 7 as negative control), respectively.

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 BioVendor 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 μ l of 100 mmol/l concentrated carbonate buffer (pH 9.5) containing 0.25 μ g purified 31A1 mouse monoclonal IgG followed by incubation overnight at 4 $^{\circ}$ C. The plates were then washed with PBS-T and blocked with 200 μ l of 1% (wt/vol) BSA in PBS containing 0.05% NaN_3 /well overnight at 4 $^{\circ}$ C. After washing 2 times with PBS-T, 100 μ l 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 $^{\circ}$ C. After washing 4 times with PBS-T, 100 μ l of HRP-conjugated 26A1 mouse IgG Fab' was added into each well and the samples were incubated for 30 min

at 4 $^{\circ}$ C. The wells were washed 8 times with PBS-T and 100 μ l 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 μ l of 0.5 mol/l H_2SO_4 . 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 μ l 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 μ g 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 μ g 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 μ l 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

STD (ng/mL)	
30.	2.600
15.	1.165
7.5	0.554
3.75	0.266
1.88	0.130
0.94	0.067
0.47	0.035
0	0.007

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.

Table 2
Validation results.

Assay value (ng/ml)	S.D. (ng/ml)	C.V. (%)	n
1. Intra-assay reproducibility			
13.8	0.71	5.1	24
3.4	0.17	5.0	24
1.2	0.07	6.1	24
2. Inter-assay reproducibility			
14.1	0.96	6.8	6
3.4	0.15	4.6	6
1.2	0.08	7.0	6
3. Specificity test			
Human HTGL	100%		
Human LPL	<0.1%		
Human EL	<0.1%		

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 dioleoylglycerol 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 performed with a Model 7080 Hitachi automatic clinical analyzer. EL concentration using the sandwich ELISA was measured as we previously reported [12].

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 $\pm$ 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 exhibited as 5.1% in the high, 5.0% in the middle and 6.1% in the low controls (Table 2). Additionally, the inter-assay results for the CVs were 6.8% in the high, 4.6% in the middle and 7.0% in the low controls. Thus, we can consider the ELISA system to be reliable concerning imprecision.

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.

Table 3
Dilution linearity of post-heparin plasma without standard HTGL added for the assay.

Dilution rate of post heparin plasma	Dilution 1; doubling diluted post heparin plasma	HTGL concentration ng/ml
2	1	47.34
4	0.5	46.58
8	0.25	24.12
16	0.125	10.98
32	0.0625	5.49
64	0.03125	2.88
128	0.015625	1.51

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.

Fig. 6. Correlation between HTGL concentration and other plasma proteins in post-heparin plasma. We assayed HTGL concentration, HTGL activity, TG, HDL-C, LDL-C, sdLDL, RLP-C, RLP-TG and EL concentration in PHP from 123 human subjects. The values in the upper column show the correlation coefficient between the other assay values and HTGL concentration assay values.

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 our 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 HTGL activity, which was associated with higher HDL-C concentration [15–17]. It should be noted that this is the first study investigating the correlation between HTGL and EL, both of which are considered to belong to the same gene family. The clinical significance of this relation remains as an outstanding issue but this finding may suggest that these 2 lipolytic enzymes may be regulated at least in part by common factors. Several studies show that EL is increased in metabolic syndrome and it is observed by using inflammation markers, one of which shows inflammation up-regulates EL [18,19]. In relation to this fact, it is reported that there is a weak but significant inverse correlation between EL and adiponectin levels [20].

However, studies including our own case show that inverse correlations exist between adiponectin levels and HTGL activity [21,22]. Moreover, it is recognized that HTGL activity is increased in metabolic syndrome or insulin resistance state [23–25]. These previous findings suggest that there is a similarity between changes in EL and HTGL in metabolic disorders and support our present finding that that positive correlation was observed between EL and HTGL concentration.

In conclusion, we developed a sandwich ELISA using newly generated monoclonal antibodies that specifically detect human HTGL in both PHP and cultured media of CHO transfected with HTGL cDNA. The conditions, such as the limits of quantification, and the range of linearity and imprecision for HTGL quantification, were suitable for both experimental and clinical uses. From the study of a healthy reference HTGL range in PHP, we found that its concentrations were approximately between 42 and 1200 ng/ml. It is possible that patients with low HTGL and high plasma HTGL concentrations associated with high or low HDL-C concentrations will be identified by measuring HTGL concentration using this new assay system. Using a combination of this assay system with an HTGL activity assay system identifies defective types of HTGL deficiency. As described above, this method could be useful for further clinical studies for measuring HTGL concentration in PHP.

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