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

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Abstract There is no established method for measuring human hepatic triglyceride (TG) lipase (HTGL) concentration in serum. In this study, we developed new monoclonal Abs (MoAbs) (9A1 mouse MoAb and 141A1 rat MoAb) that react with HTGL both in serum and in postheparin plasma (PHP) and established a novel ELISA system for measuring serum HTGL and PHP-HTGL concentrations. To confirm the specificity of MoAbs, we performed immunoprecipitation-immunoblotting analysis. Both 9A1 mouse MoAb and 141A1 rat MoAb were able to immunoprecipitate not only recombinant HTGL and PHP-HTGL but also serum HTGL, demonstrating that HTGL exists in serum obtained without heparin injection. This method yielded intra- and interassay coefficients of variation of <6% and showed no cross-reactivity with LPL or endothelial lipase. In clinical analysis on 42 male subjects with coronary artery disease, there were strong positive correlations of serum HTGL concentration to PHP- HTGL concentration (r = 0.727, P < 0.01). Serum HTGL concentrations showed positive correlations to serum TGs (r = 0.314, P < 0.05) and alanine aminotransferase (r = 0.406, P < 0.01), and tendencies toward positive correlations to LDL cholesterol, small dense LDL, and γGTP. These results suggest that this new ELISA method for measuring serum HTGL is applicable in daily clinical practice.—Miyashita, K., K. Nakajima, I. Fukamachi, Y. Muraba, T. Koga, Y. Shimomura, T. Machida, M. Murakami, and J. Kobayashi. A new enzyme-linked immunosorbent assay system for human serum hepatic triglyceride lipase. J. Lipid Res. 2017. 58: 1591–1597.

Supplementary key words lipase/lipoprotein • lipase/hepatic • lipoprotein/metabolism

Hepatic triglyceride (TG) lipase (HTGL), a lipolytic enzyme that is a secreted glycoprotein, is synthesized by hepatocytes and bound to hepatic K+-ATPase proteoglycans at the surface of liver sinusoidal capillaries. It is widely accepted that HTGL plays a major role in lipoprotein metabolism as a lipolytic enzyme that hydrolyzes TGs and phospholipids in chylomicron remnants, IDLs, and HDLs (1, 2). Patients with HTGL deficiency present with hypercholesterolemia or hypertriglyceridemia and accumulation of VLDLs, chylomicron remnants, IDLs, TG-rich LDLs, and HDLs (3–8).

Previously, we reported methods for measuring HTGL activity (9, 10) and quantification of HTGL mass (11) in postheparin plasma (PHP). However, it is too labor intensive to collect large numbers of PHP samples in daily clinical practice. To our knowledge, there is no established method for measuring serum HTGL concentration. This prompted us to develop methods for quantification of HTGL in serum without heparin injection.

Here, we established a new ELISA method using two distinct monoclonal Abs (MoAbs) raised against human HTGL for quantification of serum HTGL concentration.

MATERIALS AND METHODS

Preparation of purified recombinant HTGL

Purified recombinant human HTGL was prepared as reported previously (11, 12). We generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using the primers that we reported previously (11, 12). We generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using the primers that we reported previously (11, 12). We generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using the primers that we reported previously (11, 12). We generated recombinant human HTGL as follows. Full-length human HTGL cDNA was identified in human liver cDNA (Clontech) by PCR using the primers that we reported previously (11, 12).

Abbreviations: ALT, alanine aminotransferase; AST, aspartate aminotransferase; CAD, coronary artery disease; EL, endothelial lipase; HDL-C, HDL cholesterol; HTGL, hepatic triglyceride lipase; LDL-C, LDL cholesterol; MoAb, monoclonal Ab; PBST, PBS with 0.05% Tween 20; PHP, postheparin plasma; RLP-C, remnant-like particle cholesterol; sDLL-DL-C, small dense LDL cholesterol; TG, triglyceride.

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transfectants. We purified HTGL protein from the concentrated conditioned medium of human HTGL/HEK 293 using anti-FLAG (M2) MoAb-conjugated affinity gel (Sigma). We estimated the purity of the recombinant human HTGL protein by SDS-PAGE.

Preparation of MoAbs

The purified recombinant HTGL was emulsified with complete Freund’s adjuvant (BD) and injected subcutaneously into BALB/c mice or Wistar rats (Charles River Laboratories). The spleen cells extracted from the immunized mice or rats were fused with mouse myeloma cells (X63-Ag8.653) in the presence of 50% polyethylene glycol according to standard procedures. Many MoAbs were selected based on their reactivity with the purified recombinant HTGL by ELISA, and an inhibition assay was then performed to select MoAbs that reacted with HTGL in serum without heparin injection. Briefly, the purified recombinant HTGL (50 μl/well), diluted to 0.2 μg/ml with PBS, was added to ELISA plates followed by incubation at 4°C overnight. The wells were washed twice with 300 μl of PBS with 0.05% Tween 20 (PBST), then 300 μl of PBST with 1% BSA (BSA-PBST) were added, and the plates were incubated for 1 h at room temperature. The selected MoAbs (100 μl) incubated overnight at 4°C with 100 μl of the purified recombinant HTGL, serum, and PHP, diluted to 74.25 ng/ml (16-fold and 16-fold, respectively) with BSA-PBST, respectively, were added to ELISA plates. These mixtures were incubated for 60 min at room temperature. After washing the plates, secondary Abs (anti-mouse IgG goat IgG or anti-rat IgG goat IgG) conjugated with HRP (Southern Biotech) were added and incubated for 60 min at room temperature. After washing the plates twice with 300 μl of PBS with 0.05% Tween 20 (PBST), then 300 μl of PBST with 1% BSA (BSA-PBST) were added, and the plates were incubated for 1 h at room temperature. The selected MoAbs (100 μl) incubated overnight at 4°C with 100 μl of the purified recombinant HTGL, serum, and PHP, diluted to 74.25 ng/ml (16-fold and 16-fold, respectively) with PBST with 1% BSA, respectively, were added to ELISA plates. These mixtures were incubated for 60 min at room temperature. After washing the plates, the peroxidase activity was measured by determining the absorbance at 450 nm using tetramethyl benzidine solution (Kem-En-Tec). We then selected two MoAbs the reactivity of which against purified recombinant HTGL was markedly inhibited with the purified recombinant HTGL, serum, and PHP. These MoAbs were named 9A1 mouse MoAb and 141A1 rat MoAb.

Reactivity of 141A1 rat MoAb and 9A1 mouse MoAb against recombinant HTGL, serum HTGL, and PHP-HTGL by inhibition assay using ELISA

Recombinant HTGL adjusted to a concentration of 200 ng/ml with 100 mM carbonate buffer (pH 9.5) was added to 96-well ELISA plates (Nunc) and incubated at 4°C overnight. After washing the plates twice with PBS, 200 μl/well of PBS containing 1% BSA were added and blocking was carried out for 1 h. After removal of the blocking solution, HTGL, serum, or PHP was mixed with the culture supernatant of the primary selected clones (141A1 and 9A1) and then reacted with the plates. After washing the plates four times with washing solution, we added 50 μl per well of peroxidase-labeled anti-mouse IgG goat Ab (Southern Biotech) to the mouse MoAb or 50 μl per well of peroxidase-labeled anti-rat IgG goat Ab (Southern Biotech) to the rat MoAb followed by incubation at room temperature for 15 min. After washing five times with the washing solution, we added 50 μl/well of peroxidase substrate solution followed by 50 μl/well of 1.5 N sulfuric acid after 10 min, and the absorbance at a wavelength of 450 nm was measured. By this method, we screened many clones and selected hybridomas potentially producing Abs that could inhibit the reaction with recombinant HTGL in serum.

Reactivity of MoAbs against HTGL, LPL, and EL by ELISA

We evaluated the specificity of MoAbs by ELISA. To test the cross-reactivity of MoAbs against LPL and endothelial lipase (EL), we purchased recombinant human LPL protein from BioVendor and generated recombinant EL. Briefly, ELISA plates were coated with HTGL, LPL, and EL (50 μl/well) and diluted to 200 ng/ml with carbonate buffer (pH 9.5) overnight at 4°C. The plates were then washed with PBST and blocked with 300 μl of BSA-PBST per well overnight at 4°C. After washing with PBST, serially diluted MoAbs (~1 μg/ml) with PBST with 1% BSA were added to the wells and incubated for 30 min at room temperature. After washing the plates, secondary Abs conjugated with HRP were added and incubated for 30 min at room temperature. After washing the plates, the peroxidase activity was measured.

Reactivity of MoAbs against serum HTGL and PHP-HTGL by immunoprecipitation

Immuoaffinity gels with MoAbs coupled to Affi-Gel 10 (Bio-Rad) were prepared according to the manufacturer’s instructions. The purified recombinant HTGL, serum, and PHP were diluted to 25 ng/ml (2-fold and 40-fold, respectively) with PBST. These samples (8 ml) were incubated with 100 μl of immunoaffinity gel overnight at 4°C. After washing, the immunoaffinity gels were boiled in SDS-Tris buffer, with or without mercaptothanol (reducing or nonreducing conditions, respectively), and then separated by SDS-PAGE and transferred onto nitrocellulose membranes (GE Healthcare). The membranes were blocked with BSA-PBST, incubated with HRP-conjugated MoAbs (Fab’) prepared according to standard procedures (13). HTGL signals were visualized using ECL reagent (Takara Bio). We used anti-LRG-48A1 mouse MoAb as a negative control.

Developed ELISA system

The wells of 96-well ELISA plates coated with 100 μl of 9A1 mouse MoAb, diluted to 10 μg/ml with carbonate buffer (pH 9.5), were incubated overnight at 4°C. The plates were then washed with PBST and blocked with BSA-PBST. After washing with PBST, the samples were diluted appropriately (serum, 50-fold; PHP, 1,000-fold) with BSA-PBST and the purified recombinant HTGL was serially diluted with BSA-PBST as a standard. The above diluted samples were placed into wells and then incubated overnight at 4°C. After washing with PBST, 100 μl of HRP-conjugated 141A1 rat MoAb were added to each well and incubated for 30 min at 4°C. The wells were washed five times with PBST and 100 μl of tetramethyl benzidine solution (Kem-En-Tec) were added to each well as a substrate, followed by incubation in the dark for 30 min at room temperature. The reaction was terminated by adding 100 μl of 0.5 mol/1 H2SO4. We measured absorbance of the solution at 450 nm with an ELISA reader (E-Max; Molecular Devices).

Other methods

LPL and EL concentrations were measured by ELISA as reported previously (12, 14, 15). HTGL activities were measured using the method reported previously (9, 10). Briefly, lipase activities were assayed using dioleoylglycerol solubilized with detergent and measuring the increase in absorbance at 546 nm (subwavelength: 660 nm) due to the production of quinone diimine dye. Automated assay of lipase activity was performed with a Hitachi Model 7080 automatic clinical analyzer.

Preparation of blood samples

Study subjects. Forty-two male subjects with coronary artery disease (CAD) (Table 1) were enrolled in this study. In addition, 19 male volunteers (Table 1, supplemental Table S2) were also enrolled to determine the normal range of serum HTGL concentration. The CAD patients were admitted for percutaneous coronary intervention. Exclusion criteria were aspartate aminotransferase (AST) >100 alanine aminotransferase (ALT) >100, use of insulin-sensitizing agents and insulin injection (these were discontinued at least 1 month before the study), or endocrine disorders. The
investigation conformed to the principles outlined in the Declaration of Helsinki. The study had the approval of the Ethical Committee of Hidaka Hospital (Takasaki, Japan). Written informed consent was obtained from all participants.

PHP was obtained at 15 min after heparin (30 USP units/kg) was injected. All plasma samples were kept frozen at −80°C until analysis. Plasma concentrations of HDL cholesterol (HDL-C), LDL cholesterol (LDL-C), and TG (Denka-Seiken, Japan) were measured enzymatically.

Remnant-like particle cholesterol (RLP-C) was determined by an immunoseparation method (JIMRO II, Otsuka, Japan) (16). Small dense LDL-C (sdLDL-C) was determined by the method of Ito et al. (17).

RESULTS

Identification of recombinant human HTGL

We purified recombinant HTGL from the supernatant of HEK 293 cells that had been transfected with the hHTGL plasmid. Silver staining after SDS-PAGE indicated that the purified recombinant HTGL contained a single protein corresponding to a molecular mass of 65 kDa, corresponding to mature HTGL (Fig. 1). We used this purified recombinant HTGL as an immunogen to develop MoAbs.

Specificity of MoAbs

Two MoAbs (9A1 mouse MoAb and 141A1 rat MoAb) were selected from among many Abs that showed reactivity against recombinant HTGL. The reactivity of the obtained MoAbs against purified recombinant HTGL was inhibited by serum and PHP by the inhibition assay (data not shown). The specificities of MoAbs against LPL and EL were also confirmed by ELISA. MoAbs did not show any reactivity against LPL and EL (supplemental Table S1). The reactivities of MoAbs against serum HTGL and PHP-HTGL were also investigated by the immunoprecipitation method. The two selected MoAbs were able to immunoprecipitate not only recombinant HTGL and PHP-HTGL but also serum HTGL (Fig. 2). These results indicated that these MoAbs reacted specifically with serum HTGL and PHP-HTGL.

Analytical performance of the developed ELISA system

The standard curve showed that the absorbance was proportional to the amount of added purified recombinant HTGL (Fig. 3). The measurement range of this ELISA was 0.078–5.0 ng/ml. Dilution linearity for HTGL concentration assay was observed in three kinds of samples with different concentrations both for serum and PHP (Fig. 4A, B).

TABLE 1. Clinical profile of the study subjects

|                     | CAD Patients (n = 42) | Volunteers (n = 19) |
|---------------------|----------------------|---------------------|
| Age (years)         | 71.3 ± 11.4          | 45.7 ± 12.5         |
| Body mass index (kg/m²) | 23.4 ± 4.1       | NA                  |
| TGs (mg/dl)         | 110.2 ± 65.5         | 145 ± 102           |
| HDL-C (mg/dl)       | 47.4 ± 15.4          | 61 ± 15             |
| LDL-C (mg/dl)       | 97.3 ± 36.2          | 121 ± 31            |
| Hypertension        | 27                   | NA                  |
| Diabetes mellitus   | 13                   | NA                  |
| Statin use          | 21                   | 0                   |

NA, not available.

This ELISA system was confirmed not to react with LPL or EL protein (supplemental Table S1). Serum and PHP with different concentrations of HTGL were measured. The assay coefficients of variation were 4.2–7.1% for serum and 2.2–4.9% for PHP.

Correlations of serum or PHP-HTGL concentration with several clinical parameters

The serum HTGL concentration in 19 male volunteers measured using the method described here was 70.6 ± 33.0 ng/ml. To clarify the clinical significance of serum HTGL, we conducted correlation analysis between serum HTGL concentration and several metabolic parameters in the 42 male subjects with CAD (Table 1). There were strong positive correlations of serum HTGL concentration with PHP-HTGL concentration (r = 0.727, P < 0.01). Serum HTGL concentration was positively correlated to TG concentration and tended toward positive correlations with LDL-C and sdLDL-C, but showed no significant correlations to RLP-C or HDL-C. Similarly, PHP-HTGL concentrations were positively correlated to TGs and tended toward positive correlations to sdLDL-C. PHP-HTGL concentration also tended toward an inverse correlation to HDL-C, whereas serum HTGL concentration did not. We also investigated the correlations between PHP-HTGL...
concentrations measured by the current method and their activities by the method reported previously (9, 10), and the results indicated that these two parameters were closely correlated (Fig. 5).

**Correlations of serum HTGL concentration with liver enzymes**

Serum HTGL concentration was positively correlated to ALT ($r = 0.406, P < 0.05$) and tended toward a positive correlation with $\gamma$GTP ($r = 0.301, P = 0.053$) (Table 2). There were no correlations between serum HTGL and serum LPL concentrations.

**Correlations of serum LPL concentrations to clinical parameters**

By comparison, we investigated the correlations of serum LPL concentrations with lipids and lipoproteins in the 42 male subjects with CAD. Serum LPL concentration was positively correlated to HDL-C, inversely correlated to TG, and tended toward an inverse correlation to RLP-C.
There were no correlations of serum LPL concentration to sdLDL-C.

**DISCUSSION**

In this study, we developed a new ELISA system using 9A1 mouse MoAb and HRP-conjugated 141A1 rat MoAb for measuring serum HTGL and PHP-HTGL concentrations. There is no established method for measuring serum HTGL concentration without heparin injection, because there have been no reports of MoAbs that react specifically with both serum HTGL and PHP-HTGL. Here, we succeeded in developing MoAbs that react specifically with serum HTGL. The results of immunoprecipitation analysis with MoAbs indicated the presence of HTGL in serum. Previously, we reported an ELISA system for measuring PHP-HTGL using two distinct MoAbs raised against recombinant human HTGL, which was not able to detect serum HTGL (11). However, it is labor intensive to collect large numbers of PHP samples in daily clinical practice. One advantage of the newly developed ELISA system is that serum HTGL concentration can be measured without heparin injection. We assume that serum HTGL would reflect the physiological roles of this enzyme more precisely than PHP-HTGL given that heparin injection may affect lipoprotein metabolism.
In CAD patients, regardless of serum HTGL or PHP-HTGL, the common findings were their positive correlations with TG and tendency toward positive correlations with sdLDL-C. The observation that the PHP-HTGL concentrations showed a positive correlation with serum TG was consistent with our previous reports (10, 18) and similar correlations were observed in the case of serum HTGL in the present study. The correlation between HTGL concentrations and sdLDL-C observed here indicated that HTGL is involved in the production of sdLDL particles (19).

The current findings that PHP-HTGL concentration showed a tendency toward inverse correlations with serum HDL-C \((R = -0.285)\) were comparable with our previous study (11). On the other hand, there were no significant correlations of serum HTGL with HDL-C, which was an unexpected finding. We do not have a precise explanation for that, so far, and further studies are needed to find a solution.

We also analyzed the correlations of serum LPL concentration with several lipoproteins and found a positive correlation of serum LPL concentration to HDL-C and an inverse correlation to TG.

In the present study, the observation that serum HTGL concentration is positively correlated with ALT is interesting. Previous studies showed that HTGL activity increases or tends to increase in individuals with fatty liver (20–22). The association of HTGL with these disorders is of interest. Unlike wild-type mice, HTGL KO mice were reported to show no fat accumulation in the liver even after high-fat diet loading (23). This suggests that HTGL is not simply associated with the development of fatty liver but may have a causal effect on the development of this disease. This, in combination with the observation that fat accumulation in the liver is responsible for the development of diabetes (24), suggests that HTGL may indirectly contribute to the formation of this atherogenic disease. Consistent with these observations, high HTGL activity was suggested to be related to the formation of nonalcoholic fatty liver disease beyond insulin resistance (25).

Whether HTGL is pro- or anti-atherogenic is an important but unsolved issue. One approach for understanding how this enzyme affects the development and progression of atherosclerosis is to clarify whether complete HL deficiency is an atherogenic disease. However, it is difficult to recruit large numbers of study subjects because of the rarity of this disease. Indeed there have been very few reports regarding this issue from North America and Northern Europe (26–28). Another approach would be a cohort study to investigate how baseline HTGL level is correlated with the future incidence of CVD. For this purpose, it would be convenient to be able to measure HTGL levels in large numbers of subjects without heparin injection.

In conclusion, we have established a new ELISA method using newly generated MoAbs for quantification of serum HTGL, as well as PHP-HTGL. This method is applicable for measuring HTGL in large numbers of samples as serum can be obtained simply without labor-intensive procedures. It will be possible to conduct future epidemiological studies using the current method to determine how HTGL value would affect the occurrence of future cardiovascular events over a certain period, similar to prospect studies on the effects of LPL protein on coronary heart disease events (29).

| TABLE 2. Correlations of serum HL concentrations to several clinical parameters (n = 42) |
|-------------------------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|----------------|
|                              | AST            | ALT            | HDL-C          | LDL-C          | TG             | Serum LPL       | Serum HTGL      | PHP-HTGL       | RLP-C          |
| ALT                           |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.551          | 1              |                |                |                |                |                |                |
| \(P\)                        | <0.01          |                |                |                |                |                |                |                |
| HDL-C                         |                |                |                |                |                |                |                |                |
| \(R\)                        | -0.015         | -0.262         | 1              |                |                |                |                |                |
| \(P\)                        | 0.927          | 0.094          |                |                |                |                |                |                |
| LDL-C                         |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.476          | 0.142          | 0.102          | 1              |                |                |                |                |
| \(P\)                        | <0.01          | 0.371          | 0.521          |                |                |                |                |                |
| TG                            |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.458          | 0.308          | -0.427         | 0.385          | 1              |                |                |                |
| \(P\)                        | <0.01          | <0.05          | <0.01          | <0.05          |                |                |                |                |
| Serum LPL                     |                |                |                |                |                |                |                |                |
| \(R\)                        | -0.179         | -0.308         | 0.46           | 0.046          | -0.329         | 1              |                |                |
| \(P\)                        | 0.258          | <0.05          | <0.01          | 0.773          | <0.05          |                |                |                |
| Serum HTGL                    |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.222          | 0.406          | -0.14          | 0.275          | 0.314          | -0.103         | 1              |                |
| \(P\)                        | 0.157          | <0.01          | 0.375          | 0.078          | <0.05          | 0.518          |                |                |
| PHP-HTGL                      |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.186          | 0.43           | -0.285         | 0.208          | 0.447          | -0.232         | 0.727          | 1              |
| \(P\)                        | 0.238          | <0.01          | 0.067          | 0.186          | <0.01          | 0.139          | <0.01          |                |
| RLP-C                         |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.506          | 0.252          | -0.277         | 0.489          | 0.828          | -0.283         | 0.238          | 0.437          | 1              |
| \(P\)                        | 0.001          | 0.108          | 0.075          | <0.01          | <0.01          | 0.07           | 0.128          | <0.01          |                |
| sdLDL-C                       |                |                |                |                |                |                |                |                |
| \(R\)                        | 0.553          | 0.251          | -0.158         | 0.579          | 0.787          | -0.17          | 0.263          | 0.273          | 0.847          |
| \(P\)                        | <0.01          | 0.109          | 0.319          | <0.01          | <0.01          | 0.283          | 0.093          | 0.08           | <0.01          |
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