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Measurement of homocysteine thiolactone hydrolase activity using high-performance liquid chromatography with fluorescence detection and polymorphisms of paraoxonase in normal human serum

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Abstract

We developed a non-radioactive and sensitive assay method for measurement of the HTL hydrolase (HTLase) activity in biological samples, using OPA as a fluorescent post-labeling agent, L-homocysteine thiolactone (L-HTL) as the substrate, and HPLC to achieve rapid and selective separation of the substrate and product. The method was applied to measure the activity of HTLase in human, rabbit, rat and mouse serum samples. In addition, the correlation between the serum HTLase activity and PON1 polymorphisms in Japanese subjects was also investigated. The serum HTLase activity in humans, as determined by measurement of the enzyme activity in 22 subjects, was found to be in the range of 0.89–2.06 nmol/min mg protein, with a mean activity of 1.44 nmol/min mg protein.

Keywords: Homocysteine thiolactone hydrolase; HPLC; Polymorphism

1. Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid generated through demethylation of methionine. Under normal conditions, the intracellular concentration of Hcy is maintained at a low level as a result of remethylation reactions and catabolism via the transsulfuration pathway. However, several hereditary and acquired conditions can alter this normal state of homocysteine metabolism [1–3]. A plasma Hcy concentration greater than 15 μM is termed "hyperhomocysteinemia" [4], and recent clinical studies have indicated an association between hyperhomocysteinemia and premature cardiovascular disease. It has been suggested that Hcy may impair the vasomotor-regulatory and thrombotic properties of the vascular endothelium [5–7]. However, it remains unknown as to which aspect of Hcy metabolism is harmful to human cells and promotes arteriosclerosis.

Hcy can exist in the form of a stable cyclic compound, namely, homocysteine thiolactone (HTL). HTL formation can occur as the result of a metabolic error-editing process, in which Hcy is mis-activated by methionyl-tRNA synthetase to form enzyme-bound homocysteinyl adenylate, and free HTL is released from the latter [8]. This conversion occurs excessively in mammalian cells, including human cancer, but is minimal in normal mammalian cells [9]. Accumulation of HTL has been suggested to be involved in the mechanisms of atherogenesis [10]. In cultured human cells and in human serum, HTL reacts with the lysine residues of proteins by nucleophilic addition, and such homocysteinylation of cellular and extracellular proteins can cause cell damage. In fact, Ferguson reported that the concentration of the homocystamide low-density lipoprotein (LDL) adduct, an acylation product of HTL and the ε-amino groups of apo-B lysyl residues, may influence atherogenicity [11].

On the other hand, Jakubowski reported the presence of HTL hydrolase (HTLase) in the human serum, which hydrolyzes HTL to Hcy [12]. Human serum HTLase, a 45-kDa

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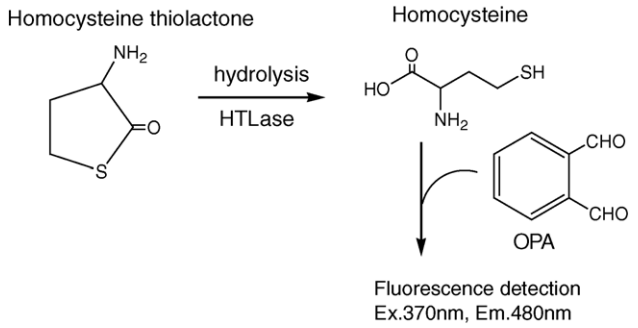

Fig. 1. Principle of the assay for HTLase activity.

protein component of high-density lipoprotein (HDL) that requires calcium for activity and stability, also hydrolyzes the organophosphate paraoxon. The N-terminal amino acid sequence of HTLase has been reported to show complete homology to that of human paraoxonase 1 (PON1), a product of the *PON*1 gene [12]. PON1, also a component of HDLs, has been shown previously to exhibit antioxidant activity and to be associated with a decreased risk of cardiovascular diseases.

Recently, we reported a liquid chromatographic method for the simultaneous determination of Hcy and HTL in biological samples [13]. This method is based on the principle that the lactone ring in the HTL molecule is cleaved by alkali to produce Hcy, which then reacts with OPA in the absence of an added thiol reagent to form a stable fluorescent derivative (Fig. 1). During our attempt to investigate the role of HTL in the mechanism of homocysteine toxicity, we developed a new and sensitive assay technique for determining the HTLase activity in biological samples using OPA as a fluorescent post-labeling agent, L-HTL as the substrate, and HPLC to achieve rapid and selective separation of the substrate and product. We then applied this method to measure the activity of HTLase in human, rabbit, rat and mouse serum samples. In addition, we also investigated the correlation between HTLase activity and PON1 polymorphisms in Japanese subjects.

2. Materials and methods

2.1. Materials

L-Homocysteine thiolactone and L-homocystine were obtained from Sigma Chemical Co. (St. Louis, MO, USA). OPA was purchased from Nacalai Tesque, (Kyoto, Japan). Tris(2-carboxyethyl)phosphine (TCEP) was obtained from Tokyo Kasei (Tokyo, Japan). All the other chemicals used were of analytical grade.

Blood samples were obtained by venepuncture from volunteers aged 21–53 years, comprising both females ($n = 11$) and males ($n = 11$), after obtaining their informed consent. Serum was separated from the samples by centrifugation.

2.2. Instrumentation

The chromatographic system consisted of an L-6000 pump (Hitachi, Tokyo, Japan), a PU-2080 pump equipped with an LG-2080-02 valve unit (JASCO, Tokyo, Japan), an AS 2057 autosampler (JASCO), an L-7300 column oven (Hitachi), a D-2500 integrator (Hitachi) and an L-7480 fluorescence detector (Hitachi).

2.3. Chromatographic conditions

The ultrafiltrated serum sample was injected into a Develosil C30-UG-5 column (150 mm × 4.6 mm, Nomura Chemical Co. Ltd., Aichi, Japan). To protect the analytical column, a Develosil C30-UG-5 guard column (10 mm × 4 mm) was fitted between the analytical column and the autosampler. The column oven was kept at 30 °C. The mobile phase, consisting of 50 mM phosphoric acid–NaOH (pH 2.0) containing 0.5 mM sodium octanesulfonate, was filtered through a cellulose nitrate filter (0.45 μm, Toyo Roshi, Tokyo, Japan) and degassed just prior to use; it was run at a flow rate at 1.0 ml/min. OPA and NaOH were dissolved in separate bottles, and mixed using the PU-2080 pump equipped with an LG-2080-02 valve unit. The eluate was mixed in a three-way tee with OPA–NaOH. The reaction coil was made up of Teflon tubing (0.5 mm i.d. × 10 m). The fluorescence intensities were monitored at an excitation wavelength of 370 nm and emission wavelength of 480 nm.

2.4. Standard solutions

Ten millimolar stock solution of HTL in 30 mM HCl and 5 mM homocystine stock solution in 100 mM HCl were prepared, and stored at −20 °C. Working standard solutions were prepared daily from each stock solution by dilution in water.

2.5. HTLase assay

HTLase was assayed by detecting Hcy, the hydrolysis product of HTL obtained following its addition to the sample as substrate. After separation of the substrate and product by HPLC, quantitation of Hcy was conducted by post-labeled fluorescence detection using the OPA reagent.

The assay mixture (50 μl) consisted of 12.5 mM HTL, 2.5 mM $CaCl_2$, 100 mM HEPES–KOH buffer (pH7.4), and 10 μl of the human serum sample. After preincubation of the assay mixture at 37 °C for 3 min prior to the addition of the serum sample, the reaction was initiated by the addition of the sample, and the assay mixture was incubated in a 37 °C water bath. The reaction was terminated after 30 min by the addition of 50 μl of 5 mM TCEP in 100 mM phosphate buffer (pH 6.0). After further incubation for 10 min at 37 °C, 900 μl of 200 mM acetate buffer (pH 4.0) was added to the reaction mixture. An aliquot of the mixture was ultracentrifuged at 5000 × *g* for 20 min using Ultrafree-MC (UFC3LGC00, Millipore), and the filtrate was transferred to an autosampler

vial after being adequately diluted with 200 mM of acetate buffer (pH 4.0).

The net peak area of Hcy produced enzymatically from the HTL was obtained by subtracting the peak area of Hcy formed non-enzymatically and that contained as an impurity in HTL. The net peak area of Hcy was calculated from the calibration curve and expressed as the amount of Hcy produced/min.

2.6. Paraoxonase assay

PON activity was measured using paraoxon as the substrate [14]. This assay protocol was based on the colorimetric estimation at 410 nm, of *p*-nitrophenol released as a result of enzymatic hydrolysis of paraoxon. A molar extinction coefficient (ε) of *p*-nitrophenol at pH 8.0 in 20 mM Tris–HCl buffer of 17,100 M^{-1} cm^{-1} was used for the calculation.

2.7. Arylesterase assay

Arylesterase activity was measured using phenylacetate as the substrate [14]. The initial rate of hydrolysis was measured spectrophotometrically at 270 nm. This assay protocol is widely used by researchers worldwide for estimating the esterolytic activity of both lipases and esterases. A molar extinction coefficient (ε) of phenol at pH 8.0 in 20 mM Tris–HCl buffer of 1310 M^{-1} cm^{-1} was used for the calculation.

2.8. Partial purification of rabbit serum HTLase

Partial purification of HTLase was conducted by a modification of the methods described by Gan et al. [14] and Jakubowski [12], and all the purification steps were carried out at 4 °C. Rabbit serum (30 ml) was dialyzed overnight against 6 l of 50 mM Tris–HCl buffer (pH 8.0) containing 3 M NaCl, 1 mM $CaCl_2$ and 5 μM EDTA, applied to 100 ml of Blue Sepharose™6 Fast-Flow pre-equilibrated with the same buffer in a batchwise operation, and allowed to stand for 30 min at room temperature. The Blue Sepharose was then washed with the buffer, until an absorbance of the supernatant solution of 0.3 was obtained at 280 nm. After further washing with 25 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, the Blue Sepharose slurry was transferred to a 2.8 cm × 50 cm column pre-equilibrated with the same buffer, and the esterase was eluted with 50 mM Tris–HCl buffer (pH 8.0) containing 0.1% deoxycholate, 1mM $CaCl_2$ and 20% glycerine. Eluents containing an arylesterase activity of greater than 20 μmol/min ml were pooled as the Blue Sepharose fraction. The Blue Sepharose fraction was dialyzed overnight against 6 l of PON1 purification buffer (25 mM Tris–HCl buffer (pH 8.0) containing 1 mM $CaCl_2$, 20% glucerine and 0.2% Tergitol NP 10), and applied to the DE52 column (1.7 cm × 20 cm) pre-equilibrated overnight with the purified buffer and slurry transferred at the ratio of 1 ml for an arylesterase activity of 280 μmol/min. This column was eluted with the same volume as the column bed of the purified buffer containing zero, 40, 50 and 60 mM NaCl, respectively, and corrected as to a 1-ml fraction. The eluted fractions with the highest specific activities of HTLase, PON1 and arylesterase were pooled and stored at 4 °C. The activities were allowed to stabilize for at least 1 month at a temperature of 4 °C. Throughout the study, partially purified enzyme from rabbit serum was used as the standard sample.

2.9. Protein determination

The protein concentration was measured using the BCA protein assay reagent (PIERCE, USA) with bovine serum albumin as the standard.

2.10. PON1 genotyping

DNA was extracted using a QIAamp DNA Blood Mini kit (QIAGEN, Germany). The PON polymorphisms were studied by a multiplex PCR-based DNA assay method reported by Motti et al. [15], using mismatch primers that introduce a unique recognition site for the endonuclease *Hin*fI in the PCR products when the R allele of PON1-192 or the L allele of PON1-55 is present. The multiplex-PCR was performed in a 50-μl volume reaction mixture containing 0.1 μg of DNA template, 0.12 μM of both PON1-192 primers, 0.12 μM of both PON1-55 primers, 600 μM of each dNTP, 7 mM $MgCl_2$, and KOD dash polymerase (TOYOBO, Japan). For the PON1-55 polymorphism, the primers used were as follows: 5′-GAGTGATGTATAGCCCCAGTTTC-3′ (forward) and 5′-AGTCCATTAGGCAGTATCTCCG-3′ (reverse). For the PON1-192 polymorphism, the primers used were 5′-TTGAATGATATTGTTGCTGTGGGACCTGAG-3′ (forward), and 5′-CAGCCACGCTAAACCCAAATACATCTCCCAGAA-3′ (reverse). The primers were obtained from Amersham Pharmacia (USA). DNA was amplified under the following conditions for PCR; initial step at 94 °C for 1 min, followed by 40 cycles of at 94 °C for 20 s, 65 °C for 2 s, and 74 °C for 15 s, with a final extension step of at 74 °C for 1 min. After digestion with *Hin*fI, the products were separated by PAGE and visualized by ethidium bromide staining.

3. Results

3.1. Chromatographic conditions

The fluorometric detection system employed for the measurement of Hcy in this study was the same as that employed in our previous study [13], except for some modifications, that is, the OPA reagent and alkaline solution were prepared separately and delivered together using a low-pressure gradient unit, because of the stability of the OPA. Under this condition, OPA reagent could be used for the HTLase assay for at least 48 h.

Complete separation of Hcy from the reaction mixture was achieved using a reversed-phase C_{30} column with 50 mM H_3PO_4–NaOH buffer (pH 2.75) containing 0.5 mM sodium

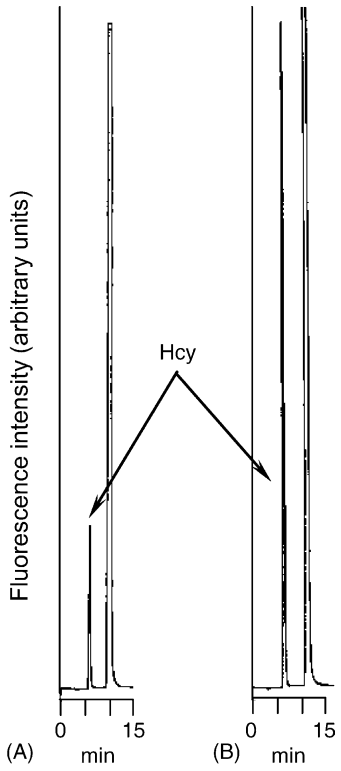

Fig. 2. Typical chromatograms in the assay of HTLase activity: (A) blank containing assay mixture alone; (B) human serum sample. The analytical conditions are described in Section 2 (chromatographic conditions).

octanesulfonate, in contrast to the pH 2.0 phosphate buffer containing methanol (98:2, v/v) employed in our previous study. Under the above condition, Hcy eluted at approximately 6.9 min and separated completely from HTL, and the retention time was also found to be better reproducible (data not shown) as compared to that obtained using the ODS column. A typical chromatogram is depicted in Fig. 2.

The calibration curve obtained by plotting the Hcy peak area against the concentration of Hcy was linear over the range of 0.01–10 μM, with a correlation coefficients (r) of 0.999. The minimum detectable level of Hcy, defined as a signal-to-noise ratio of 2, was 100 fmol.

To check the selectivity, blank plasma samples from human donors and spiked substrate (HTL) standard samples were analyzed in accordance with the method described in Section 2. A minor, but acceptable interference from the substrate standard was detected at the retention time of Hcy, because of an impurity contained in the HTL, although this impurity was always lower than 0.1% relative to the peak of the substrate (HTL). In the HTLase assay, this impurity peak area was therefore subtracted from that of the serum sample. No interference constituents from endogenous substances in the serum sample was observed, because OPA reacts specifically with only compounds having both a primary amine and thiol moieties in the molecule. Each chromatographic run was completed within 15 min.

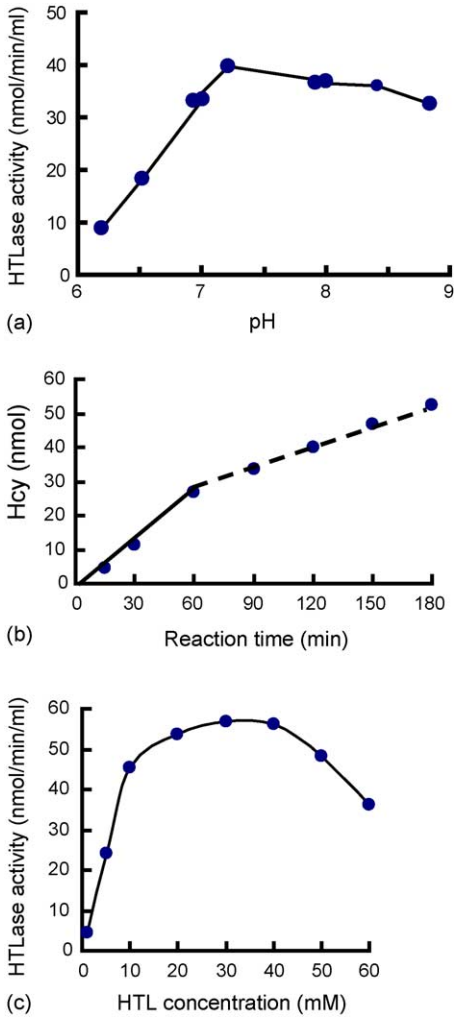

Fig. 3. Effects of (a) pH; (b) reaction time; and (c) HTL concentration on HTLase activity. Sample: partially purified enzyme from rabbit serum (a) pH 6.2–7.0: 0.1 M PIPES–KOH buffer; pH 7.0–8.0: 0.1 M HEPES–KOH buffer; pH 8.0–9.0: 0.1 M Tris–HCl buffer. The experimental conditions are described in Section 2 (HTLase assay).

3.2. Optimization of the HTLase assay condition

Since rabbit and mouse serum contain 5∼10-fold more greater HTLase activity than human serum [12,16], rabbit serum was used as the source of HTLase for our preliminary experiments. The effects of pH, reaction time and HTL concentration on the enzymatic reaction were investigated by conducting the assay under the conditions described in Section 2. The pH dependence of the enzyme activity was investigated using 0.1 M PIPES–KOH buffer (pH 6.1–7.0), 0.1 M HEPES–KOH buffer (pH 7.0–8.0) and 0.1 M Tris–HCl buffer (pH 8.0–9.0), all containing 2.5 mM $CaCl_2$, which is essential for maintaining the stability and activity of HTLase [12]. The catalytic activity of the enzyme was found to be maximal a pH 7.4, obtained using 0.1 M HEPES–KOH buffer (Fig. 3a). The enzyme reaction was found to be linearly correlated with time for 60 min at 37 °C (Fig. 3b). Based on this result, the

Table 1
Homocysteine concentration and HTLase activity in human serum

	n	Mean (range)	
		Homocysteine (μmol/l)	HTLase (nmol/min mg protein)
Male	11	10.0(7.9–11.9)	1.48(1.03–1.89)
Female	11	9.6(6.0–12.5)	1.41(0.89–2.06)
Total	22	9.8(6.0–12.5)	1.44(0.89–2.06)

Table 2
HTLase activities in human and animal sera

	n	HTLase (nmol/min mg protein)
Human	22	1.4 ± 0.3
Rabbit	3	14.6 ± 4.1
Rat	3	2.8 ± 0.7
Mouse	5	3.5 ± 0.6

optimum reaction time was determined to be 30 min for the present method. The dependence of the hydrolytic velocity on the concentration of HTL in the reaction buffer is shown in Fig. 3c. A Lineweaver–Burk plot yielded a K_m value of HTLase for HTL of 5.56 mM. From this data, the final concentration of HTL for the assay was chosen as 12.5 mM.

3.3. HTLase activity in human and animal sera

To test the reproducibility of the method, we divided our human serum pool into 10 aliquots and stored them at −20 °C. The inter-day CV of our method was 4.6%.

We applied our method to assay the HTLase activity in normal human serum samples obtained from 22 healthy blood donors (11 males and 11 females, ranging in age between 21 and 53 years). The mean values are shown in Table 1; no significant sex-related difference was observed.

We also applied our method to compare the HTLase activity in rabbit, rat, mouse and human serum samples. Table 2 shows the activity of HTLase in the human and animal serum samples. Rabbit serum contained roughly 10-fold greater HTLase activity than human serum as originally described by other investigators [12,16].

3.4. HTLase activity and the PON1 genotype

Two common polymorphisms at codon L55M and Q192R in PON1 have been reported to be associated with an increased risk for cardiovascular disease [17]. Therefore, we studied the association between HTLase activity and the *PON*1 genotype. The results of examination of serum samples obtained from healthy subjects are presented in Table 3. The activity of HTLase was lowest in the subjects with the 55MM genotype, and highest in those with the 192RR genotype. Similar associations were observed between the PON activity and the *PON*1 genotypes. On the other hand, while similar arylesterase activities were noted in subjects with any genotype other than the 55MM genotype, subjects with the 55MM genotype showed reduced arylesterase activity in addition to reduced HTLase and PON1 activity. Fig. 4 shows a good correlation ($r = 0.828$) between the HTLase activity and PON activity, and low HTLase activity was associated with the M55 and Q192 allels. Similar correlations were reported in the black and Caucasian populations [18]. Further, a weak correlation ($r = 0.655$) between the HTLase activity and arylesterase activity in human serum was obtained; however, regression analysis revealed no correlation between the PON

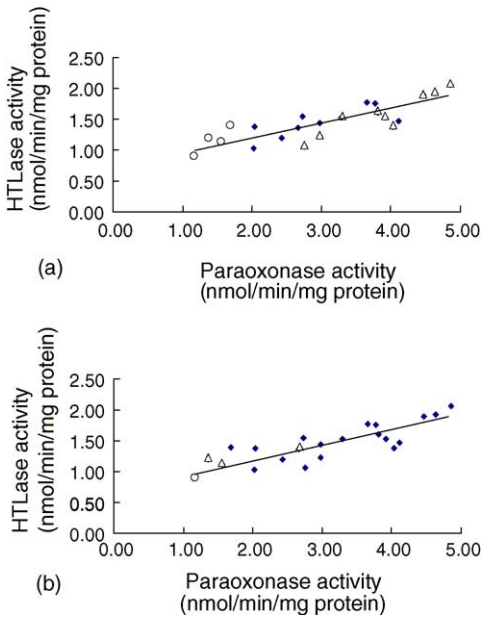

Fig. 4. Correlation between the HTLase activity and PON activity. Serum samples were obtained from 22 healthy volunteers. Polymorphisms of the 192 allele (a) and the 55 allele (b) are indicated. (◯): QQ, (⧫): QR, (△): RR. (b) (◯): MM, (△): ML, (⧫): LL.

Table 3
Association between the activities of HTLase, PON and arylesterase and the *PON*1 genotype

	*PON*1 genotype					
	55MM	55ML	55LL	192QQ	192QR	192RR
Distribution	1(4.5%)	3(13.6%)	18(81.8%)	4(18.2%)	9(40.9%)	9(40.9%)
HTLase[a] activity	0.89	1.22	1.51	1.15	1.44	1.58
PON[a] activity	1.15	1.86	3.34	1.44	2.93	3.86
Arylesterase[b] activity	0.75	1.08	1.02	1.03	1.04	0.97

[a] nmol/min mg protein.
[b] μmol/min mg protein.

activity and arylesterase activity in normal human serum. Further, no strong correlation was observed between any esterase activity and the Hcy concentration in normal human serum.

4. Discussion

In this paper, we present a non-radioactive, highly sensitive assay technique for serum HTLase activity developed by us, using HPLC with fluorescence detection. Good reproducibility of the assay method was obtained using the C_{30} column. Therefore, our chromatographic system is expected to be useful for assay of the HTLase activity in biological samples, for enzyme purification from samples showing relatively low activity and with limited starting materials, and also for determining the enzyme kinetics.

The results of characterization of HTLase partially purified HTLase from rabbit serum in the present study were consistent with those of a previous study for characterizing HTLase from human serum [12]. In regard to the influence of calcium on the HTLase activity, it was found that the addition of EGTA, a calcium-chelating agent, to rabbit serum resulted in a 3% decrease of the HTLase activity. The K_m (5.56 mM) of rabbit serum HTLase for HTL was lower than that of human serum HTLase for the same substrate [12], although the K_m values were relatively high as for physiologically substrates. The mean value of HTLase activity in human serum ($n = 22$) was 1.44 nmol/min mg protein. This value is slightly higher than that reported by Jakubowski et al., who reported a corresponding value of 0.95 nmol/min mg protein [12]. In addition, a difference of the serum HTLase activity has also been reported between black and white human populations [18].

The correrations between the HTLase activity and *PON*1 polymorphisms were also studied. The frequencies of the 55 and 192 *PON*1 alleles in the Japanese population determined in this study were consistent with those reported from previous studies for Japanese populations [19] and black populations [18]. The serum HTLase activity has been shown to be clearly dependent on the polymorphism of *PON*1, and it is possible that since Japanese subjects have a high frequency of the 55LL and 192RR polymorphisms, associated with an elevated HTLase activity, the rate of development of arteriosclerosis in the Japanese population is more controlled as compared to that in other populations.

Numerous epidemiological studies have established that an elevated level of Hcy is an independent risk factor for atherosclerotic vascular disease. Regulation of the Hcy level is dependent on the nutrient intake, especially of folate and vitamins B6 and B12 [3]. Since a proportion of Hcy is metabolized to HTL, low HTLase activity is considered to promote the development of arteriosclerosis. To clearly elucidate the role of HTL in the mechanism of Hcy toxicity, further studies using a large number of clinical samples are needed.

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