papers on methodology

Establishment of enzyme-linked immunosorbent assays for lipoprotein lipase with newly developed antibodies

Masako Kawamura, Takanari Gotoda, Natsuko Mori, Hitoshi Shimano, Koichi Kozaki, Kenji Harada, Masako Shimada, Toshimori Inaba, Yoshiro Watanabe, Yoshio Yazaki, and Nobuhiro Yamada[1]

The Third Department of Internal Medicine, Faculty of Medicine, University of Tokyo, Hongo 7-3-1, Tokyo 113, Japan

Abstract We developed eight new antibodies against lipoprotein lipase (LPL), which included polyclonal antibodies raised against recombinant human LPL produced by transformant cells and two synthetic peptides corresponding to either amino (N)- or carboxy (C)-terminus of human LPL. With these antibodies, we established three effective sandwich enzyme-linked immunosorbent assays (ELISAs) for LPL, which enabled us to examine LPL mass not only in the postheparin plasma from human, rat, mouse, and guinea pig but also in the media and lysates of cultured cells. All of the developed antibodies showed high affinities for LPL, but their binding to LPL did not always influence the lipolytic activity of the enzyme. Interestingly, although the anti-C-terminus antibody should bind to a common epitope of human and mouse LPL, its binding selectively suppressed only human LPL activity. Because amino acid sequence surrounding the epitope is common to both LPLs, difference in the sequence outside the epitope will contribute to the selective suppression of LPL activity by the antibody. Our results also suggested that both termini of LPL would be exposed on the surface of the molecule because they were fully accessible to antibodies and that the N-terminus of LPL would be functionally less important because binding of the anti-N-terminus antibody did not affect human LPL activity. The ELISAs were further utilized to demonstrate the presence of C-terminus truncated LPL protein in the postheparin plasma of an LPL-deficient patient, to map an epitope of the anti-C-terminus antibody within residues 433–436, and to gain insight into the structure–function relationship of the LPL molecule. Availability of effective antibodies that have different epitope specificities and different inhibitory effects on LPL function will be of great use in immunological analysis of LPL.—**Kawamura, M., T. Gotoda, N. Mori, H. Shimano, K. Kozaki, K. Harada, M. Shimada, T. Inaba, Y. Watanabe, Y. Yazaki, and N. Yamada.** Establishment of enzyme-linked immunosorbent assays for lipoprotein lipase with newly developed antibodies. *J. Lipid Res.* 1994. **35:** 1688–1697.

Supplementary key words polyclonal antibody • monoclonal antibody • synthetic peptide • ELISA • Chinese hamster ovary cells • in vitro expression • LPL deficiency

Lipoprotein lipase (LPL) plays a central role in plasma triglyceride metabolism through catabolism of triglyceride-rich lipoprotein particles such as chylomicrons and very low density lipoproteins (VLDL) (1). Recently, we have observed markedly enhanced conversion of VLDL to low density lipoproteins in transgenic mice overexpressing human LPL. The mice were resistant against diet-induced hypercholesterolemia as well as hypertriglyceridemia, indicating that LPL plays an important role also in plasma cholesterol metabolism in vivo (2).

In order to detect and measure LPL protein, a number of specific antibodies have been produced against the enzyme and also several sandwich enzyme-linked immunosorbent assays (ELISAs) have been developed previously (3–8). The antigens utilized for the development of these antibodies were always native enzyme proteins, although they came from a variety of sources that included postheparin plasma, milk, heart, and adipose tissue from species. Accordingly, development of antibodies required much labor, especially the preparation of a sufficient amount of purified antigens. Furthermore, because most epitopes recognized by these antibodies have not been determined, it is usually difficult to selectively detect a specific portion of the LPL molecule with these antibodies. Thus, ELISAs with such antibodies may be inappropriate for the reliable measurement of abnormal LPL proteins, such as truncated LPL variants that were

Abbreviations: LPL, lipoprotein lipase; N-, amino-; C-, carboxy-; ELISA, enzyme-linked immunosorbent assay; VLDL, very low density lipoproteins; CHO, Chinese hamster ovary; FFA, free fatty acids; KLH, keyhole limpet hemacyanin; PL, pancreatic lipase.

[1]To whom correspondence should be addressed.

found in patients with familial LPL deficiency (9) and also even in the normal populations (10).

To overcome these problems in the present study, we developed new anti-LPL antibodies. We immunized rabbits with the antigens that had not been used previously. These included two synthetic peptides corresponding to either amino (N)- or carboxy (C)-terminal 16 amino acids of mature human LPL as well as recombinant human LPL, which was massively synthesized in the Chinese hamster ovary (CHO) cells transfected with human LPL cDNA. In addition, we developed one polyclonal and four monoclonal antibodies against bovine LPL. These antibodies were used to construct sensitive ELISAs for LPL.

MATERIALS AND METHODS

Expression of human LPL in CHO cells

A 1,581-bp cDNA fragment encompassing the entire coding region of human LPL (11) was subcloned in the sense orientation into the Rc/CMV vector (Invitrogen, San Diego, CA) as described elsewhere (12). The expression plasmid encoding human LPL (designated as pCMV-LPL) was transfected into wild-type Chinese hamster ovary cells (CHO-K cells) by the calcium phosphate coprecipitation technique (13) with some modifications. Briefly, 250 μl solution containing 250 mM $CaCl_2$ and 8.8 μg of the pCMV-LPL purified by the cesium chloride gradient ultracentrifugation was added dropwise to the same volume of 2 × HBS buffer (280 mM NaCl, 10 mM KCl, 50 mM HEPES, 1.5 mM $Na_2HPO_4 \cdot 2H_2O$, 12 mM dextrose, pH 7.0). The mixture was kept still at room temperature for 30 min to make DNA and calcium phosphate coprecipitates, and was then added to the medium of the CHO-K cells (2.8×10^6 cells/6-cm dish) that were precultured for 24 h in DMEM with 10% FCS. After 4 h incubation, the cells were washed once with PBS free of $CaCl_2$ and subjected to a glycerol shock for 90 sec with 15% glycerol in 1 × HBS. Subsequently, the cells were washed with PBS again and allowed to grow for 24 h in Ham's medium with 10% NCS. At this stage, the cells were subcultured by fivefold dilution with the complete medium supplemented with 600 μg/ml of geneticin (G418). After a 3-week treatment with G418, resistant colonies were obtained. Of them, 48 independent clones were picked up and further cultured in individual 6-cm dishes.

Measurement of LPL activity

LPL activity was measured by the method of Nilsson-Ehle and Schotz (14) with some modifications. The substrate mixture (10 ml) contained 50 μCi tri[9,10(n)-^{3}H]oleoylglycerol; 56.6 μmol unlabeled glycerol trioleate, 0.2 M Tris-HCl (pH 8.6), 0.1% Triton X-100, 2% BSA, and 2 ml of human plasma as a source of apolipoprotein C-II. Each sample (100 μl) was added to the same volume of the substrate mixture and was incubated at 37°C for 30 min. The reaction was stopped by the addition of 3.25 ml of methanol–chloroform–heptane 1.41:1.25:1 (v/v/v), and subsequently by 1.05 ml of 0.1 M potassium carbonate–borate buffer (pH 10.5). Free fatty acids (FFA) were extracted from the mixture by vortexing for 15 sec and were separated by centrifugation at 3000 rpm at 15°C for 20 min. The resulting methanol–water upper phase (1 ml) was added to 10 ml of Ready Safe (Beckman). Finally, radioactivities were counted to calculate FFA molecules liberated by the lipolytic reaction.

Northern blot analysis of LPL mRNA

Total RNAs (10 μg) were isolated from each CHO transformant, electrophoresed on a 1% agarose gel with 6% formaldehyde, transferred to a nylon membrane, and hybridized with the human LPL cDNA probe in Rapid Hybridization Buffer (Amersham) at 65°C for 1 h. The blot was washed twice with 0.5 × SSC–0.1% SDS (1 × SSC = 150 mM NaCl and 15 mM sodium citrate) at 65°C for 20 min.

Purification of LPL by heparin-Sepharose affinity chromatography

To obtain recombinant human LPL, culture medium was collected every 24 h from the CHO cells expressing human LPL. The collected medium (300 ml) was adjusted to 0.4 M on salt concentration and was applied to a heparin-Sepharose affinity column. After application, the column was washed with 8 vols of solution containing 0.4 M NaCl, 5 mM Veronal (pH 7.4), 0.5 mg/ml EDTA, 0.5 mg/ml NaN_3, and 0.2% Triton X-100. Subsequently, the column was washed again with 200 ml of 0.8 M NaCl, 5 mM Veronal (pH 7.4), 0.5 mg/ml EDTA, and 0.5 mg/ml NaN_3. LPL bound to the heparin-Sepharose column was then released with an elution buffer containing 1.8 M NaCl, 5 mM Veronal (pH 7.4), 10% glycerol, 0.5 mg/ml EDTA, and 0.5 mg/ml NaN_3. The 1.8 M fractions were co-eluted with LPL activity and were pooled and stored at −80°C.

Fresh bovine milk was a kind gift from Morinaga Milk Industry Co., Ltd., (Tokyo, Japan). Bovine skim milk was obtained from fresh milk by delipidation with centrifugation at 9,600 rpm for 30 min at 4°C. Bovine LPL was purified from skim milk with a heparin-Sepharose affinity column as described above.

Development of four polyclonal antibodies against LPL

To develop antibodies against the purified recombinant human LPL as well as bovine milk LPL, an initial dose (250 μg) of the respective LPL proteins emulsified in complete Freund's adjuvant was injected subcutaneously into a Japanese White rabbit. The rabbits received an addi-

tional injection of the same dose of purified antigen every 2 weeks. From the ninth week, 2 ml of blood was collected from each rabbit every week and finally 50 ml of blood was subjected to the purification with LPL-Sepharose CL-4B affinity column chromatography.

In parallel experiments, two peptides of 16 amino acids corresponding to the N-terminus and the C-terminus of the mature human LPL were synthesized and conjugated with keyhole limpet hemacyanin (KLH). The KLH-conjugated peptides (500 μg) were injected to immunize rabbits, and antisera were raised and purified with LPL-Sepharose CL-4B columns.

Development of monoclonal antibodies against bovine LPL

Monoclonal antibodies were developed against purified bovine LPL essentially as described elsewhere (15). Positive clones were screened with an ELISA whereby the culture media of hybridoma cells were examined upon their binding capacity to immobilized LPL. Of 1728 hybridomas obtained, 108 clones were shown to secrete antibodies that reacted with an LPL antigen (500 ng) immobilized on each well of the ELISA plates. Four clones secreting antibodies with the strongest reactivities were selected and designated 3G3, 8H5, 5B12, and 3B8, respectively, and were subcloned by the limiting dilution method. Immunoblot analysis showed that the monoclonal antibodies produced by these four clones can specifically react with purified LPL. To obtain ascites fluid rich in antibodies, each clone of hybridoma cells (5×10^6 cells/mouse) was injected intraperitoneally into a 20-week-old BALB/c male mouse primed with 0.5 ml of pristane 10 days earlier. Ascites fluid was harvested 10–14 days after injection and was centrifuged at 3000 rpm for 30 min at room temperature to remove cells. Immunoglobulins in ascites were purified by precipitation with 40–50% ammonium sulfate and were labeled with biotin.

Antibody binding assay

Microtiter plates were coated with 0.5 μg of bovine LPL that was suspended in 50 μl of PBS. After incubation at 4°C for 16 h, the plates were washed 7 times with PBS containing 0.05% Triton X-100 and blocked with 100 μl of PBS containing 1% gelatin. The purified polyclonal and monoclonal antibodies (1 mg IgG/ml) were serially diluted with PBS and an equal volume (50 μl) of diluted antibodies was added to each well of the microtiter plates. After a 16-h incubation at 4°C, the plates were washed 7 times, and then 50 μl of peroxidase-labeled rabbit anti-mouse or goat anti-rabbit IgG (1:2000 dilution) was added to each well. After incubation at room temperature for 1.5 h, the wells were washed again and 100 μl of substrate solution was applied to each well. The plates were examined with a Dynatech MR-700 plate reader (Dynatech Laboratories, VA) to measure color intensity of the wells.

Development of ELISAs for LPL

The wells of polystyrene microtiter plates were coated with aliquots (50 μl) of first antibodies that were diluted with PBS to a final concentration of 10 μg IgG/ml. The plates were incubated for 16 h at 4°C, washed 7 times with PBS containing 0.05% Triton X-100, and were incubated at room temperature for 2 h with PBS containing 1% gelatin to block the uncoated binding sites left in the plates. The plates were then washed more 7 times. Samples were mixed in a buffer containing 1 M NaCl, 0.01% Triton X-100, and protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 1 mM EDTA, and 0.05 mM aprotinin), applied to the wells, and incubated at 4°C for 16 h. The plates were again washed 7 times, and 50 μl of biotinylated second antibody (5 μg IgG/ml) was applied to each well of the respective plates. After a 16-h incubation at 4°C and subsequent washing, 50 μl of streptoavidin-peroxidase (1:10000 dilution) was added to each well and incubated at room temperature for 1.5 h. After washing, color reactions were developed on the plates by the addition of *o*-phenylenediamine substrate solution (0.08% *o*-phenylenediamine, 0.01% H_2O_2, 0.15 M citrate buffer, pH 5.0) and stopped in 5 min with 4 N HCl. Optical density of the color reactions was analyzed at 492 nm by the microtiter plate reader. For each ELISA with the respective pairs of antibodies, a standard calibration curve was delineated with purified bovine milk LPL as a standard.

Other materials and methods

One established monoclonal antibody (MAb 5D2) (5), which had been raised against bovine milk LPL, was purchased from Oncogene Sciences (Mineola, NY) and used as a control antibody in some experiments. Mutant human LPLs with various levels of C-terminal truncation were previously expressed transiently in Cos-1 cells (16). The profile of the LPL-deficient patient homozygous for the Trp^{382} to stop mutation has been reported elsewhere (12). To obtain postheparin plasma, fasting blood was drawn 10 min (human) or 3 min (rat, mouse, and guinea pig) after administration of a bolus injection of heparin (10 U/kg for human, 100 U/kg for other species).

RESULTS

In vitro expression of human LPL in CHO cells

Of the 48 clones examined, the clone designated N46 secreted the highest lipolytic activity into media (6.2 μmol FFA/h per mg protein), which was much higher than that of wild-type CHO-K cells (0.85 μmol FFA/h per mg protein). The secreted lipolytic activity accumulated within medium and reached a maximum level in 24 h. Thus, the culture media of the N46. cells (designated CHO-LPL

cells) were replaced every 24 h, collected, and stocked.

Northern blot analysis of total RNA isolated from the CHO-LPL cells showed a single major band with an approximate length of 2.3 kb, which was completely absent in RNA from CHO-K cells and thus must be the signal for the expressed human LPL mRNA transcript (**Fig. 1**). To confirm that the detected lipolytic activity was actually derived from LPL, the collected media from CHO-LPL cells were investigated both biochemically and immunologically. First, affinity for heparin was examined with a heparin-Sepharose column. The medium from CHO-LPL cells was applied to the column which was subsequently eluted with buffers of various salt concentrations. The peak of lipolytic activity was not only co-eluted with a buffer of 1.6 M NaCl but also augmented with the addition of normal human plasma containing apolipoprotein C-II (**Fig. 2**), which is a unique characteristic of LPL (1). Second, the peak fraction eluted with a heparin-Sepharose column was examined by Western blot analysis together with two types of control LPL molecules (**Fig. 3**). Staining of the electrophoresed 8% SDS-polyacrylamide gel with Coomassie Blue visualized a major band in the lane of culture medium of CHO-LPL cells (lane designated "Recombinant LPL") which migrated intermediate between the signals for control LPL ("Human milk LPL" and "Bovine milk LPL"). Furthermore, Western blot analysis showed that these signals can immunoreact with an antisera raised against bovine milk LPL, while such a major band was almost undetectable in the lane of culture medium of wild-type CHO-K cells either by SDS-PAGE or by Western blot analysis (data not shown). These results established that the CHO-LPL cells actively synthesized and secreted recombinant human LPL

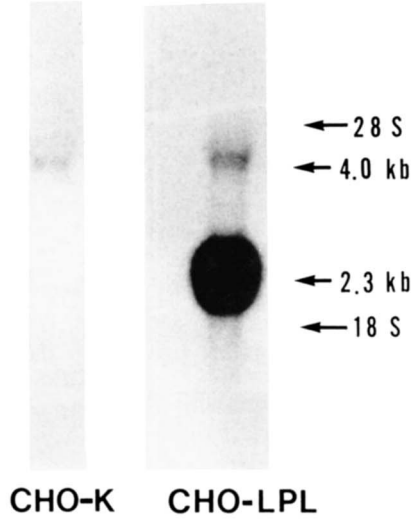

Fig. 1. Northern blot analysis of total RNAs isolated from CHO-K and CHO-LPL cells.

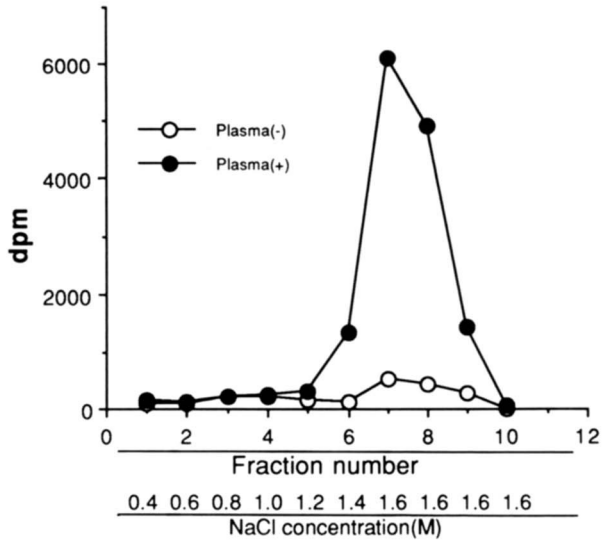

Fig. 2. Isolation of recombinant human LPL by heparin-Sepharose affinity chromatography. Culture medium of CHO-LPL cells was applied to a heparin-Sepharose CL6B affinity column, from which LPL was eluted with buffers of various salt concentrations. Experiments were performed in the absence (○) or in the presence (●) of normal plasma as a source of apolipoprotein C-II.

molecules. The estimated molecular weight (55,600) of the recombinant LPL was slightly smaller compared to that of human milk LPL (Fig. 3), which may reflect possible difference in their contents of carbohydrates.

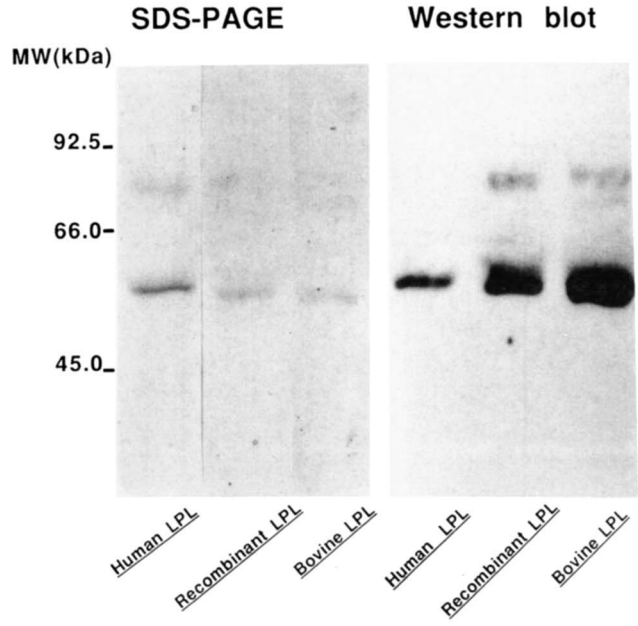

Fig. 3. Visualization of LPL proteins by SDS-PAGE and Western blot analysis. An equal amount (50 μg) of purified protein was applied to each lane. Human LPL, recombinant LPL, and bovine LPL correspond to proteins purified from human milk, culture medium of CHO-LPL cells, and bovine milk, respectively.

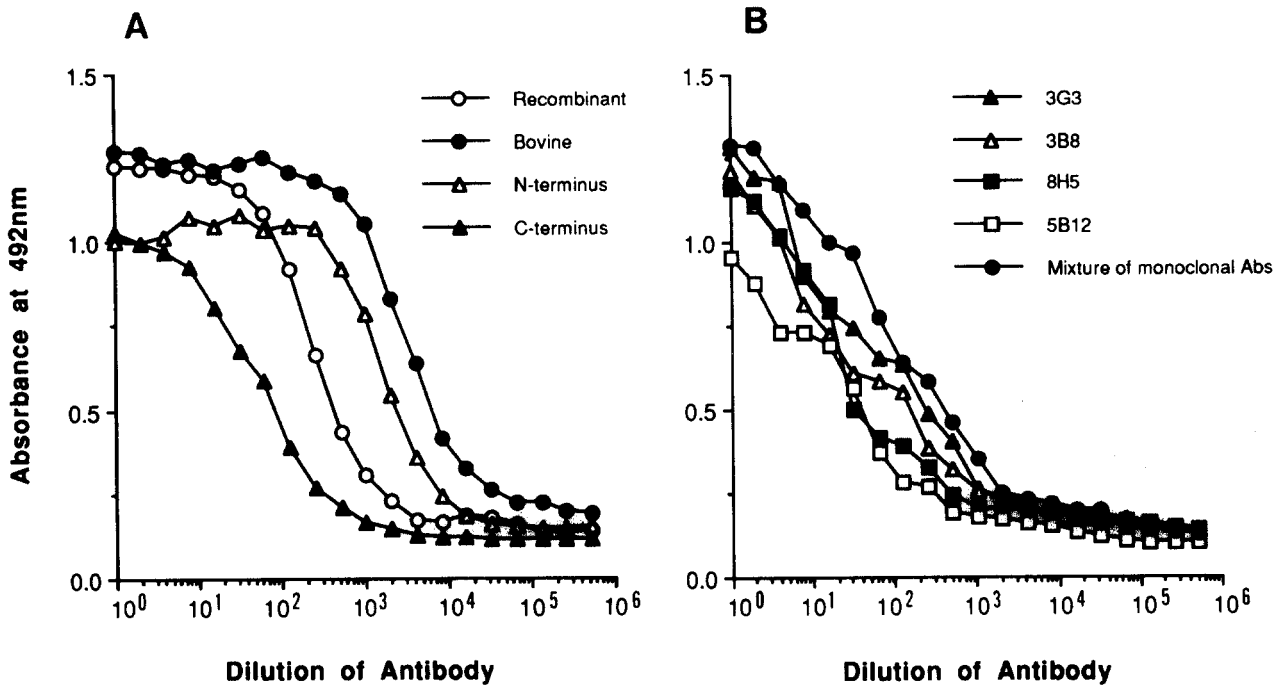

Fig. 4. Binding affinity of antibodies to immobilized bovine LPL antigen. Dilution curves were delineated for polyclonal (A) and monoclonal (B) antibodies.

Affinity of antibodies for purified LPL

Four polyclonal and four monoclonal antibodies were developed. To determine a combination of antibody affinity and titer, serially diluted antibodies were examined upon their ability to bind to LPL purified from bovine milk. **Figure 4** shows the binding curves for the respective antibodies, with dilution of antibody on the horizontal lines on a log scale. Although all of the polyclonal antibodies showed high affinities, as might be expected, the one raised against bovine milk LPL had the highest binding affinity for purified bovine LPL (Fig. 4A). Monoclonal antibodies showed higher affinities in order of 3G3 > 3B8 > 8H5 > 5B12 (Fig. 4B). Interestingly, a mixture of equal amounts of these four monoclonal antibodies had a higher affinity than any single antibody, probably reflecting the diversity of the epitopes recognized by these four monoclonal antibodies.

Antibodies' inhibitory effects on LPL activity

Antibodies were examined upon their inhibitory effects on the lipolytic activity of LPL from the human postheparin plasma. As shown in **Fig. 5,** antisera raised against either bovine milk LPL or recombinant human LPL markedly suppressed the LPL activity in a concentration-dependent manner, as the control antibody MAb 5D2 did. The anti-C-terminus antibody also suppressed human LPL activity moderately. The suppression of LPL activity must be a function of these specific antibodies, because nonspecific mouse or rabbit IgG alone did not affect the activity. By contrast, the anti-N-terminus antibody and the mixture of four monoclonal antibodies virtually failed to suppress the activity.

The suppressive effects of antibodies on LPL activity derived from postheparin plasma of rat, mouse, and guinea pig, as well as from purified bovine milk were also studied (**Table 1**). The results showed that the two an-

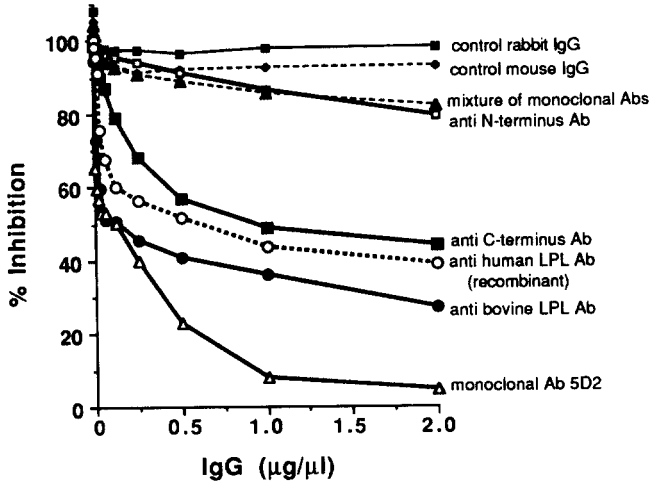

Fig. 5. Inhibition of human postheparin plasma LPL activity by specific antibodies. Inhibition curves were delineated for the respective antibodies or for mouse and rabbit control IgG. Each antibody was added to 20 μl of human postheparin plasma in concentrations indicated.

tisera raised against bovine milk LPL and recombinant human LPL and MAb 5D2 could suppress the activity of LPL from multiple species. Considering the significant homology of C-terminal amino acids of LPL from different species (16), it was surprising that the anti-C-terminus antibody suppressed only human LPL activity. None of the developed antibodies seemed to cross-react with hepatic lipase, because these antibodies did not precipitate the postheparin plasma lipolytic activity of LPL-deficient patients (data not shown).

Establishment of ELISA systems

All the developed antibodies were biotinylated and tried for construction of ELISA systems. Of the pairs of different antibodies, three pairs had the greatest ability to precisely detect and measure LPL enzyme mass. The ELISAs established were the ones that used any of anti-N-terminus antibody, anti-C-terminus antibody, and a mixture of monoclonal antibodies as the first antibody in combination with the biotinylated anti-bovine LPL polyclonal antibody as the second antibody. In each case, a standard calibration curve was delineated for weighed LPL standard purified from bovine milk (**Fig. 6**). Each standard curve was almost linear for the concentrations between 0 and 1000 ng/ml, assuring the sensitivity of the ELISAs within this range.

LPL enzyme mass in the postheparin plasma

The three ELISAs established were used to measure LPL enzyme mass in the postheparin plasma obtained from human, rat, mouse, and guinea pig (**Table 2**). Although the values determined for an identical sample varied slightly among assays, the ratio of LPL masses in human, rat, mouse, and guinea pig was almost constant in the three ELISAs. Thus, the antibodies used for the ELISAs should react not only with human and bovine LPL but also with LPL from other species.

LPL enzyme mass in culture media and cell lysates of CHO cells

To confirm the availability of established ELISA for analysis of cultured cells, both the media and the cell lysates of CHO cells were assayed for LPL mass with an ELISA using the mixture of monoclonal antibody together with anti-bovine LPL polyclonal antibody (**Fig. 7**). Compared with wild-type CHO-K cells, CHO-LPL cells had about 8- to 10-fold higher amounts of LPL mass in medium and about 3- to 3.5-fold higher mass in cell lysates, which was consistent with the difference between LPL activities derived from these cells. Addition of heparin always caused a shift of LPL enzyme mass and activity from cell homogenate to culture medium, suggesting that heparin added to the medium can facilitate secretion of LPL from CHO cells.

Detection of C-terminus truncated LPL

We previously expressed a series of C-terminus truncated human LPL in Cos-1 cells in vitro (16). The truncated LPL secreted into culture medium was used in this study to examine the epitope specificity of the anti-C-terminus antibody. The mass of the truncated LPL was comparatively measured by two ELISAs that used either anti-N-terminus antibody or anti-C-terminus antibody as the first antibody (**Fig. 8**). The results showed that the anti-C-terminus antibody could fully detect LPL-436 (which lacked C-terminal amino acids after position 436) and other less severely truncated LPLs while it detected LPL-434 and LPL-435 only incompletely. Because the antibody had been developed against C-terminal 16 amino acids of human LPL (positions 433–448), the results may indicate that the anti-C-terminus antibody would recognize an epitope mapped within amino acid residues 433–436. However, we can never exclude the possibility that the antibody preparation may also recognize epitopes in more distant locations.

TABLE 1. Suppression of LPL activity by antibodies

Source of LPL	Suppression of LPL Activity[a]					
	Anti Bovine LPL	Anti Recombinant Human LPL	Anti N-Terminus	Anti C-Terminus	Mixture of Monoclonal Abs	MAb5D2
PHP human	↓↓↓	↓↓~↓↓↓	→~↓	↓↓↓	→~↓	↓↓↓
PHP rat	↓↓	→~↓	→	→	→	→~↓
PHP mouse	↓↓~↓↓↓	→~↓	→	→	→	→
PHP guinea pig	→	→~↓	→	→	→	→~↓
Bovine milk	↓↓↓	↓~↓↓	→	→	→	↓↓~↓↓↓

All experiments were performed three times with an identical concentration (0.5 μg/μl) of the respective antibodies. Abbreviations: LPL, lipoprotein lipase; Abs, antibodies; PHP, postheparin plasma.

[a] Suppression rates were represented as follows: →<10%; ↓ 10–25%; ↓↓ 25–50%; ↓↓↓ >50%.

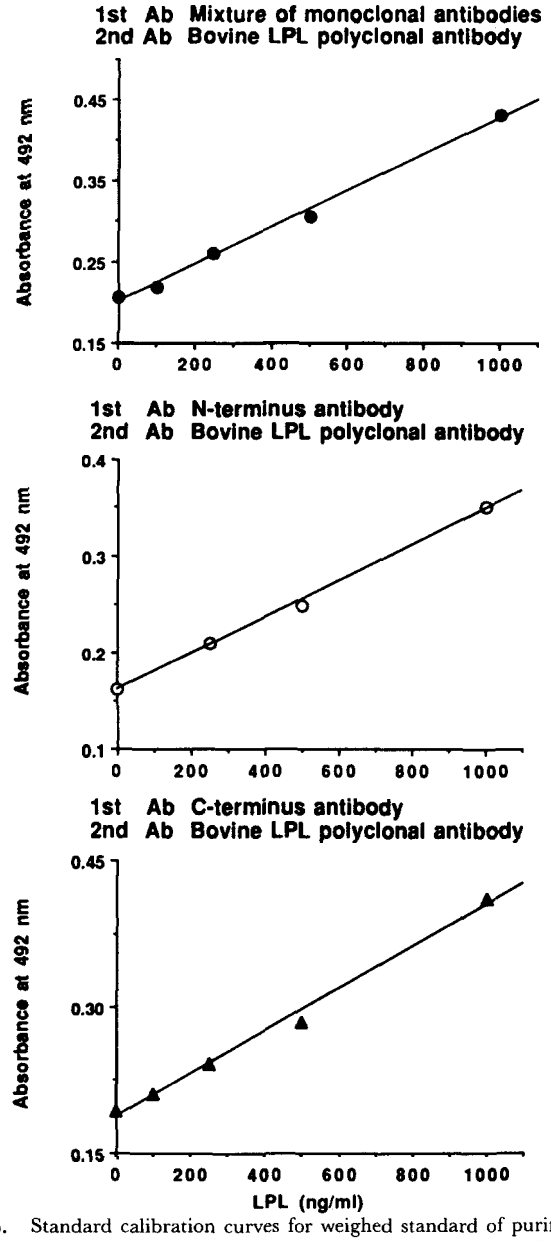

Fig. 6. Standard calibration curves for weighed standard of purified bovine LPL delineated with three types of ELISAs. The ELISAs utilized a mixture of monoclonal antibodies, anti-N-terminus antibody, and anti-C-terminus antibody as the first antibody, respectively, and anti-bovine milk LPL polyclonal antibody as the second antibody.

We have also previously reported an LPL-deficient patient homozygous for a Trp^{382} to stop mutation, who had a markedly decreased level of postheparin plasma LPL mass measured by an ELISA with anti-N-terminus antibody (12). The mass value (22 ng/ml) was very low and thus might be considered a background level. To prove the significance of the low mass value and to demonstrate the presence of C-terminus truncated LPL in this patient, the LPL mass was re-examined by other two ELISAs that used the mixed monoclonal antibodies and anti-C-terminus antibody as each first antibody. The former ELISA showed the mass to be 28.6 ng/ml, which was almost compatible with the previously reported value. However, the other ELISA with anti-C-terminus antibody showed the mass to be almost nothing, establishing the presence of a low but significant level of C-terminus truncated LPL in the plasma of the patient.

DISCUSSION

Northern blot analysis of RNA from CHO-LPL cells showed a signal for the expressed human LPL mRNA with an approximate length of 2.3 kb (Fig. 1). Interestingly, despite several washes with high stringency, another much weaker band with an approximate length of 4.0 kb was found in the lanes for both CHO-K and CHO-LPL. It was previously reported that CHO cells can synthesize and secrete a lipase that was shown to be identical to LPL by both the biochemical and immunological evidences (17). Also, it is known that rat LPL mRNA is a single species of 4.0-kb transcript (18, 19), which is expressed faintly in adult rat ovary (18). Thus, we suppose that the 4.0-kb band is a signal for hamster LPL mRNA endogeneously expressed in CHO-K cells. Because densitometric analysis showed that the intensity of the 4.0-kb band was less than 1/20 of that of the 2.3-kb band, the endogeneous expression could be ignored in experiments with CHO-LPL cells.

To our knowledge, this is the first report of a study of the development of antibody with synthetic peptides of LPL as an antigen. Our results suggested that both the N-

TABLE 2. LPL enzyme mass in the postheparin plasma from human, rat, mouse and guinea pig

First Antibody Used	Human[a]	Rat[b]	Mouse[b]	Guinea Pig[b]
	(LPL mass, ng/ml)			
Mixture of monoclonal Abs	409.2	824.5	982.5	518.2
Anti N-terminus Ab	514.2	913.5	1118.0	587.6
Anti C-terminus Ab	491.5	889.4	1069.8	528.5

Second antibody, anti bovine LPL polyclonal antibody. Specific activity: human = 43.0 ± 5.4; rat = 94.0 ± 5.1; mouse = 57.8 ± 3.8; guinea pig = 84.9 ± 5.6 μEq FFA/h per μg (n = 3).

[a] After an overnight fast, a bolus injection of heparin (10 U/kg) was administered to a normal subject, from whom blood was collected 10 min after the heparin injection.

[b] After an overnight fast, a bolus injection of heparin (100 U/kg) was administered to the animals and blood was collected 3 min after the injection.

and C-termini of LPL molecule are quite accessible to antibodies and thus should exist on the outer surface of the molecule. In addition, taking account of the fact that 10 out of the N-terminal 16 amino acids and 13 out of the C-terminal 16 amino acids are shared between human and bovine LPL (20), the observation that the two antibodies against both ends of mature human LPL can cross-react with bovine LPL (Fig. 4) may imply that amino acid residues conferring epitope specificity of these antibodies are likely some of those shared residues. Because most of the epitopes of previously developed antibodies have not been determined, availability of the antibodies that react selectively with either terminus of LPL will be helpful in exploring the structure–function relationship of LPL.

Elucidation of a three-dimensional structural model of human pancreatic lipase (PL) (21) and the proposed significant structural homology between human LPL and PL (22) predicted that both ends of human LPL would exist far distant from the catalytic center of the molecule (20, 22). As for N-terminus antibody, this may provide an explanation for the inability of the antibody to significantly affect the catalytic action of LPL despite its high reactivity with the molecule. Also, it has previously

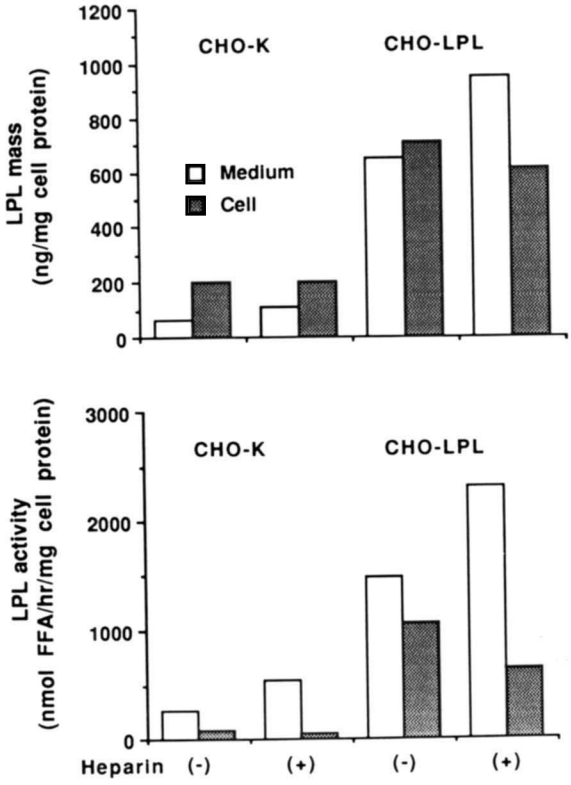

Fig. 7. Measurement of LPL mass and activity in culture medium and cell lysates of CHO-K and CHO-LPL cells. Confluent CHO cells were cultured in fresh medium for 24 h in the absence (−) or presence (+) of heparin (5 U/ml). After removal of medium, the cells were washed three times with PBS and lysed in 25 mM ammonium buffer (pH 8.5) with sonication. Open bars and hatched bars represent the values of medium and cell lysates, respectively.

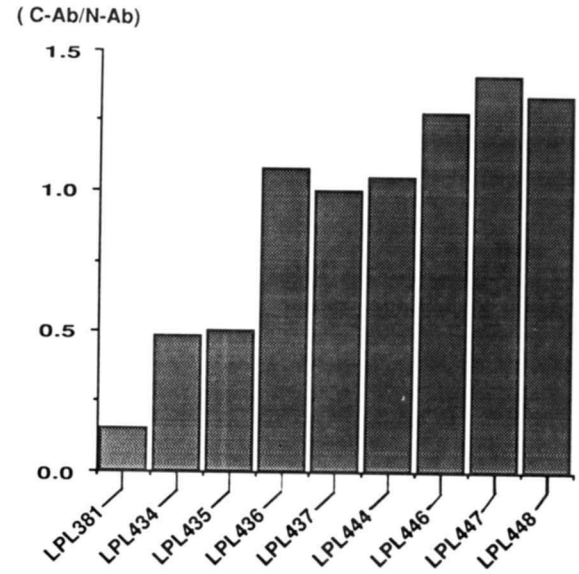

Fig. 8. Measurement of the mass of C-terminus truncated LPL by two ELISAs with either anti-C-terminus or anti-N-terminus antibody. The mass of each truncated LPL was measured six times by the two ELISAs comparatively. The mean mass value obtained with the C-terminus antibody was divided by that with the N-terminus antibody, and the ratio is represented on the vertical axis. The length of amino acids of each C-terminus truncated LPL is shown on the horizontal axis by the numbers affixed to "LPL". Because human LPL consists of 448 amino acids, for example, LPL-436 represents a mutant LPL of 436 amino acids with deletion of C-terminal 12 amino acids.

been reported that an amino acid substitution at the N-terminus of human LPL (Asp^9 to Asn change) did not affect LPL function (23). Taken together, it seems unlikely that the N-terminus of LPL plays an important role in the expression of LPL function. In contrast, the C-terminus must be involved in the expression of function, because the binding of C-terminus antibody highly suppressed human LPL function (Table 1), as well as because a common polymorphic form of human LPL lacking the last two amino acids (10) had altered levels of lipolytic function (16, 24). We previously reported that the C-terminal region of human LPL is closely associated with the expression of both enzyme mass and activity, and suggested the functional importance of Val^{436} (16). In this context, it was interesting that a possible epitope of the anti-C-terminus antibody was mapped within amino acid residues 433–436. The C-terminus antibody may have possibly suppressed LPL activity through effect on Val^{436}. Nevertheless, amino acid sequences outside the epitope may also influence the suppression of LPL activity by the C-terminus antibody, because binding of the antibody did not suppress the activity of mouse LPL (Table 1), which has the same amino acid sequence as residues 433–443 of human LPL (16).

Contrasting with the commercially available monoclonal antibody MAb 5D2 (5), which was also raised against bovine milk LPL, none of our four monoclonal antibodies

inhibited the LPL activity from human postheparin plasma (Fig. 5). This probably resulted from the use of different methods for the selection of positive hybridoma, by immunosuppression of LPL activity (5) or by immunobinding to immobilized LPL. It is likely that the epitopes recognized by our four monoclonal antibodies differ from those of the other monoclonal antibodies developed previously, which always immunoprecipitated lipolytic activity of LPL (3, 5–7). It may be strange that a mixture of monoclonal antibodies made in mice would react with mouse LPL (**Table 2**). However, the ELISA with the mixed monoclonal antibodies could reproducibly detect the immunoreactive mass in the mouse plasma, even when the mouse plasma was mixed at various ratios with human plasma (data not shown). We suppose that the antibodies' inability to neutralize mouse LPL activity (Table 1) allowed these reactive but not neutralizing antibodies to be made in mice. Similar examples have previously been reported in other cases (25).

In the present study, we developed new antibodies against LPL and established sensitive ELISAs for the measurement of the enzyme mass. Availability of effective antibodies that have different epitope specificities and different inhibitory effects on LPL function will be of great use in immunological analysis of LPL.

Manuscript received 9 March 1993, in revised form 17 December 1993, and in re-revised form 23 March 1994.

REFERENCES

1. Garfinkel, A. S., and M. C. Schotz. 1987. Lipoprotein lipase. *In* Plasma Lipoproteins. A. M. Gotto, Jr., editor. Elsevier Science Publishers, New York, NY. 335–357.
2. Shimada, M., H. Shimano, T. Gotoda, K. Yamamoto, M. Kawamura, T. Inaba, Y. Yazaki, and N. Yamada. 1993. Overexpression of human lipoprotein lipase in transgenic mice. *J. Biol. Chem.* **268:** 17924–17929.
3. Goldberg, I. J., J. R. Paterniti, Jr., D. S. France, G. Martinelli, and J. A. Cornicelli. 1986. Production and use of an inhibitory monoclonal antibody to human lipoprotein lipase. *Biochim. Biophys. Acta.* **878:** 168–176.
4. Goers, J. W. F., M. E. Pedersen, P. A. Kern, J. Ong, and M. C. Schotz. 1987. An enzyme-linked immunoassay for lipoprotein lipase. *Anal. Biochem.* **166:** 27–35.
5. Babirak, S. P., P-H. Iverius, W. Y. Fujimoto, and J. D. Brunzell. 1989. Detection and characterization of the heterozygote state for lipoprotein lipase deficiency. *Arteriosclerosis.* **9:** 326–334.
6. Semenkovich, C. F., C. C. Luo, M. K. Nakanishi, S-H. Chen, L. C. Smith, and L. Chan. 1990. In vitro expression and site-specific mutagenesis of the cloned human lipoprotein lipase gene. *J. Biol. Chem.* **265:** 5429–5433.
7. Ikeda, Y., A. Takagi, Y. Ohkaru, K. Nogi, T. Iwanaga, S. Kurooka, and A. Yamamoto. 1990. A sandwich-enzyme immunoassay for the quantification of lipoprotein lipase and hepatic triglyceride lipase in human postheparin plasma using monoclonal antibodies to the corresponding enzymes. *J. Lipid Res.* **31:** 1911–1924.
8. Peterson, J., W. Y. Fujimoto, and J. D. Brunzell. 1992. Human lipoprotein lipase: relationship of activity, heparin affinity, and conformation as studied with monoclonal antibodies. *J. Lipid Res.* **33:** 1165–1170.
9. Santamarina-Fojo, S. 1992. Genetic dyslipoproteinemias: role of lipoprotein lipase and apolipoprotein C-II. *Curr. Opin. Lipidol.* **3:** 186–195.
10. Hata, A., M. Robertson, M. Emi, and J-M. Lalouel. 1990. Direct detection and automated sequencing of individual alleles after electrophoretic strand separation: identification of a common nonsense mutation in exon 9 of the human lipoprotein lipase gene. *Nucleic Acids Res.* **18:** 5407–5411.
11. Gotoda, T., M. Senda, T. Gamou, Y. Furuichi, and K. Oka. 1989. Nucleotide sequence of human cDNA coding for a lipoprotein lipase (LPL) cloned from placental cDNA library. *Nucleic Acids Res.* **17:** 2351.
12. Gotoda, T., N. Yamada, M. Kawamura, K. Kozaki, N. Mori, S. Ishibashi, H. Shimano, F. Takaku, Y. Yazaki, Y. Furuichi, and T. Murase. 1991. Heterogeneous mutations in the human lipoprotein lipase gene in patients with familial lipoprotein lipase deficiency. *J. Clin. Invest.* **88:** 1856–1864.
13. Wigler, M., R. Sweet, G. K. Sim, B. Wold, A. Pellicer, E. Lacy, T. Maniatis, S. Silverstein, and R. Axel. 1979. Transformation of mammalian cells with genes from prokaryotes and eukaryotes. *Cell.* **16:** 777–785.
14. Nilsson-Ehle, P., and M. C. Schotz. 1976. A stable radioactive substrate emulsion for assay of lipoprotein lipase. *J. Lipid Res.* **17:** 536–541.
15. Yoon, T. H., N. Mori, K. Kitamura, S. Ishibashi, H. Shimano, H. Mokuno, T. Gotoda, F. Takaku, and N. Yamada. 1991. Characterization of monoclonal anti-rabbit apolipoprotein E antibodies and chemical composition of lipoproteins separated by anti-apolipoprotein E immunoaffinity chromatography. *J. Biochem.* **109:** 204–210.
16. Kozaki, K., T. Gotoda, M. Kawamura, H. Shimano, Y. Yazaki, Y. Ouchi, H. Orimo, and N. Yamada. 1993. Mutational analysis of human lipoprotein lipase by carboxy-terminal truncation. *J. Lipid Res.* **34:** 1765–1772.
17. Rojas, C., S. Enerback, and G. Bengtsson-Olivecrona. 1990. Synthesis and secretion of active lipoprotein lipase in Chinese-hamster ovary (CHO) cells. *Biochem. J.* **271:** 11–15.
18. Semenkovich, C. F., S-H. Chen, M. Wims, C-C. Luo, W-H. Li, and L. Chan. 1989. Lipoprotein lipase and hepatic lipase mRNA tissue specific expression, developmental regulation, and evolution. *J. Lipid Res.* **30:** 423–431.
19. Mori, N., T. Gotoda, S. Ishibashi, H. Shimano, K. Harada, T. Inaba, F. Takaku, Y. Yazaki, and N. Yamada. 1991. Effects of human recombinant macrophage colony-stimulating factor on the secretion of lipoprotein lipase from macrophages. *Arterioscler. Thromb.* **11:** 1315–1321.
20. Wang, C-S., J. Hartsuck, and W. J. McConathy. 1992. Structure and functional properties of lipoprotein lipase. *Biochim. Biophys. Acta.* **1123:** 1–17.
21. Winkler, F. K., A. D'Arcy, and W. Hunziker. 1990. Structure of human pancreatic lipase. *Nature.* **343:** 771–774.
22. Derewenda, Z. S., and C. Cambillau. 1991. Effects of gene mutations in lipoprotein and hepatic lipases as interpreted by a molecular model of the pancreatic triglyceride lipase. *J. Biol. Chem.* **266:** 23112–23119.
23. Lohse, P., P. Lohse, O. Beg, J. D. Brunzell, S. S. Fojo, and H. B. Brewer, Jr. 1991. Familial chylomicronemia:

identification of a unique patient homozygote for two separate mutations in the LPL gene. *Arterioscler. Thromb.* **11:** 1415a.

24. Kobayashi, J., T. Nishida, D. Ameis, G. Stahnke, M. C. Schotz, H. Hashimoto, I. Fukamachi, K. Shirai, Y. Saito, and S. Yoshida. 1992. A heterozygous mutation (the codon for $Ser^{447} \rightarrow$a stop codon) in lipoprotein lipase contributes to a defect in lipid interface recognition in a case with type I hyperlipoproteinemia. *Biochem. Biophys. Res. Commun.* **182:** 70–77.

25. Ziltener, H. J., B. Fazakas de St. Groth, K. B. Leslie, and J. W. Schrader. 1988. Multiple glycosylated forms of T cell-derived interleukin 3 (IL-3). *J. Biol. Chem.* **263:** 14511–14517.