Development of Antibody Against Epitope of Lipoprotein(a) Modified by Oxidation
Evaluation of New Enzyme-Linked Immunosorbent Assay for Oxidized Lipoprotein(a)

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Background—Recently, the biological effects of oxidized lipoprotein(a) [Lp(a)] have been reported to be more potent than Lp(a), the arteriosclerosis-relevant lipoprotein. Thus, investigations with oxidized Lp(a) are expected to provide viewpoints different from the conventional ones based on Lp(a).

Methods and Results—An anti-Lp(a) monoclonal antibody (161E2) was produced against synthetic peptide antigen (Arg-Asn-Pro-Asp-Val-Ala-Pro). This epitope was characterized as having various properties because its external exposure was induced as a result of oxidative modification. Using 161E2 antibody, we developed a new enzyme-linked immunosorbent assay to measure Lp(a) modified by oxidative stress. The present data demonstrated that oxidized Lp(a) that contains the epitope of 161E2 antibody was present in the serum of humans. Therefore, we used this new enzyme-linked immunosorbent assay to evaluate the role of oxidized Lp(a) in patients with hypertension, which induces oxidative stress. Interestingly, hypertensive patients with complications showed a significantly higher level of oxidized Lp(a) in serum than did normotensive subjects (P<0.01), whereas there was no significant difference in native Lp(a) between normotensive and hypertensive subjects. Importantly, positive immunostaining with 161E2 monoclonal antibody was found in the human arteriosclerotic tissue.

Conclusions—We developed a new antibody against an epitope in Lp(a) as a result of oxidation treatment but not in native Lp(a). The present data demonstrated in vivo the presence of oxidized Lp(a) in the atherosclerotic tissue and its elevation in hypertensive patients. The presence of oxidized Lp(a) may be important in understanding the role of Lp(a) in cardiovascular disease. (Circulation. 2000;102:1639-1644.)

Key Words: lipoprotein(a) ■ immunoassay ■ monoclonal antibody ■ atherosclerosis ■ hypertension

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Isolation of Plasminogen, LDL, and Lp(a)

Three healthy female subjects with elevated serum Lp(a) levels (30, 45, and 21 mmol/L) served as donors for Lp(a) isolation. The phenotypes of Lp(a) were 27, 29, and 34 kringle 4 repeats. As previously reported, the following density fractions were isolated:

1. d<1.02, to remove VLDL and IDL;
2. d=1.02 to 1.05, to obtain LDL;
3. d=1.05 to 1.2, to obtain Lp(a)-rich fraction; and
4. d>1.21, to obtain plasminogen from the plasma protein fraction.

Lp(a)-enriched fraction was subjected to lysine-Sepharose 6B affinity chromatography (Pharmacia). The purity of Lp(a) was evaluated with SDS-PAGE on 2.5% to 16% polyacrylamide gradient gels (Tefco gel) and agarose gel. The absolute protein mass of the isolated Lp(a) was determined by densitometry, and phospholipid, cholesterol (both free and esterified), and triglyceride analyses were also performed with a commercial kit (Shino-Test). The total concentration of protein mass plus lipid for the isolated Lp(a) was taken as the Lp(a) concentration. Plasminogen was also prepared from the human serum protein fraction (d>1.21).

Evaluation of Anti–Modified Lp(a) Monoclonal Antibody (161E2) Against Various Modified Lp(a)

The oxidation of Lp(a) was carried out with copper ions and lipoxygenase as oxidizing agents. Lp(a) was oxidized through incubation with CuCl₂, (0, 0.1, 0.2, 0.5, 1.0, 2.0, 4.0, 6.0, and 10.0 μg/mL; Wako Chemical) or lipoxygenase (0, 1.0, 3.0, 5.0, and 20.0 μg/mL; Sigma Chemical Co) for 12 hours at 37°C. The degree of oxidation was quantified through 2 methods:

1. The increase in relative mobility on agarose gel (Helena Laboratory) or
2. The formation of thiobarbituric acid–reactive substances (TBARS; Wako Chemical). During this preparation, we confirmed that Lp(a) was intact and not degraded. Glycation of purified Lp(a) was also performed with the incubation Lp(a) (1 mg/mL) with 200 mmol/L glucose in sterile PBS that contained 1 mmol/L Na₂EDTA (pH 7.4) at 37°C for 7 days. The degree of glycation of Lp(a) was examined according to the agarose gel electrophoresis method. The purity of Lp(a) (1 mg/mL) was also measured after 6 days of storage at room temperature and at 37°C and after freeze/thaw cycle repeated 10 or 30 times.

Electrophoresis and Immunoblotting

Lp(a) and modified Lp(a) were dissolved in 0.9% NaCl of a concentration of 0.5 mg/mL and subjected to electrophoresis. The phenotype of Lp(a) was evaluated with a high-resolution SDS–agarose gel electrophoretic method. We used apo(a) isofrom standard human serum (Immuno AG) with different 5-banded phenotypes as a molecular weight reference to control for gel-to-gel variation.

ELISA Procedure for Modified Lp(a) and Native Lp(a)

Because this epitope exists at ≥2 locations in all apo(a), we used anti–modified Lp(a) monoclonal antibody for both the solid-phase antibody and labeled antibody to improve specificity. The monoclonal antibody was labeled with horseradish peroxidase (Roche) as Immuno Pure Maleimide Activated Horseradish Peroxidase Kit (Pierce Chemical). Polystyrene microtiter plates (Nunc) were coated with anti–modified Lp(a) monoclonal antibody (161E2; 15 μg/mL) or anti-Lp(a) polyclonal antibody (15 μg/mL; Immuno AG) and then distributed into 96-well microwell microtiter plates. After washing, 3,3'-5,5'-tetramethylbenzidine was added to each well. The enzyme reaction was allowed to proceed for 30 minutes at room temperature. The reaction was stopped by stop solution, and the absorbance was read at 450 nm. Lp(a) standard serum (Daiichi Chemical) was used to calibrate the native Lp(a)

Methods

Production of Anti–Modified Lp(a) Monoclonal Antibody

With a peptide synthesizer (model 430A; Applied Biosystems), the peptide was synthesized according to the t-butoxycarbonylamino acid solid-phase method (Figure 1). The degree of peptide conjugation to BSA or KLH was 19 peptides/BSA molecule or 1289 to 3250 peptides/KLH molecule, respectively. BALB/c mice were injected with 50 μg KLH-peptide in Freund’s complete adjuvant. This intraperitoneal injection was followed 2 weeks later with an injection of 15 μg KLH-peptide in Freund’s incomplete adjuvant. Thereafter, the mice were boosted at 2-week intervals with Lp(a) in saline for 3 months. Mice were then administered a final intravenous injection of Lp(a) 3 days before fusion. Fusion was carried out with 1×10⁸ myeloma cells (NS-1) and 1×10⁹ spleen cells. After fusion, cells were resuspended in S clone medium (Sanwa Chemical) supplemented with thymidine (7.6 mg/mL), aminopterin (0.18 mg/mL), and hypoxanthine (13.6 mg/mL) and then distributed into 96-well microtiter plates (Nunc). Two weeks later, the wells of the microtiter plates were coated with 300 ng/100 μL BSA-peptide, and the microtiter plates were tested by ELISA.

Because this epitope is hidden on the native Lp(a) molecule, we successfully developed a new enzyme-linked immunosorbent assay (ELISA) system with this antibody that can distinguish native and modified Lp(a), including oxidized Lp(a). Here, we demonstrate the presence of Lp(a) modified by oxidative stress in human serum and its elevation in patients with hypertension and oxidized Lp(a) in the atherosclerotic lesions of humans.

Figure 1. Position of peptide antigen in apo(a) detected by monoclonal antibody (Arg-Asn-Pro-Asp-Val-Ala-Ala-Pro).

Because this epitope exists at ≥2 locations in all apo(a), we used anti–modified Lp(a) monoclonal antibody for both the solid-phase antibody and labeled antibody to improve specificity. The monoclonal antibody was labeled with horseradish peroxidase (Roche) as Immuno Pure Maleimide Activated Horseradish Peroxidase Kit (Pierce Chemical). Polystyrene microtiter plates (Nunc) were coated with anti–modified Lp(a) monoclonal antibody (161E2; 15 μg/mL) or anti-Lp(a) polyclonal antibody (15 μg/mL; Immuno AG) and then distributed into 96-well microwell microtiter plates. After washing, 3,3'-5,5'-tetramethylbenzidine was added to each well. The enzyme reaction was allowed to proceed for 30 minutes at room temperature. The reaction was stopped by stop solution, and the absorbance was read at 450 nm. Lp(a) standard serum (Daiichi Chemical) was used to calibrate the native Lp(a)
supine. BP measurement was repeated a standardized setting in the clinic in the morning with the patient pressure (BP) was measured with a standard sphygmomanometer in blood was taken during the morning after an overnight fast. Blood or hyperlipidemia were excluded from this study. Antecubital venous patients were divided into a group of 160 patients without complications (WHO I) and a group of 63 patients with complications (WHO II/III). Patients with hepatic disease, lung disease, diabetes mellitus, or anti-mouse Ig HRP-labeled polyclonal antibody (DAKO). polyclonal antibody, anti-goat Ig HRP-labeled polyclonal antibody, anti–modified Lp(a) monoclonal antibody (161E2), anti–modified Lp(a) polyclonal antibody (International Enzyme), anti–modified Lp(a). Lane 1, Reacted with anti-plasminogen polyclonal antibody. Lane 2, Reacted with anti-apoB polyclonal antibody. Lane 3, Reacted with anti-Lp(a) polyclonal antibody. Lane 12, Reacted with anti–modified Lp(a) monoclonal antibody (161E2). ELISA. The assay calibrator was Lp(a) with 17, 27, and 34 kringe 4 repeats. Calculation of the concentration of modified Lp(a) was based on the concentration of BSA-peptide that contributed to 16 peptides per molecule of BSA as the standard.

Measurement of Modified Lp(a) in Human Serum
One hundred thirteen normotensive control subjects (59 men and 54 women, 59±2 years old) and 223 essential hypertensive patients who had never been treated (117 men and 106 women, 61±2 years old) were studied. The normotensive and hypertensive groups were divided into 3 stages of hypertension according to the guidelines of the World Health Organization (WHO)/International Society of Hypertension in 1993. Furthermore, untreated hypertensive patients were divided into a group of 160 patients without complications (WHO I) and a group of 63 patients with complications (WHO II/III). Patients with hepatic disease, lung disease, diabetes mellitus, or hyperlipidemia were excluded from this study. Antecubital venous blood was taken during the morning after an overnight fast. Blood pressure (BP) was measured with a standard sphygmomanometer in a standardized setting in the clinic in the morning with the patient supine. BP measurement was repeated ≥3 times in a blinded fashion.

Immunohistochemical Staining by Anti–Modified Lp(a) Monoclonal Antibody (161E2)
The blood vessels were removed 2 hours after death at the autopsy. Immediately after removal, tissue rings from the aortic arch were fixed in buffered formalin and paraffin embedded, according to standard procedures. Subsequently, immunohistochemical staining was performed with anti-apoB polyclonal antibody (Biorstride), anti-Lp(a) polyclonal antibody (International Enzyme), anti–modified Lp(a) monoclonal antibody (161E2), anti-rabbit Ig HRP-labeled polyclonal antibody, anti-goat Ig HRP-labeled polyclonal antibody, or anti-mouse Ig HRP-labeled polyclonal antibody (DAKO).

Statistical Analysis
All values are expressed as mean±SEM. ANOVA with subsequent Bonferroni’s test was used to determine the significance of differences in multiple comparisons. Multiple regression analyses were used to assess the relation between BP and other parameters. Values of P<0.05 were considered statistically significant.

Results
Characterization of Anti–Modified Lp(a) Monoclonal Antibody (161E2)
We selected a peptide that consisted of 9 residues from kringe 4 type 2 of apo(a) (Arg-Asn-Pro-Asp-Ala-Val-Ala-Ala-Pro) by using prediction methods for the position of antigenic sites in proteins (Figure 1). Hybrids obtained from the fusion between the splenocytes of BALB/c mice immunized with KLH-peptide and the mouse hybridoma cell line P3U1 were screened by solid-phase ELISA against BSA-peptide, plasminogen, and LDL and subcloned several times. To evaluate whether native Lp(a), Lp(a) oxidized with CuCl2, plasminogen, and LDL reacted with the monoclonal antibodies, we performed Western blot analysis. The 2 monoclonal antibodies reacted with oxidized Lp(a) but did not react with native Lp(a), plasminogen, or LDL (Figure 2). Moreover, we tested the effects of various oxidants on the signal detected by anti–modified Lp(a) monoclonal antibody (161E2). Electrophoretic mobility of native and oxidized Lp(a) on agarose gel as a parameter of the different concentrations of CuCl2 or lipoxygenase is shown in Figures 3 and 4. After the treatment of Lp(a) with either CuCl2 or lipoxygenase, a change in mobility of Lp(a) could be detected in agarose gel electrophoresis. In addition, the treatment with CuCl2 at concentrations of >0.5 μg/mL induced a change in mobility detected with agarose gel electrophoresis, accompanied by an increase in TBARS (lipid peroxide) level (Figure 3).

Development of Oxidized Lp(a) ELISA Method
Differences in the signals of the modified Lp(a) ELISA method between native Lp(a) and modified Lp(a) were investigated after the subjection of purified Lp(a) to various treatments for modification, including glycation, freeze/thaw cycles, and 6-day storage at 37°C or room temperature. Glycation of Lp(a) was confirmed in the experiments, on the basis of a change of mobility in agarose gel electrophoresis.
No differences in the parameters were observed between native Lp(a) and Lp(a) subjected to various treatments for modification. Only oxidized Lp(a) could be detected with the 161E2 monoclonal antibody (Figure 5).

Therefore, we developed a new ELISA to detect oxidized Lp(a) by using antioxidized Lp(a) monoclonal antibody (161E2) as the capture antibody and the detection antibody. To increase the precision and the comparability of the oxidized Lp(a) ELISA method, we designed it for use with a relatively low dilution of plasma (1:100) and calibrator (1:100). A standard curve obtained with BSA-peptide is shown in Figure 6. The working range of the assay was between 0 and 0.345 nmol/L BSA-peptide, and the assay was linear within this range. The intra-assay and interassay coefficients of variation for mean values of triplicate determinations (n=10) of high-level oxidized Lp(a) serum (0.115 nmol/L BSA-peptide) were 1.2% and 5.0%, respectively; those for low-level oxidized Lp(a) serum (0.035 nmol/L BSA-peptide) were 4.5% and 7.2%, respectively. Figure 7 shows the results of our investigation of whether the ELISA serves as a quantitative assay for purified oxidized Lp(a) with the different phenotypes. Using serial dilutions of oxidized Lp(a) with the phenotypes of 27, 29, and 34 kringle 4 repeats, we found the linearity of the response curve constructed with the oxidized Lp(a) ELISA method. Levels of each oxidized Lp(a) (BSA-peptide) were 0.23, 0.2, and 0.18 nmol/L. These data suggest that the assay with the 161E2 antibody is quantitative for oxidized Lp(a) independent of the phenotypes.

Serum Native Lp(a) and Oxidized Lp(a) Concentrations in Normotensive Subjects and Untreated Hypertensive Patients

Using the new ELISA for oxidized Lp(a), we measured native and oxidized Lp(a) concentrations in normotensive subjects and untreated hypertensive patients, because hypertension is well known to stimulate oxidative stress.22,23 Oxidized Lp(a) concentration in untreated hypertensive patients was significantly associated with systolic, but not diastolic, BP (P<0.05, r=0.13). In contrast, there was no significant association between BP and serum Lp(a) concentration. To further analyze the relationship between serum oxidized Lp(a) concentration and BP, we evaluated the effect of hypertensive complications on serum native Lp(a) and oxidized Lp(a) concentrations in hypertensive subjects, according to WHO guidelines. There was no apparent difference in serum native Lp(a) concentration among the normotensives, hypertensives without complications, and hypertensives with complications. Moreover, the oxidized Lp(a) concentration in hypertensive patients with complications (WHO II/III) was significantly higher than that in those without complications (WHO I) (Figure 8, P<0.01), whereas serum oxidized Lp(a) concentrations in hypertensive patients without complications showed a tendency to be higher than that in normotensive subjects. In normotensive subjects, oxidized Lp(a) level did not correlate with serum concentration of native Lp(a).

Immunocytochemical Findings

Finally, we examined the presence of oxidized Lp(a) in the vessel wall, because Lp(a) has been reported to easily

Figure 4. Right, Agarose gel electrophoresis of Lp(a) oxidized by lipoxygenase; staining with fat red 7B with agarose gel electrophoresis. Lane 1, native Lp(a). Lanes 2 to 5, Lipoxygenase (1.0, 3.0, 5.0, and 20.0 μg/mL). Left, ELISA of oxidized Lp(a) induced by lipoxygenase as antigen.

Figure 5. Western blotting of modified Lp(a) by 161E2 monoclonal antibody and anti-Lp(a) polyclonal antibody. A and F, Oxidized Lp(a). B and G, Lp(a) glycated with 200 mmol/L glucose at 37°C for 6 days. C and H, Lp(a) after freeze/thaw cycle repeated 10 times. D and I, Lp(a) after freeze/thaw cycle repeated 30 times. E and J, Lp(a) after 6 days of storage at room temperature. A to E, By anti-161E2 monoclonal antibody. F to J, By anti-Lp(a) polyclonal antibody.

Figure 6. Standard curve of ELISA using 161E2 monoclonal antibody for oxidized Lp(a). Dose-response curve was obtained with BSA-peptide (19 peptides per molecule of BSA).

Figure 7. Typical dose-response calibrator curves generated by plotting dilution rate versus absorbance (Abs) at 450 nm. Oxidized Lp(a) of 27, 29, and 34 kringle 4 repeats was diluted with a serum without Lp(a). Assay calibrator was BSA-peptide with 19 peptides. Oxidized Lp(a) levels (BSA-peptide) were 0.23, 0.2, and 0.18 nmol/L, respectively.
penetrate the vessel wall and to accumulate within the atherosclerotic lesion. Similar to previous reports, apoB and native Lp(a) showed a similar distribution in the cap region and around the necrotic core of the elevated intimal lesion. On the other hand, oxidized Lp(a) was highly localized in the cap region, which was superimposed by native Lp(a) and apoB accumulation (Figure 9).

Discussion
Recently, oxidized Lp(a) has been reported to have additional specific biological properties compared with native Lp(a). Because the biological effects of oxidized Lp(a) are more potent compared with native Lp(a), it is noteworthy to investigate the role of oxidized Lp(a) in the pathogenesis of atherosclerosis. However, the lack of a suitable antibody that reacts with oxidized Lp(a) limited the investigation of oxidized Lp(a). Fortunately, we obtained a new monoclonal antibody to react with oxidized Lp(a) but not native Lp(a). Here, we report a newly developed ELISA to detect modified Lp(a), especially oxidized Lp(a). The profile of this monoclonal antibody (161E2) against modified Lp(a) was characterized by the following features: (1) The epitope detected by the 161E2 monoclonal antibody recognized a part of the apo(a) molecule. Consequently, the 161E2 monoclonal antibody recognized a part of the apo(a) molecule. (2) It is possible to quantify oxidized Lp(a) with the peptide used as the standard, because the recognized epitope is well defined due to the antibody against the known synthetic peptide. It allows extremely good stability and large-scale preparation for ELISA. Because 161E2 antibody reacted with an epitope on kringle IV type 2, it seems to lead to a difficult problem in distinguishing between the number of modified kringle 4 repeats versus the number of Lp(a) particles. Although the former possibility is unlikely due to the linearity with dilution of Lp(a) containing different single isoforms, further studies must be necessary to define the effects of kringle size. Serial dilutions of each of these serums containing a single isoform gave a linear range of presumed concentrations. In addition, the kringle 4 repeats did not provide a large difference in the value of serum Lp(a) concentration measured by antibody against kringle 4. For example, even if their kringle repeats increase from 19 to 38, the value of Lp(a) would not be increased to 19-fold or even to 2-fold. Antibody would probably not be able to bind to all of epitopes in the kringle 4 due to the 3-dimensional structure of the protein.

During the development of atherosclerotic lesions, by-pass vein grafts, and aortic aneurysms, Lp(a) accumulates within fibrin clots attached to blood vessel walls. Because Lp(a) accumulates within the fibrin clot with time, fatty streaks are formed that develop into occlusive atherosclerotic

![Figure 8.](image)

Figure 8. Serum concentration of oxidized Lp(a) according to WHO stage in normotensive and hypertensive subjects. Nor indicates normotensive subjects (n=113); HT, hypertensive subjects; WHO I, hypertensive subjects with WHO stage I (n=223); WHO II/III, hypertensive subjects with WHO stage II/III (n=63). *P<0.01 vs normotensive. #P<0.01 vs WHO I.

![Figure 9.](image)

Figure 9. Immunohistochemical staining of atherosclerotic lesion of coronary artery with (a) anti-apoB polyclonal antibody, (b) anti-native Lp(a) polyclonal antibody, and (c) anti-oxidized Lp(a) monoclonal antibody (161E2 antibody). Oxidized Lp(a) was highly localized in deep area and subendothelial zone of cap region with superimposition of native Lp(a) and apoB, although the staining varied considerably. Coronary artery was obtained from a 73-year-old man at autopsy.
plaques. The accumulation of Lp(a) within the blood vessel is believed to promote an antifibrinolytic environment, foam cell formation, the generation of a fatty streak, and an increase in smooth muscle cells. Importantly, immunohistochemical staining with 161E2 antibody could detect a positive signal in the human atherosclerotic vessel. Because oxidized Lp(a) is a potent stimulus of monocyte adhesion to endothelial cells, the presence of oxidized Lp(a) might promote atherogenic changes in the human blood vessel. The present results failed to demonstrate which cells can be stained with this antibody. Because previous reports revealed that oxidative modification converted Lp(a) particles to a form readily recognized by macrophage scavenger receptors, the positive cells might be macrophages that had taken up oxidized Lp(a) via scavenger receptors. In addition, oxidized Lp(a) caused more pronounced stimulation of superoxide production, whereas native Lp(a) itself caused a moderate, dose-dependent stimulation of superoxide production. Accumulation of native Lp(a) may enhance the stimulation of oxidized Lp(a), a more potent atherogenic lipoprotein, in the vessel wall.

We developed a new monoclonal antibody (161E2) against the epitope in Lp(a) that was revealed externally as a result of oxidative treatment but not native Lp(a). The present data demonstrated the in vivo presence of oxidized Lp(a) in the atherosclerotic human blood vessel and its elevation in hypertensive patients compared with normotensive subjects. The presence of oxidized Lp(a) may be important in understanding the role of Lp(a) in cardiovascular disease.

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