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Development of new enzyme-linked immunosorbent assay for oxidized lipoprotein(a) by using purified human oxidized lipoprotein(a) autoantibodies as capture antibody

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Abstract

Background: Oxidized Lp(a) [ox-Lp(a)] has been reported to play more potent role than native Lp(a) in atherosclerosis. Ox-Lp(a), autoantibodies, and Lp(a) immune complexes have all been detected *in vivo*. Thus, the isolation of its autoantibodies and the investigation of ox-Lp(a) may provide a new means to explore the exact pathogenic role of ox-Lp(a). We isolated and identified human autoantibodies against ox-Lp(a) and developed a new ELISA for ox-Lp(a) by using autoantibodies as capture antibody.
Methods: Ox-Lp(a) autoantibodies were isolated and identified from healthy subjects by affinity chromatography. 2 "sandwich" ELISAs were developed for measuring ox-Lp(a) level, using autoantibodies against ox-Lp(a) or rabbit antiserum against ox-LDL as the capture antibody and quantitating with monoclonal anti-apo(a) enzyme conjugate, respectively. Ox-Lp(a) levels were studied by both the ELISAs in 100 patients with coronary heart disease (CHD) and 100 control subjects.
Results: The isolated ox-Lp(a) autoantibodies reacted with both apo(a) and apoB epitopes of Ox-Lp(a). Compared to control, plasma ox-Lp(a) levels in patients with CHD were significantly increased (ELISA using human autoantibodies: 24.3±33.4 vs. 8.4±9.3 μg/ml, $P<0.0001$; ELISA using antibodies against ox-LDL: 13.0±13.8 vs. 7.3±9.7 μg/ml, $P<0.0001$, respectively). Furthermore, a significantly positive relationship between ox-Lp(a) levels detected by 2 ELISAs was also found ($R=0.78$, $P<0.0001$).
Conclusion: We isolated human autoantibodies against ox-Lp(a), which can recognize both apo(a) and apoB epitopes of ox-Lp(a). The developed ELISA for ox-Lp(a) by using human auoantibodies may more accurately reflect the state of Lp(a) oxidation *in vivo*. Ox-Lp(a) levels increase in patients with CHD.

Keywords: Lipoprotein(a); Oxidative modification; Autoantibody; ELISA; Atherosclerosis

1. Introduction

Lipoprotein(a) [Lp(a)] is a plasma lipoprotein whose structure and composition closely resembles that of low density lipoprotein (LDL), but with an additional molecule of apolipoprotein(a) [apo(a)], a large glycoprotein, linked to apoB by a disulfide bond [1,2]. High Lp(a) levels have been established as an independent risk factor for atherocslerosis [3–5]. Oxidized Lp(a) [ox-Lp(a)] has been reported to play more potent role than native Lp(a) in atherosclerosis. Oxidized Lp(a) is a ligand for scavenger receptors [6,7] and might reasonably be expected to contribute to foam cell formation. Oxidized Lp (a) may also induce adhesion molecular expression on monocytes, promoting their recruitment and adhesion to the endothelium [8], and modified forms of Lp(a), some resembling oxidized Lp(a), have been identified in human atheromatous lesions [9]. In addition, *in vitro* oxidative modification increases the inhibitory effect of Lp(a) on plasminogen binding to cell surfaces, which could attenuate fibrinolytic activity by reducing plasminogen activation [10].

Abbreviations: Lp(a), Lipoprotein(a); LDL, low density lipoprotein; apo, apolipoprotein; ox, oxidized; MDA, malondialdehyde; CHD, coronary heart disease; ELISA, enzyme-linked immunosorbent assays; HDL, high density lipoprotein.

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Some studies reported the existence of oxidized Lp(a) [11,12] and antibodies against malondialdehyde (MDA) modified Lp(a) *in vivo* [13], and subsequently forming Lp(a) immune complexes [14,15]. We found Lp(a) immune complexes present in human plasma and significantly increase in the patients with coronary heart disease (CHD) [14]. It is obvious that Lp(a) might also trigger an immune response and play the role in the development of atherosclerosis. Theoretically, apoB and apo(a) proteins of Lp (a) molecules can both be oxidatively modified *in vivo*. Thus isolation of anti-ox-Lp(a) antibodies was of the utmost importance, not only to confirm their presence in plasma found to be positive in screening assays but also to allow their adequate characterization. Until now, only 2 enzyme-linked immunosorbent assays (ELISA) for circulating ox-Lp(a) have been developed, in which ox-Lp(a) levels were estimated by the degree of oxidized apo(a) or apoB protein of Lp(a) [11,12]. Oxidized phospholipid has also been found preferentially associated with Lp(a) [16–20]. However, neither of the above methods can simultaneously detect apo(a) and apoB epitopes of ox-Lp(a).

So we isolated and identified ox-Lp(a) autoantibodies from healthy subjects by affinity chromatography. Furthermore, we developed 2 "sandwich" ELISAs for measuring plasma ox-Lp(a) level, using isolated autoantibodies against ox-Lp(a) or rabbit antiserum against ox-LDL as the capture antibody and quantitating with monoclonal anti-apo(a) enzyme conjugate, respectively. The ox-Lp(a) levels were also studied by both the ELISAs in patients with CHD and control subjects.

2. Materials and methods

2.1. Study subjects

Autoantibodies against ox-Lp(a) were isolated from healthy human subjects. One hundred male myocardial infarction patients with an average age of 58.4± 5.2 y were drawn from a hospitalized population and were identified by typical signs, electrocardiograph and laboratory tests. The patients were studied more than 6 months after their recovery from acute attacks. Control group consisted of 100 male healthy volunteers with a mean age of 54.9±6.2 y, and were selected from routine healthy examination, physical and eletrocardiography and laboratory tests without dylipidemia, hypertension, diabetes mellitus, or any clinical evident sign of atherosclerosis. The blood samples were taken after at least 12 h of fasting and collected into EDTA (1 mg/ml)-containing tubes, and plasma were separated immediately and stored in −70°C until analysis. All the samples were measured within 1 y. The study was approved by the Ethics Committee of Jinling Hospital after all the subjects had given their informed consent their consent.

2.2. Isolation of Lp(a), LDL and plasminogen

Lp(a) was purified from plasma of healthy donors by sequential density ultracentrifugation, followed by a gel-filtration chromatography as described by Albers et al. [21]. LDL ($1.030 < d < 1.050$ g/ml) was obtained from plasma of Lp (a)-negative healthy donors after ultracentrifugation. The Lp(a) and LDL samples were checked for purity by agarose and polyacrylamide gradient gel electrophoresis. Plasminogen was also prepared from the human serum protein fraction ($d > 1.21$) [22].

2.3. Lp(a) and LDL oxidation

Lp(a) or LDL was diluted in phosphate-buffered saline (PBS) to a protein concentration of 0.5 mg/ml and incubated with 30 μmol/l $CuSO_4$ for 12 h at 37 °C, followed by extensive dialysis against 0.01 mol/l PBS with 0.1 mmol/l EDTA, pH 7.4, respectively [10]. The degree of oxidation was quantified by relative electrophoretic mobility (REM) and the generation of thiobarbituric acid-reactive substances (TBARS). MDA modification of Lp(a) was performed according to Haberland et al. [23] by incubating equal volumes of freshly isolated Lp(a) and 0.2 mol/l MDA for 3 h at 37 °C, followed by extensive dialysis against 0.01 mol/l PBS with 0.1 mmol/l EDTA, pH 7.4. The degree of modification was quantified by REM and the formation of conjugated dienes determined by spectrophotometry at 234 nm.

2.4. Isolation of the autoantibodies

The antibodies to ox-Lp(a) were isolated by an affinity chromatography protocol developed by us. 20 mg of ox-Lp(a) was firstly coupled to 4 g of CNBr-Sepharose 4B (Pharmacia Biotech). The coupling was allowed to proceed for 18 h at 4 °C, with the tube containing the gel slurry placed on a rocking platform. Then the remaining residual active groups were blocked with 0.2 mol/l glycine, pH 8.0, for 2 h at room temperature. After blocking, the slurry was transferred to a chromatography column. The excess of uncoupled ligand was removed by three cycles of washing with $NaHCO_3$ buffer (0.1 mol/l, pH 8.3) and acetate buffer (0.5 mol/l, pH 4) both containing 0.5 mol/l NaCl, and then equilibrated with 0.01 mol/l $NaHCO_3$ buffer, pH 8.3. To isolate ox-Lp(a) antibodies, 10 ml of plasma from 8 healthy subjects selected randomly was allowed to diffuse into the column, and the plasma-loaded column was incubated overnight at 4 °C. Unbound proteins were washed off with the equilibrating buffer and the bound fractions were eluted with 3 mol/l KCNS, pH 6.1. Finally, the eluted antibodies were collected and equilibrated with $NaHCO_3$ buffer.

2.5. Specificity of purified ox-Lp(a) antibodies

To determine the specificity of purified ox-Lp(a) antibodies, a modification of ELISA for antibodies against ox-Lp(a) was used. The reactivity of eluted autoantibodies with immobilized ox-Lp(a) (10 μg/ml) was tested using microtitre plates, in which a series of aliquots containing purified antibody were tested. Three aliquots of 300 μl antibody with identical concentration (0.12 mg/ml) were studied for each purified antibody. They were absorbed with 30 μl ox-Lp(a), ox-LDL, and PBS (all at concentrations of 1 mg/ml, except PBS), respectively. The samples were incubated overnight at 4 °C and then centrifuged at 5000 rpm for 10 min. Any visible precipitate and the bottom 100 μl layer of each tube were discarded. The supernatant was tested for each aliquot. The results were expressed as the percent in reduction of reactivity with ox-Lp(a) after absorption, determined by subtracting the OD of the absorbed aliquots from the OD of the unabsorbed aliquot (absorbed by PBS). The degree of reduction of reactivity with ox-Lp(a) was considered a direct indication of the reactivity of the purified antibody with each of the lipoprotein preparations used for absorption.

2.6. Production of rabbit antibodies against ox-LDL

Antibodies to ox-LDL were obtained by immunization of New Zealand White female rabbits with ox-LDL as described by Lopes-Virella et al. [24]. The resulting rabbit antiserum was first fractionated by affinity chromatography with immobilized protein G, and the IgG fractions were absorbed in a column of immobilized native LDL. The washout from the column of immobilized native LDL, containing antibodies to ox-LDL and irrelevant IgG, was used to capture ox-LDL.

2.7. Production of monoclonal antibodies

Monoclonal antibodies against apo(a) were made in our laboratory [14]. Briefly, BALB/c mice were each given an intrasplenic injection of 100 μg of Lp (a) in Freund's complete adjuvant. The same dose was repeated 2 weeks later in Freund's incomplete adjuvant. The mouse with the highest antibody titer to Lp (a) as determined by ELISA was boosted with intravenous injections of 50 μg of Lp(a) without any adjuvant for three consecutive days before the fusion. After a