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Original Research Article

Lipoprotein (a) particles characterization by Dynamic Light Scattering

Abstract

Lp(a) is a novel cardiovascular risk factor resembling an LDL particle. It includes a copy of apolipoprotein (a) [apo(a)], whose molecular weight is dependent on the number of genetically encoded kringle IV type 2 (KIV-2) repeats and inversely related with Lp(a) plasma concentration and risk. The reason for this inverse relationship is unknown and, particularly, there are no data regarding the size of Lp(a) particles carrying apo(a) with different molecular weights. The aim of the present work was to explore if a relationship existed between apo(a) molecular weight and particles size in Lp(a) samples carrying 20, 25 and 28 KIV-2 repeats (K20, K25 and K28, respectively). Dynamic Light Scattering (DLS) measurements were performed on affinity-purified Lp(a). A preliminary finding was that particles were typically distributed into three different size groups instead of the single one expected. No difference in average particle size between Lp(a) carrying different apo(a) isoforms was found. However, the percentage of medium-sized particles in each sample was found to be inversely related to the number of KIV-2 repeats (R^2=0.99), with a clear predominance in K20 (58.53%). These data deserve further investigations, as they might be potentially relevant to explain the pathogenic role of low molecular weight Lp(a) isoforms. **(199 words)**

Keywords

Lipoprotein (a), particle size, atherosclerosis, plasma, Zeta-sizer.

Introduction

Lipoprotein(a) [Lp(a)] is a plasma LDL-like particle [1] and it is an independent risk factor for atherosclerotic diseases because of its concentration-dependent pro-atherogenic, prothrombotic and antifibrinolytic properties [2, 3]. Different epidemiological studies have suggested that Lp(a) could increase the risk of cardiovascular disease and ischemic stroke especially if associated with other predisposing factors such as hypercholesterolemia, hypertension, diabetes mellitus and low HDL level [2].

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Lp(a) is present in the arterial wall of atherosclerotic lesions and its accumulation involves recruitment of macrophages [4, 5]. These cells incorporate the lipid component and are transformed into foam cells. In this environment, macrophages also release cytokines and growth factors that lead to the proliferation and migration of vascular smooth muscle cells from the intima-media and contribute to plaque formation. Lp(a) also has a direct effect on fibrinolytic factors, in particular, it stimulates the expression of PAI-1 in endothelial cells and it has been described as directly inhibiting plasmin by complexing and inactivating tissue-type plasminogen activator (tPA) [6].

Lp(a) composition is similar to that of LDL in terms of cholesterol, triglycerides, phospholipids, and apoB100. The unique and distinctive component of Lp(a) is the apolipoprotein(a) [apo(a)] glycoprotein, a member of the plasminogen gene family, with a strong structural homology with plasminogen [7]. Apo(a) is disulfide linked to the apoB100 of the LDL-like particle. It is known to be a very heterogeneous glycoprotein including domains referred to as kringle IV, kringle V, and the protease domain [8]. The apo(a) kringle IV domains can be classified into 10 types (KIV_1-KIV_{10}) on the basis of amino acid sequence [9]. Kringle IV type 2 is present in a variable number of copies (from 3 to 48), which generates Lp(a) isoform size heterogeneity (more than 25 described) in humans [10-12], with the low MW species related to high plama concentration and vice versa [13].

The size of the main lipoprotein classes is well defined [14], but recent evidence suggests that further classifications in class subtypes according to size can be relevant to determine the related risk [15]. This is probably related to the fact that different particle sizes correspond to a different lipid composition and also to a different capacity to overcome the endothelium and to be internalized and metabolized by target cells.

In the present study, we wanted to verify if affinity purification of plasma-derived Lp(a) combined with Dynamic Light Scattering (DLS) can represent a useful method to investigate the relationship between the number of KIV_2 carried by each apo(a) isoform and the size of affinity purified Lp(a) particles obtained from homozygous individuals.

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Materials and methods

Molecular weight determination of Lp(a) in patients sera

The apo(a) phenotype was analyzed with high-resolution phenotyping with sodium dodecyl sulfate agarose gel electrophoresis (SDS-agarose) under reducing conditions as outlined previously [13] with slight modifications.

Briefly, 15 µl of EDTA-plasma samples were pretreated with 30 µl of a reducing solution. The submarine electrophoretic run was performed on 1% SDS-agarose SDS-agarose gel and electrophoresis was carried out in tank buffer for 14 h at 80 V and 0.04 A. Reduced samples (20 µl) were applied in wells, at 3 cm from the cathode of the gel. The separated proteins were transferred onto a nitrocellulose membrane (Bio-Rad, Segrate, Italy) by a capillary blotting technique and tested with a polyclonal anti-human Lp(a) antiserum from rabbit (DAKO, Glostrup, Denmark) diluted 1:500 in 1% BSA overnight,. A peroxidase-conjugated goat anti-rabbit immunoglobulin (DAKO, Glostrup, Denmark, 1:1000 in TBS) was used as a secondary antibody. The membrane was developed with 50 ml of TBS, 500 µl of hydrogen peroxide, 30 mg of 4-chloro-1-naphthol (4CN) dissolved in 10 ml of cold methanol for 15 min. The reaction was then stopped washing with water. Relative band mobility was determined as referred to the mobility of apo(a) standard isoforms included in each blot (values: 35, 27, 23, 19, and 14 KIV-2 repeats; Immuno AG, Wien, Austria). Thus the estimated number of K-IV repeats in each sample was calculated.

Determination of Lp(a) concentration

Lp(a) concentration was determined with Macra Lp(a) (Trinity Biotech), a sandwich ELISA-based assay to specifically recognise the apo(a) moiety. The wells of a microtiter plate were coated with the monoclonal antibody to capture Lp(a) at RT during a 1 h incubation. After washing the wells with the supplied washing solution, the polyclonal anti-Lp(a) horseradish peroxidase (HRP) conjugate was added. After 20 min, the plate was washed and incubated with a substrate for HRP (hydrogen peroxide) and a chromogen (o-phenylenediamine). After 20 min the reaction was stopped with

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sulfuric acid and read at 492 nm. The concentration of Lp(a) was quantitatively determined by comparison with a standard curve prepared with Lp(a) standard samples of known concentrations.

Lp(a) purification

Activated Sepharose 4 CNBr (Amersham Biosciences) was resuspended and washed three times in 1 mM HCl pH 3.0, put on the end-over-end mixer for 15 min, and then coupled with 5 mg/ml rabbit anti-human lipoprotein(a) polyclonal antibody (DAKO) dissolved in Coupling Buffer (0.1 M $NaHCO_3$ pH 8.3, 0.5 M NaCl) at RT for 2 h. After centrifugation, the remaining active groups of the resin were blocked with 0.2 M glycine pH 8.0 on an end-over-end mixer at RT for 2 h. The resin was finally washed with three cycles of alternating pHs. In order to purify Lp(a), 25-50 ml of the selected plasma samples were incubated with the anti-Lp(a) affinity matrix, previously equilibrated in PBS, 10 mM NaN_3, in an end-over-end mixer at 4°C overnight. The gel was loaded into a column and washed with PBS made up to 0.35 M NaCl, pH 7.0. Lp(a) was eluted with 100 mM glycine pH 3.0 and 2 ml fractions were collected and supplied with 200 µl of 1.0 M Tris pH 8.0 to neutralise the pH, pooled, concentrated, and then dialysed against refolding buffer (30 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM DTE, 1.3 g/l ε-aminocaproic acid, 200 mM PMSF, 0.5 g/l sodium azide, Fluka) or PBS. Purified Lp(a) preparations were checked by Western blot analysis and Lp(a) concentration determined by Macra Lp(a) ELISA.

Size measurement

Lp(a) samples were concentrated to 1 mg/ml concentration. Measurement of particle size was performed by Malvern Zeta Sizer ZS90 using default settings. Buffer was used as a negative control. Measurements were repeated 3 times for each sample.

Statistical analysis

The experiments were performed twice. Statistical analysis was performed by ANOVA and Tukey post-hoc analysis using the VassarStats server (http://vassarstats.net/).

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Results and discussion

Apo(a) molecular weight determination

Among the thirteen plasmas tested in western blotting, four homozygous samples (single band phenotypes) with the following number of kringles were selected for further analysis: 20 (K20, two samples), 25, 28 (K25, K28, one sample each). This prevalence of single band phenotypes (30.8%) is not too far from that present in white Americans (23.9%), while the expected prevalence of the specific phenotypes here detected is 1.51%, 1.36% and 1.9% in the same population [16].

Affinity purification of Lp(a)

Most of the described purification procedures to isolate Lp(a) include a gradient centrifugation step. This introduces a selection bias based on particle density which could reduce the real complexity of the sample. In contrast, particles purified through a single affinity chromatography step should resemble all the plasma elements that can bind anti-Lp(a) antibodies. Lp(a) concentration in starting plasma samples was comprised between 40 and 140 μg/ml and purification yields were 3-5 μg/ml purified plasma (3.5-7.5%). Two independent purifications were repeated for each sample and sample integrity confirmed by western blotting.

Lp(a) size

DLS allows a label-free measurement of particle size. Zeta-sizer measurements were performed on Lp(a) dialysed versus refolding buffer or PBS, in order to assure the best stability of the sample, in the first case, and more physiological conditions, in the second case. Very similar results were found in both cases and the data obtained with refolding buffer are here presented and discussed.

Unexpectedly, a composite profile including 3 peaks of different size was typically found, as summarized in Table 1. Small particles diameter ranged between 11.92 and 14.02 nm; medium-sized particles between 45.70 and 56.92 nm and large particles instead between 398.77 and 502.89 nm. A size for Lp(a) particles of 28.3±0.5 nm and 25 nm has been previously determined by non-denaturing native gel electrophoresis ([17] and [14], respectively), while a smaller diameter can be deduced from

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the experiments by Fless et al. [18] (ca 20 nm). Interestingly, none of the sizes we determined for small, medium and large particles perfectly fits with these reported data, the closest values being those of the medium particle series (45.70-56.92 nm). The reason for this discrepancy lays on two factors. One is the fact that DLS measures the *hydrodynamic* diameter of particles, which includes both the molecule and the adherent solvent layer. The other is that apo(a), which is a very hydrophilic molecule, is often described as floating from the Lp(a) particle like a sort of tail, thus turning its approximate shape from that of an ideal sphere into that of an elliptical object. In DLS, the direct observations are the intensity fluctuations due to the diffusion of the particles, and this diffusion coefficient, interpreted as a hydrodynamic size using the Stokes Einstein equation, is compared to that of a hypothetical sphere moving with the same diffusion coefficient. Particularly, the direct intensity size distribution may inherently be weighted to larger sizes, due the fact that the scattering intensity is proportional to size^6. Under ideal conditions, the number distribution transformation of the DLS result should be very close to other measurements techniques, but, for polydisperse samples like ours, this can be more difficult to achieve. It is worth noticing that the hydrodynamic diameter of a particle is relevant in biological assays and in *in vitro* migration.

Why did we observe more than the single expected peak? We can hypothesise that in our case we have either identified three types of plasma particles which bind to anti-apo(a) antibodies, or that the smaller and the larger particle peaks could be artifacts due to the purification procedure.

Regarding artifacts, the small particles could derive from Lp(a) degradation, while large particles could represent aggregates due to the high Lp(a) concentration needed for DLS measurements. Stabilization of purified Lp(a) in solution, in fact, is not trivial, especially at high concentration and, in our experience, precipitation of purified Lp(a) in PBS can start occurring already at 80 µg/ml, a concentration 12.5x lower than the one required for DLS determinations.

On the other hand, the affinity chromatography column we built allowed the capture of Lp(a) to occur through its apo(a) moiety, while other methods typically used for Lp(a) purification, one of the most used being ultracentrifugation, is based on Lp(a) density. We can therefore also hypothesise that the heterogeneity we detected could be a relevant physiological feature thus far undetectable with non-DLS techniques based on material derived from non-affinity based purification methods. Because of

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the relevance microvesicles (100-1000 nm in size) are acquiring in biology [19], this issue requires further investigations. Indeed, apo(a) has been detected in plasma microvesicles [20] and LDL particles have been shown to co-purify with microvesicles [21]. Both hypothesis (aggregation and microvescicles) can explain the high SDs of large particles measurements (Table 1).

Table 1 Particle size (nm, average ± SD, n≥4)

	K20	**K25**	**K28**
Small	11.92±2.81	14.02±1.67	12.84±2.29
Medium	47.06±12.52	56.92±9.65	45.70±9.08
Large	502.89±295.62	398.77±107.89	413.66±114.32

Comparing the size of small, medium and large particles in different apo(a) isoforms (Table 1), no difference was detected (P=.40, P=.35, P=.62) and changing the buffer to PBS did not change these results. We can therefore exclude a correlation between apo(a) isoform and particles size, which was the original aim of the present work.

Along with size, we also measured a second parameter provided by the Zeta-sizer, peak intensity (Fig. 1), expressed as a percentage of total signal and hence an indicator of the relative abundance of a particle species.

Within small particles, the relative peak intensity was higher in K28 compared to K20 and K25 (P=.0019 and P=.0083, respectively). No difference was detected in medium sized lipoproteins (P=.17), while, within large particles, a difference was detected (P=.02), due to a higher proportion measured in K28 compared to K20 (P=.025). This would suggest a different relative distribution of particle sizes inbetween the different purified Lp(a) isoforms, with a prevalence of small and large particles in K28 compared to K20 and K25.

Significant differences were also observed in relative peak intensities (Fig. 1) inside each isoform for K20 and K25 (Fig. 1, P=.000 for K20, P=.000 for K25), but not for K28, where the three species seemed to occur in roughly the same percentage (P=.21). Particularly, in both K20 and K25 the most

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represented particle size was the medium-sized one (58% versus 14% and 22% of small and large particles, respectively, for K20 and 46% versus 16% and 33%, respectively, for K25).

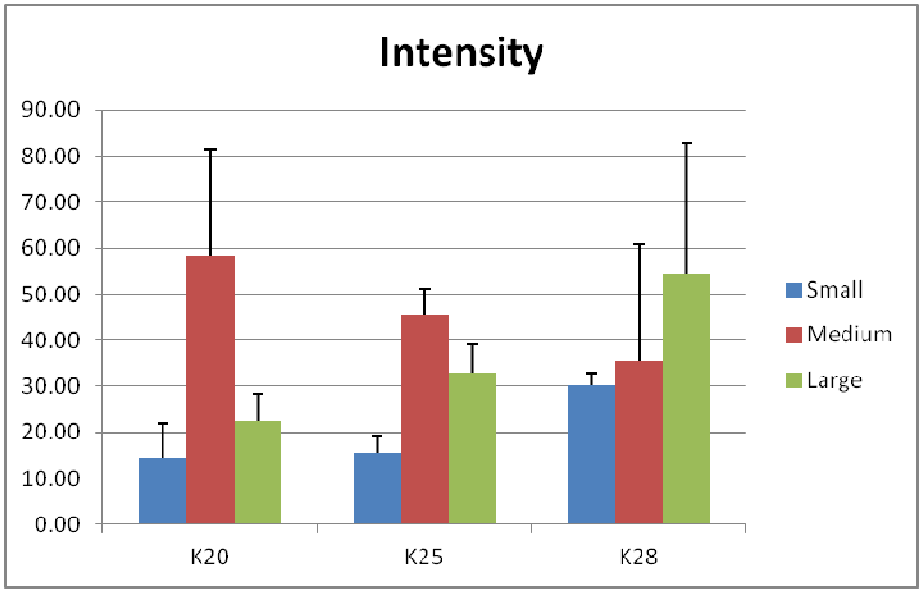

Fig. 1 Relative peak intensities of Lp(a) particles.

Interestingly, normalizing the intensity of medium and large particles by the one of small particles, the ratio among large ones is constant in the three apo(a) types, while medium particles greatly prevail in K20 and K25 samples compared with K28 (4 and 2.5 times, respectively, Fig. 2). Indeed, the inverse relationship between KIV-2 repeats number and percentage of medium particles has an R^2 of 0.99, and the direct relationship between KIV-2 repeats number and percentage of large particles has an R^2 of 0.88. R^2 becomes only 0.68 if small particles are considered.

A difference in relative particle composition as measured by DLS could depend on the physico-chemical features of Lp(a) carrying different apo(a) isoforms, leading, for example, to a lower tendency of small isoforms towards aggregation. In fact, it has been shown that, in patients, an LDL pattern that has more small dense LDL particles, called Pattern B (19.0–20.5 nm), equates to a higher risk factor for coronary heart disease than does a pattern with more of the larger and less-dense LDL particles (Pattern A, 20.6–22 nm) [22]. This is thought to be because the smaller particles are more easily able to penetrate the endothelium, whose normal gaps are 26 nm in diameter [22]. This process could also be extended to Lp(a), suggesting a higher pathogenic potential for small Lp(a) isoforms.

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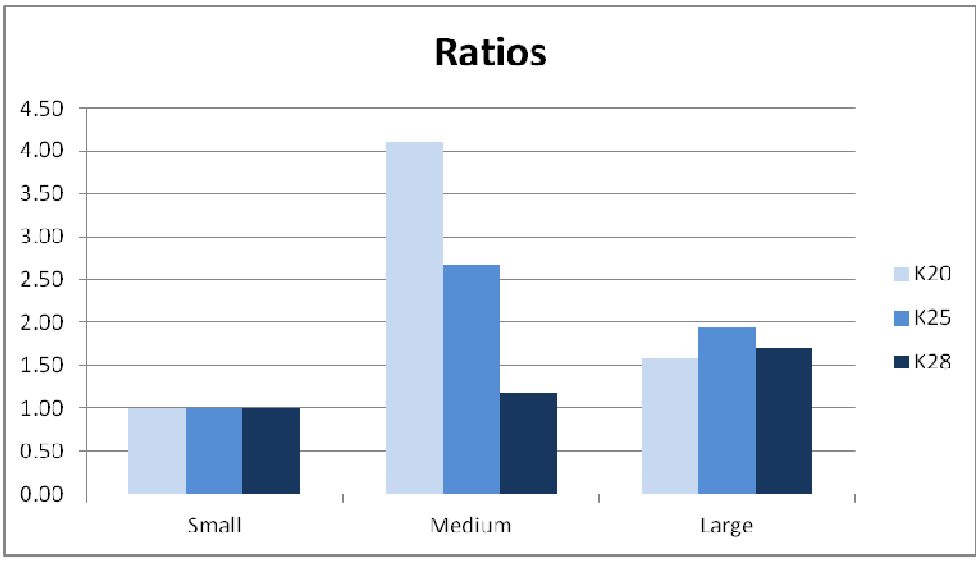

Fig. 2. Relative intensiy peaks normalized versus small particles.

It is worthwhile wondering why aggregation, if large particles indeed correspond to aggregates, is observed in purified Lp(a) at the concentration suitable for DLS measurements (1 mg/ml), considering the fact that it can be reached even *in vivo*, but this aspect has never been investigated so far according to our knowledge.

In summary, a difference in size was not detected in affinity-purified Lp(a) carrying apo(a) with different isoforms, but two serendipitous observations were reported. The first regards the co-existence of three different species with different hydrodynamic diameters in Lp(a) samples purified by affinity chromatography. The second regards the different relative abundance of these species in Lp(a) samples with different apo(a) isoforms. Particularly, a higher prevalence of medium particles, the most compatible in size with the thus-far described Lp(a), was measured in K20 than in K25 and K28 samples. More effort is needed to characterize the role of Lp(a) as a particle in atherogenesis, and our data show for the first time that DLS analysis could be relevant to study the pathogenetic mechanism of Lp(a).

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Ethical approval

All the plasma samples were obtained from donors affiliated to the Immunoheamatology and Transfusional Service of IRCCS San Matteo Foundation, Pavia, who had signed an informed consent according to the existing relevant Italian regulations.

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