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Original Article

Evidence that lipoprotein(a) is not an adhesive and proaggregating molecule for platelets *in vitro*

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Abstract *Background.* Several studies demonstrated that Lp(a) play an important role in genesis and development of the atherosclerotic lesions. Nevertheless, Lp(a) displays an adjunctive and exclusive thrombotic effect due to a supposed competition of the apo(a) protein moiety with plasminogen for activation and binding either to specific plasminogen receptors at the cell surface or to stabilized fibrin. Moreover, Lp(a), similarly to other lipoproteins, affects the functionality of platelets through its binding to cellular receptors, thus sensitizing platelets to a wide variety of stimulating agents. In the present study the potential role of Lp(a) in the adhesion and aggregation of human platelets was investigated.

Methods. Lp(a) was purified from human plasma by lysine-Sepharose affinity chromatography and was used to coat the plastic surface of microplate wells where human platelets were incubated for 1 h in the absence and in the presence of the stimulants 10 mM ADP or 0.1 U/mL thrombin.

Results. In this assay system, the adhesion of unstimulated platelets to 50 μg/mL Lp(a) was very low (3.8%), as was the adhesion to BSA (2.9 =%) and to fibrinogen (5.2%), while platelet adhesion was much higher (18.8%) when collagen was used as coating agent. Thrombin markedly increased the adhesion to BSA and to fibrinogen (19.7% and 23.3% respectively), while ADP increased the adhesion to fibrinogen but not to BSA (18.2% and 3.0% respectively). The adhesion of stimulated platelets to Lp(a) was very low (3.0% and 5.6% with ADP and thrombin respectively). Finally, Lp(a) at doses >30 μg/mL inhibited both the adhesion of thrombin-stimulated platelets to fibrinogen or BSA and the ADP-induced aggregation in a dose-dependent fashion.

Conclusion. From our results, we conclude that Lp(a) is not a physiological substrate for adhesion of platelets as we demonstrated that Lp(a) may behave as a potential anti-adhesive and anti-aggregating molecule. Therefore, the athero-thrombotic potential of Lp(a) has to be explained by other mechanisms, at the inhibition of plasminogen activation. *(Eur J Lab Med 1998;6:24-28).*

Introduction

Lp(a) is a low density lipoprotein, which contains a single copy of apolipoprotein(a) linked to apolipoprotein B100[1,2]. Epidemiological studies demonstrated that increased levels of Lp(a) in plasma are an independent risk factor for cardiovascular disease, though the mechanisms responsible for the atherogenetic and thrombotic potential of Lp(a) are unknown, as well as its physiological role[3-8]. Apo(a) resembles in structure the zymogen plasminogen, and chiefly consists of multiple tandem repeats of domains homologous to the plasminogen kringle 4, a single copy of kringle 5 and an inactive terminal protease domain[8,9]. The structural homology to plasminogen gave rise to the hypothesis that Lp(a) may display thrombotic activity since previous reports demonstrated that it is able to compete with plasminogen for the binding to platelets and to endothelial cells, thus reducing plasminogen binding and activation[10,11]. It has been reported that Lp(a), as other lipoproteins, could influence the functionality of platelets and endothelium through its binding to cellular receptors, as known for other atherogenetic lipoproteins in either native or oxidized form, thus sensitizing platelets to a wide variety of stimulating agents[12,13]. The precise mecha-

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nisms of LDL-platelets interaction have not been discovered in detail; the binding site has been supposed to be the surface glycoprotein gp IIIb-IIIa, one of the most important adhesion molecules which also represents the binding site for fibrinogen. Although apo(a) contains an arginyl-glycylaspartate (RGD) tripeptide, which is the consensus sequence for the binding of adhesive proteins to the platelet membrane glycoprotein IIb-IIIa, the binding site for Lp(a) to platelets has been identified as the glycoprotein IIb and this interaction does not involve an RGD-like sequence[14]. The evidence that the supposed binding of Lp(a) to platelets may interfere with several platelet functions has been recently provided as it was demonstrated that Lp(a) inhibits both collagen-induced aggregation and adhesion of platelets[15]. Therefore, in the present study we further investigated whether Lp(a) could behave as adhesive molecule for platelets: purified Lp(a) obtained with an original procedure[16] was employed to coat microvessel culture plastic surfaces where human platelet suspensions were left to adhere in the absence and in the presence of ADP and thrombin. Purified Lp(a) was further incubated with prepared human platelets to investigate possible effects on ADP-induced aggregation.

Materials and Methods

Materials. p-nitrophenyl-phosphate, bovine serum albumin (BSA), human fibrinogen (type I) and Triton X-100 were purchased from Sigma, collagen from Menarini; ADP from Boehringer; thrombin from Calbiochem; purified human albumin from Behring Institute, Marburg, Germany. Dulbecco's phosphate buffered saline (PBS) was from Gibco Ltd, Paisley, Scotland, the composition of PBS was 0.2 g/L KCl, 0.2 g/L KH_2PO_4, 0.047 g/L $MgCl_2$, 8 g/L NaCl, 1.15 g/L Na_2HPO_4 (pH 7.4). Sterile 96-well microtiter plates with flat-bottomed wells (type Immulon 4) were from Dynatech. Anti-Lp(a) horseradish peroxidase (HRP)-conjugated polyclonal antibodies were from Strategic Diagnostics, Newark, Delaware, USA. Other materials and reagents were of the highest purity available.

Purification of Lp(a). Lipoprotein(a) was purified to homogeneity as previously described[16]. Briefly, 15 g of lysine-Sepharose (Pharmacia, Uppgala, Sweden) were swollen in 50 mL of distilled water, packed into a refrigerated 40 x 2.6 cm I.D. chromatographic column and extensively washed with 500 mL of 50 mM $(NH_4)HCO_3$, pH 7.95. Hundred mL of serum obtained from a single healthy volunteer homozygous for a previously identified medium size isoform were filtered and the filter was injected in a single passage into the column. Non specific proteins were initially washed-out with 500 mL of 50 mM $(NH_4)HCO_3$, pH 7.95, and non-Lp(a) kringle-containing proteins exhibiting low affinity for lysine-Sepharose were eluted with 50 mL of 0.5 M sodium phosphate buffer, pH 7.4. The Lp(a) fraction bound to lysine-Sepharose (Lp(a) Lys+) was finally eluted with 50 mL of 0.5 M sodium phosphate buffer, pH 7.4 and extensively dialyzed against PBS. The high degree of purity of the material was proved in a previous investigation[16]. Total protein concentration of the purified material was measured with the method of Lowry[17] and final concentration was therefore expressed in terms of Lp(a) protein mass instead of total lipoprotein mass. The clinical significance of Lp(a) species not retained by lysine-Sepharose (Lp(a) Lys–) is still uncertain; Lp(a) Lys– is identical in structure and composition to Lp(a) Lys+[18] and its measurement does not provide additional improvements in the prediction of coronary artery disease in the general population[19]. Moreover, it has been recently reported that the site implicated in the binding of entire Lp(a) particles to lysine-Sepharose is not involved in the binding to fibrinogen, suggesting that lysine binding is not synonymous with fibrinogen binding[20].

Preparation of platelet suspensions. A sample of 10 mL blood was drawn by venepuncture from healthy volunteers with almost undetectable levels of plasma Lp(a) and 8.4 mL aliquots of blood were added to 1.6 mL of ACD (citric acid 15 g/L, 20 g/L dextrose, 25 g/L sodium citrate) in plastic test tubes. Platelet-rich plasma (PRP) was prepared by centrifugation at about 120 g for 10 min. PRP was re-centrifuged at 700 g for 15 min and platelets were gently suspended (5×10^7/mL) in a buffer consisting of 145 mM NaCl, 5 mM KCl, 14 mM HEPES, 0.5 mM Na_2HPO_4, 6 ml glucose and 0.2% BSA, pH 7.4 (buffer A). The platelet suspension was kept at room temperature and used within 1 h.

Assay of adhesion. Platelet adhesion was performed according to a previously described colorimetric method[21]. Ninetysix-wells culture microplates were pre-coated by overnight incubation at +4 °C with one of the following solutions: (a) 2 mg/mL BSA, in phosphate-buffered saline (PBS), (b) 0.2 mg/mL human fibrinogen, in PBS, (c) 20 µg/mL collagen, diluted in 0.9% NaCl, (d) 50 µg/ml Lp(a), diluted in PBS. Immediately before use, the plates were washed twice with 0.9% NaCl, using an automatic plate washer (Easy Washer 2, SLT Labs Instruments). The wells were supplemented with 25 mL of the test agonist (ADP and thrombin, 3 x the final desired concentration in buffer A supplemented with 3 mM $CaCl_2$ and 3 mM $MgSO_4$). The plate was then brought to 37 °C, and 50 mL of the platelet suspension (2.5×10^6 platelets), pre-warmed at 37 °C, were added to each well using a multichannel pipette. Incubation was carried out for 60 min, using a humidified thermostat (37 °C, 5% CO_2). At the

end of the incubation, the plate was transferred to an automatic washer and subjected to 2 *washing* cycles with PBS at room temperature. Adherent platelets were measured by the acid phosphatase assay[21]. The percentage of cells was calculated on the basis of a standard curve, obtained with a known number of platelets of the same subject. The intra-assay reproducibility and intra individual variability were respectively 7% and 4%.

Assay of aggregation

0.15 mL of PRP were incubated with 0.15 mL of either purified Lp(a) at different concentrations or PBS. After 10 min incubation at 37 °C, 25 μL of agonist were added to the platelet suspension. Aggregation in PRP was induced by ADP 3 μM (final concentration) and measured in a four-channel aggregometer (Aggrecorder II, DIC, Kyoto, Japan). Measurements were performed in quadruplicate and results were expressed as percent of total PRP aggregation after 5 minutes.

Results and Discussion

Figure 1 shows the extent of platelet adhesion in different conditions of coating and stimulation. Considering the adhesion to BSA, unstimulated platelets and ADP-stimulated platelets showed little adhesion (2.9 ± 0.3% and 3.2 ± 0.3% respectively), as expected since BSA is presumed to be a "neutral" surface. Unexpectedly, thrombin-stimulated platelets showed a substantial increase of adhesion to BSA (19.7 ± 1.1%). The apparent discrepancy between ADP and thrombin can be explained considering

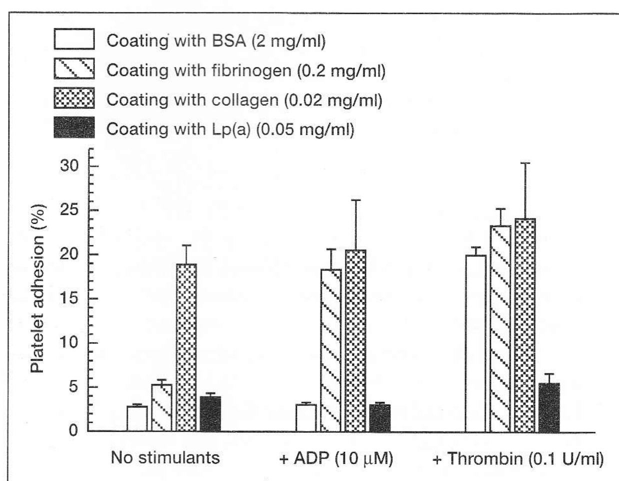

Figure 1. Platelet adhesion to different coating substrates. Microplate wells were coated ovenight with tbe indicated protein substrates er microplate washing, platelets were incubated for 1 h in the absence (no stimulants) and in the presence of ADP or thrombin at the indicated concentrations. The mean values ± SD. of 8 assays done in two separate experiments are reported.

that, with stimuli such as thrombin and collagen, the fibrinogen can be secreted from the platelet α-granules, but stimuli such as ADP and epinephrine are not fully competent for α-granules secretion in the absence of aggregation[21,22]. Therefore, ADP-stimulated platelets can bind only to fibrinogen-coated surfaces, while thrombin-activated platelets can bind also to BSA-coated surfaces presumably because they release fibrinogen and/or other compounds that may act as an adhesive substrate.

The data of Figure 1 show that the adhesion to fibrinogen was agonist-dependent, in agreement with previous observations[21] and with the current view that in ADP and thrombin-stimulated platelets gp IIb/IIIa, the major platelet fibrinogen receptor, undergoes not only agonist-stimulated membrane expression but also functional activation[23]. On collagen-coated surfaces, a marked adhesion was observed even in the absence of agonists, in agreement with the fact that collagen receptors are constitutively expressed on the platelet membrane and mediate cell activation and secretion[24].

Culture wells were also coated with pure Lp(a), prepared according to a recently developed chromatographic procedure[16]. Effective coating of plastic surface was confirmed using anti-Lp(a) HRP-conjugated polyclonal antibodies. The adhesion of unstimulated platelets to purified Lp(a) was very low (3.8 ± 0.5%), a value intermediate between the adhesion to BSA and to fibrinogen and much lower than the adhesion to collagen. This indicates that Lp(a) does not work as an agonist of platelet adhesion like collagen. Moreover, the adhesion to Lp(a) of ADP-stimulated cells was very low (3.0 ± 0.2%), a value comparable to that of the adhesion to albumin, indicating that Lp(a) does not work as an adhesion molecule for activated platelets, like fibrinogen. Finally, data of Figure 1 show that not even thrombin-stimulated platelets showed an appreciable adhesion to Lp(a)-coated surfaces (5.6 ± 1.1%). This finding was quite unexpected, considering that the adhesion of thrombin-stimulated platelets to Lp(a) was even lower than the adhesion to a "neutral" surface like BSA. The latter finding may suggest that Lp(a) is an anti-adhesion molecule in our assay systems or, at least, that Lp(a) is a molecule that is more "inert" than BSA as regards the platelet adhesion.

In order to further investigate a possible role of Lp(a) as an inhibitor of cell adhesion, an experiment was designed where Lp(a) was mixed with a molecular excess of fibrinogen or albumin in the coating mixture. We observed that Lp(a) dose-dependently inhibited the adhesion of platelets to plastic demonstrating that the lipoprotein effectively binds to plastic in our coating procedure. Presence of Lp(a) in the coating mixture also inhibited the adhesion of thrombin-activated platelets to BSA

and the dose-dependence of this inhibition is similar to the dose-dependence of inhibition of adhesion to plastic. Also the adhesion to fibrinogen was inhibited by Lp(a), but the effective doses were slightly higher than the doses capable of inhibiting the adhesion to plastic and to BSA.

Although our study was mainly concentrated on the effects of Lp(a) on platelet adhesion, we observed that purified Lp(a) also inhibited in a dose-dependent fashion the ADP-induced aggregation of platelets in our assay system. The pre-incubation of platelets with Lp(a) 50 μg/mL and 125 μg/mL resulted in a respective decrease of 52.2 ± 7.6% and 96.3 ± 1.2% of the aggregation as compared to platelets pre-incubated with PBS. Moreover, the addition of Lp(a) 125 μg/mL when the aggregation assay was already triggered, resulted in almost total disgregation of platelets; this latter effect was partially reversible after addition of a further 3 μM ADP stimulus.

Three possible explanation can be advanced for these findings: a) Lp(a) binds to plastic more effectively and more rapidly than BSA and fibrinogen, thus eliminating any anchoring surface for activated platelets, b) Lp(a) acts as an inhibitor of platelet activation by ADP and by thrombin, or c) on the basis of the reported binding of apolipoprotein(a) to gp IIb/IIIa[14,15], Lp(a) may compete with fibrinogen for this receptor, acting as an inhibitor of surface adhesion and aggregation of activated platelets. The first explanation seems unlikely, because the inhibition of platelet adhesion to fibrinogen and to BSA occurs at doses of Lp(a) much lower than the doses of competing proteins, so that it is conceivable that, in the coating process, both fibrinogen (or BSA) and Lp(a) bind to the plastic to the same extent. Also the second explanation seems unlikely, because Lp(a) is used as a coating reagent, while during the assay platelets come in contact with soluble thrombin. Therefore, the most probable explanation of our findings is that Lp(a) may compete with fibrinogen for binding to platelet fibrinogen receptor. This is in agreement with previous reports showing that Lp(a) binds to platelets almost exclusively through glycoprotein IIb[13,14].

Although the precise mechanism of the inhibitory effect of Lp(a) remains to be investigated, taken together our data suggest that Lp(a) is not a physiological substrate for adhesion of platelets and that, conversely, this lipoprotein may act as an effective anti-adhesive and anti-aggregatory substrate when it is surface-bound. In the complex phenomena of atherogenesis and thrombogenesis *in vivo*, Lp(a) may have multiple roles, but our findings suggest that the thrombotic potential of Lp(a) cannot be explained on the basis of a direct activation of platelet adhesion and aggregation and thus, the explanation of epidemiological findings has to be found in other mechanisms like the inhibition of plasminogen activation as previously suggested[10,11,13].

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