Lipoprotein(a) Binds to Recombinant Nontypeable *Haemophilus influenzae* Aspartase

Wenlong Li[1], Liping Xu[2], Yakun Zhang[1], Wencheng Bai[1], Lulei Zhou[1], Yuxin Li[1], Na Liu[1], Ling Liu[1], Runlin Han[1, 3, *]

[1]Research Center of Plasma Lipoprotein Immunology, Inner Mongolia Agricultural University, Huhhot, China
[2]College of Basic Medicine, Inner Mongolia Medical University, Huhhot, China
[3]Key Laboratory of Animal Clinic Diagnosis and Treatment (Ministry of Agriculture of the People's Republic of China), Inner Mongolia
  Agricultural University, Huhhot, China

Email address:
han-runlin@163.com (Runlin Han)

Abstract: The respiratory pathogen nontypeable *Haemophilus influenzae* (NTHi) can recruit plasminogen (Plg) on the cell surface by its Plg receptor aspartase (ASP) and utilize host Plg and fibrinolytic system to achieve its adherence and immune invasion. Lipoprotein(a) [Lp(a)] consists of one molecule low-density lipoprotein (LDL) and one molecule apolipoprotein(a) [Apo(a)]. Apo(a) shares a high degree of homology with the human Plg, and both of them contain lysine-binding sites (LBS), which enables them to interact with various cell-surface receptors or fibrin(ogen). However, the definite physiological function of Lp(a) remains vague. Here, we present evidence that Lp(a) via its Apo(a) may bind to the Plg receptor ASP. Recombinant aspartase (rASP) and C-terminal lysine-deleted variant of ASP (rASPΔK) were used in the current study. The rASP specifically bound to Lp(a), but rASPΔK did not, indicating that C-terminal lysine residue of rASP was responsible for the interaction. In addition, rASP interacted with Lp(a), but not with LDL, revealing that LBS of Apo(a) was involved in the binding. Our results also showed that Lp(a) could inhibit the binding of Plg to rASP. Plasma Lp(a) might play a role in anti-NTHi infection by binding to its Plg receptor ASP.

Keywords: Lipoprotein(a), Apolipoprotein(a), Nontypeable *Haemophilus influenzae*, Plasminogen, Recombinant Aspartase

1. Introduction

Plasminogen (Plg) and the fibrinolytic system can be utilized and exploited by a number of Gram-positive and Gram-negative bacteria, including *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Helicobacter pylori*, *Neisseria meningitides*, and *Haemophilus influenzae* (*H. influenzae*), for dissemination and invasion within the host [1-4]. Aspartase (ASP) and protein E (PE) have been identified as two plasminogen receptors (PlgRs) on the surface of *H. influenzae* [5,6]. ASP stimulates the activation of Plg, mediated by tissue-type plasminogen activators (tPA), and then contributes to the formation of bacterium-bound proteolytic plasmin (Pm) activity in the spread of *H. influenzae* through tissue barriers [2,5]. *H. influenzae* can be subtyped into encapsulated and unencapsulated strains [7]. Unencapsulated strains, referred to Nontypeable *H.*

influenzae (NTHi), are normal inhabitants of human nasopharynx and rarely cause systemic infection, but they are responsible for otitis media, sinusitis, chronic bronchitis, and other milder infections [7-9].

Lipoprotein(a) [Lp(a)] contains one molecule of apolipoprotein(a) [Apo(a)] covalently bound to apolipoprotein B 100 (apoB 100) of low-density lipoprotein (LDL) via a disulfide bridge [10]. Lp(a) acts as a unique risk factor for cardiovascular diseases and the elevated plasma concentration of Lp(a) leads to atherogenesis and thrombosis since it contains abundant LDL [11-13].

The apo(a) component of Lp(a) confers distinctive structural properties to this lipoprotein and exhibits high sequence similarity to zymogen plasminogen, both possessing weak and strong LBS in their kringle domains [1,14,15]. Apo(a) contains two types of plasminogen-like kringle domains (KIV and KV), followed by a serine

protease domain which is catalytically inactive [14, 16]. The KIV-like domain in Apo(a) can be classified into ten types (KIV$_1$ to KIV$_{10}$), and KIV$_{10}$ have strong LBS that resembles Kringle 4 in Plg [17,18]. Both Plg and Apo(a) bind to various cell-surface receptors or fibrin(ogen) surface by using their LBS in the kringle domains [19-22]. Research has demonstrated that Lp(a) inhibits the binding of plasminogen to fibrin and tPA-fibrin compound, and suppresses the activation of Plg [23-25]. The Plg activation plays an important role in host fibrinolytic system [26]. We previously observed that the competitive Plg binding between Lp(a) and several bacterial PlgRs, like inosine 5'-monophosphate dehydrogenase (IMPDH) of *Staphyylococcus aureus* and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of group A *Streptococcus* [27, 28].

Pathogen infection and inflammation exert a great effect on the plasma lipoproteins metabolism [29]. We have hypothesized that Lp(a) might be a potent anti-infective molecule in the human immune system since Lp(a) might competitively inhibit pathogens from employing host Plg [30]. However, there has been no research focused on the relationship between host Lp(a) and NTHi infection.

The present study demonstrates that human plasma Lp(a) binds specifically to the recombinant NTHi aspartase (rASP) via its Apo(a) component, which results in partial inhibition of the binding of rASP to Plg.

2. Materials and Methods

2.1. Bacterial Strain and Growth Conditions

The strain of nontypeable *Haemophilus influenzae* (ATCC 49247) was obtained from the National Center for Clinical Laboratories (Beijing, China). The cultivation of NTHi was performed as reported previously [31]. Briefly, NTHi, stored at -70°C in 25% glycerin, was subcultured onto chocolate agar and incubated in a humid atmosphere at 37 °C and 5% CO_2 for 24 h. The bacterial lawn was inoculated into brain-heart infusion (BHI) liquid broth supplemented with 4 μg/mL β-NAD (Sigma-Aldrich, Saint Louis, USA) and 10 μg/mL Hemin (Sigma-Aldrich, Saint Louis, USA). The bacterial culture was grown at 37 °C, with shaking at 200 rpm. *Escherichia coli* (*E. coli*) BL21 (DE3) was grown in Luria-Bertani (LB) broth. The bacterial strains used in the study are listed in Table 1.

2.2. Expression of Recombinant Proteins in E. coli

Recombinant NTHi aspartase (rASP) (~53 kDa) and C-terminal lysine-deleted variant of ASP (rASPΔK) were expressed in *E. coli* BL21 (DE3) and purified by using 6×Histidine-tag expression and purification system (IBA-GmbH, Goettingen, Germany), as described previously [32]. In brief, genome DNA was extracted from NTHi (ATCC 49247). The DNA fragments encoding ASP (Genbank: NC009566; Region: 57522..58940) (or C-terminal lysine-deleted variant of ASP) were amplified by PCR, digested with BsaI, and cloned into the plasmid pASK-IBA37, designed for periplasmic expression. The plasmid and specific primers for PCR are listed in Table 1.

E.coli BL21 (DE3) harboring plasmid constructs were grown in the presence of ampicillin (Bio Basic Inc., Markham, Canada). Protein expression was induced with anhydrotetracycline for 3 h, and the recombinant proteins were extracted from the periplasm by FastBreak Cell Lysis Reagent (Promega, Madison, USA) and were purified by affinity chromatography with TALON® Metal Affinity Resins (Clontech laboratories Inc., Mountain View, USA). The purity of recombinant proteins was analyzed by SDS-ployacrylamid gel electrophoresis (SDS-PAGE), and the protein concentration was quantified with Pierce BCA Protein Assay Kit (Thermo Scientific, Rockford, USA).

Table 1. *Strains, plasmids, and primers used in the present study.*

Strains, plasmids, or primers	Source or application
Stains	
nontypeable *Haemophilus influenzae* (ATCC 49247)	National Center for Clinical Laboratories (Beijing, China)
E.coli BL21 (DE3)	Shanghai Sangon
Plasmid	
pASK-IBA37	IBA-GmbH
Primers	
ASPF: 5′-atggtaggtctcagcgcatgactcaatttagaaaagaagtagat-3′	for *ASP* Cloning
ASPR: 5′-atggtaggtctcatatcatttatttaatttcgctttgtaagttgg-3′	for *ASP* Cloning
ASPΔKF: same as ASPF	for *ASPΔK* Cloning
ASPΔKR: 5′-atggtaggtctcatatcaatttaatttcgctttgtaagttgg-3′	for *ASPΔK* Cloning

2.3. Assay of Aspartase Activity of Recombinant Proteins

Aspartase activity was measured by determination of fumarate formation using the method described previously by Williams and Lartigue [33], with several modifications. In brief, purified recombinant protein (1 μg dissolved in 1 μL PBS) was added to 299 μL reaction medium (pH 7.0) (containing 50 mM Sodium L-aspartate, 50 mM Tris, 2 mM MgSO$_4$·7H$_2$O, and 0.1 mM EDTA). An aliquot of 300 μL reaction medium was used as a control group. The absorbance at 240 nm was determined by a Multi-Detection Microplate Reader (model Synergy HT, BioTek, Winooski, USA). The molar extinction coefficient of fumarate was reported by Emery to be 2.53×10^3 M^{-1}·cm^{-1} [34]. A unit of aspartase was defined as the amount producing 1 μmol of fumarate per min at 30°C. Aspartase specific activity was

expressed as units per mg of protein under the standard assay conditions.

2.4. Enzyme-Linked Immunosorbent Assay (ELISA)

Purified rASP or rASPΔK solution (2 μg in 100 μL PBS) was immobilized onto a 96-well microtiter plate (Greiner Bio-One, Frickenhausen, Germany) at room temperature for 1.5 h. After washing three times with 200 μL TBST (Tris-buffered saline containing 0.05% Tween 20, pH 7.4), aliquots of 200 μL blocking buffer (1% BSA in TBST) were added, and the samples were incubated for 1.5 h at room temperature. Then, different amounts (100 ng, 50 ng, and 10 ng) of Lp(a) (Biomedical Technologies Inc., Stoughton, USA) in 100 μL TBST were supplemented into the wells. After incubation for 1.5 h at room temperature, the unbound Lp(a) was removed by washing three times with TBST, while the bound Lp(a) was identified by a goat polyclonal anti-Apo(a) antibody (Biomedical Technologies Inc., Stoughton, USA)(1:4000 diluted in 100 μL TBST). After three washes with TBST, HRP-conjugated donkey anti-goat IgG (R&D Systems, Minneapolis, USA) (1:1000 diluted in 100 μL TBST) was used as a secondary antibody for the detection. The reaction of color development with the addition of TMB substrate (Promega, Madison, USA) was stopped by the addition of the stop solution (8.5 M acetic acid, 2.5 M H_2SO_4). The absorbance at 450 nm was determined by using the Multi-Detection Microplate Reader.

The binding of rASP and rASPΔK to human plasma [the concentration of plasma Lp(a) was 20 mg/L)] was aslo analyzed by ELISA. Plasma Lp(a) [1:20 diluted in TBST, plasma Lp(a) employed at a concentration of 100 ng/100 μL] bound to recombinant proteins was examined by the same method described above.

The binding of rASP to different amounts of LDL (100 ng, 50 ng and 10 ng) was performed as described above. LDL (Biomedical Technologies Inc., Stoughton, USA), bound to immobilized rASP, was identified by a goat anti-LDL polyclonal antibody (Sigma-Aldrich, Saint Louis, USA) and HRP-conjugated donkey anti-goat IgG secondary antibody.

The binding of rASP and rASPΔK to different amounts of Plg (100ng, 50 ng and 10ng) were also investigated. Plg (R&D Systems, Minneapolis, USA), bound to immobilized rASP, was identified after incubation with a mouse anti-Plg monoclonal antibody (Abcam, Cambridge, UK) (1:2000 dilution in TBST). HRP-conjugated goat anti-mouse IgG (Bios, Beijing, China) was employed as a secondary antibody (dilution 1:2000 in TBST). The procedures that followed were performed as described above.

2.5. Asp-affinity Pull Down Assay and Western Blot

Affinity-chromatography columns were packed with 0.2 mL of the TALON® metal resin and equilibrated with buffer W (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 0.01% NaN$_3$, pH 8.0). Purified rASP and rASPΔK (100 μg in 150 μL Buffer W) were applied respectively onto two columns to allow binding through C-terminal affinity tags. Besides, 150 μL PBS was

applied onto another column. After washing with 8 column volumes (8 CV) of buffer W, aliquots of 2 mL human plasma were passed over the three columns. The columns were washed with 12 CV of buffer W. The complexes of rASP protein and their ligands were eluted in 300μL fractions with 1.5 CV of buffer E (50 mM NaH$_2$PO$_4$, 300 mM NaCl, 150 mM Imidazole, 0.01% NaN$_3$, pH 7.0). "rASP + Plasma", "rASPΔK + Plasma", and "PBS + Plasma" represented the elution fraction of the different columns. The total protein present in each sample was precipitated with 10% trichloroacetic acid (TCA) for 1 h on ice. After centrifugation at 4 °C, the pellets were resuspended in 1 M Tris-HCl buffer (pH 8.0). Plasma Lp(a) were separated by 6% SDS-PAGE, and then immunoblotting was conducted.

For immunodetection of Apo(a), elution samples were subjected to 6% SDS-PAGE and transferred to a nitrocellulose membrane at 400 mA for 3 h (wet blotting). The nitrocellulose membrane was blocked with TBST supplemented with 5% skim milk at room temperature for 2 h and washed with TBST. Bound Apo(a) was detected with a goat anti-Apo(a) antibody (1:20000 diluted in TBST, supplemented with 2% skim milk) followed by HRP-conjugated donkey anti-goat IgG as a secondary antibody (1:5000 diluted in TBST, supplemented with 2% skim milk). Bound Apo(a) was detected with HRP-conjugated Tactin (Thermo Scientific, Rockford, USA) (1:8000 diluted in TBST). Detection of reactive antibodies was performed by using a chemiluminescence substrate (Tiangen, Beijing, China), and the chemiluminescence signals were visualized on Syngene G:Box Chemi XT4 (Syngene, Cambridge, UK).

2.6. Binding-Inhibition ELISA

The effect of 6-aminocaproic acid (EACA) (Sigma-Aldrich, Saint Louis, USA) on Lp(a) binding to immobilized rASP was analyzed by binding-inhibition ELISA. EACA, utilized at different concentrations (0 mM, 0.1mM, and 1mM), were combined with a constant quantity (100 ng) of Lp(a) in 100 μL TBST. Then the mixtures were added to immobilized rASP. After incubation and extensive washing, each bound Lp(a) was identified by specific antibodies.

In addition, Lp(a), utilized at different amounts (0 ng, 50 ng, and 100 ng), was combined with a constant quantity (100 ng) of Plg in 100 μL TBST. Then, the mixtures were added to immobilized rASP for the determination of the effect of Lp(a) on the binding of Plg to rASP. After incubation and extensive washing, each bound Plg was identified by specific antibodies.

3. Statement of Ethical Standards

The blood was taken from volunteers after the provision of written informed consent and authorization in compliance with the regulation of the Inner Mongolia Agricultural University (Hohhot, China). The experiments with human plasma were approved by the Academic Board and the Science and Technology Department of the Inner Mongolia Agricultural University. All participants signed a written

informed consent form. The concentration of Lp(a) was determined in the Inner Mongolia Autonomous Region People's Hospital.

4. Statistical Analyses of the Data

All tests were performed in triplicates, and the results were presented as mean ± standard deviation. Unless stated otherwise, the statistical significance of the results was established by Student's *t*-test for paired data (GraphPad Software, San Diego, USA). Differences with *p*<0.05 were considered statistically significant (*P<0.05, **P<0.01, ***P<0.001).

5. Results

5.1. Purification of rASP and rASPΔK

rASP and rASPΔK were purified by affinity chromatography with TALON® Metal Affinity Resins. The samples collected from different purification procedures were analyzed by 12% SDS-PAGE. The results indicated that both rASP and rASPΔK were purified successfully (Fig. 1).
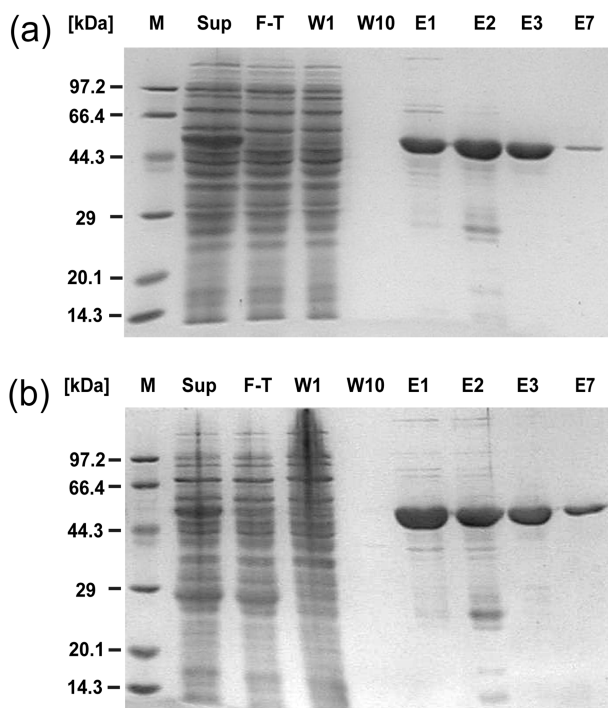
Fig. 1. *Purification of recombinant proteins.*

a The purification of rASP. b The purification of rASPΔK. "M" represents the molecular mass markers (kDa); "Sup" refers to the supernatant after lysis by *E.coli* BL21; "F-T" denotes the resolution passed over the column; "W1, W10" represent the 1st, 10th wash fraction; "E1, E2, E3, E7" refer to the 1st, 2nd, 3rd, and 7th eluate fraction, respectively. The common ~53 kDa bands depicted the target proteins.

5.2. ASP Activity Assay of Recombinant Proteins

Aspartase activity of the purified recombinant proteins was determined spectrophotometrically at 240 nm. The specific activity of rASP and rASPΔK were 59.81 ± 6.30 U/mg and 104.38 ± 18.08 U/mg, respectively. The results showed that the recombinant proteins had the ability to catalyze the formation of fumarate and possessed efficient biological activity of ASP. The specific activity of the mutant ASP is higher than the wildtype protein, which has been reported by early researches [35].

5.3. Lp(a) Binds to C-terminal Lysine of rASP via LBS

To test the hypothesis that ASP binds to Apo(a), binding of rASP to LDL or Lp(a) was assayed by ELISA, respectively. rASP was incubated with different amounts of Lp(a) or LDL. The results evidenced that Lp(a) bound to rASP in a dose-dependent manner. In contrast, LDL did not associate with rASP (Fig. 2). This indicated that Lp(a) binds to rASP via Apo(a), but not LDL.

In order to determine the region of rASP responsible for binding to Lp(a), a variant of ASP (rASPΔK) which lacked C-terminal lysine residue was also expressed and purified. The binding of rASPΔK to Lp(a) was analyzed by using rASP as a positive control. The results revealed that rASPΔK cannot bind to Lp(a) (Fig. 3a). Thus, as we speculated, the C-terminal lysine residue of ASP plays a key role in this process.

To further characterize the relevance of rASP for Lp(a) binding, we assayed binding of both purified Lp(a) and human plasma. The data showed that they had no difference in the binding of recombinant proteins (Fig. 3b). The results indicated that the source of Lp(a) exerted no effect on the Lp(a) binding assay, and rASPΔK did not bind to Lp(a). Human plasma was also used for stimulating the physiological environment *in vitro*.

These data were further confirmed by Asp-affinity pull down assay and western blot analysis. Lp(a) was present only in the elution of "rASP + Plasma" **(**Fig. 3c); the elution of "PBS + plasma" was used as a negative control. These findings implied that rASP binds to plasma Lp(a) via its C-terminal lysine.
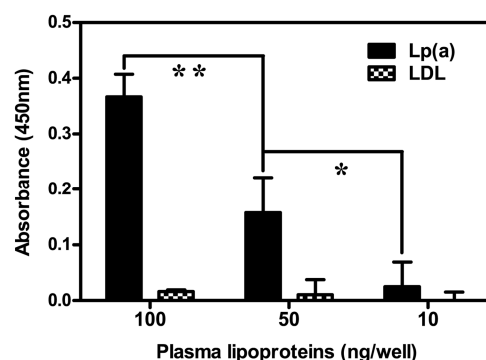
Fig. 2. *rASP binds to Lp(a), but not to LDL.*

rASP binding to plasma lipoproteins was assayed by ELISA. Lp(a) or LDL, used in increasing amounts, were bound to immobilized rASP. Lp(a) (or LDL) binding to a BSA-coated surface was utilized as a negative control, which should be eliminated. The results are presented as means ± SD, each carried out in triplicates. *P<0.05, **P<0.01, ***P<0.001.
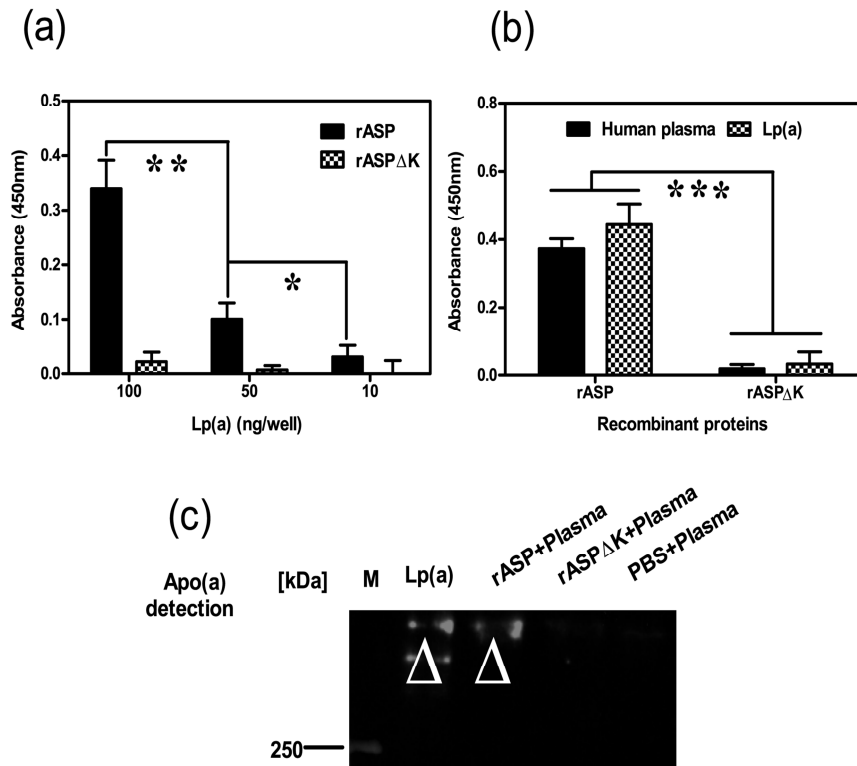
Fig. 3. *Interaction of recombinant proteins with plasma Lp(a).*

a Lp(a) binding to rASP and rASPΔK was assayed by ELISA. Lp(a), used in increasing amounts, was bound to immobilized recombinant proteins. b Binding of recombinant proteins to human plasma and Lp(a); c The results of Asp-affinity pull down assay and western blot analysis for Lp(a). "Δ" represents the Lp(a) detected in "rASP + Plasma". For ELISA, Lp(a) binding to a BSA-coated surface was utilized as a negative control, which should be eliminated. The results are presented as means ± SD, each carried out in triplicates. *P<0.05, **P<0.01, ***P<0.001.

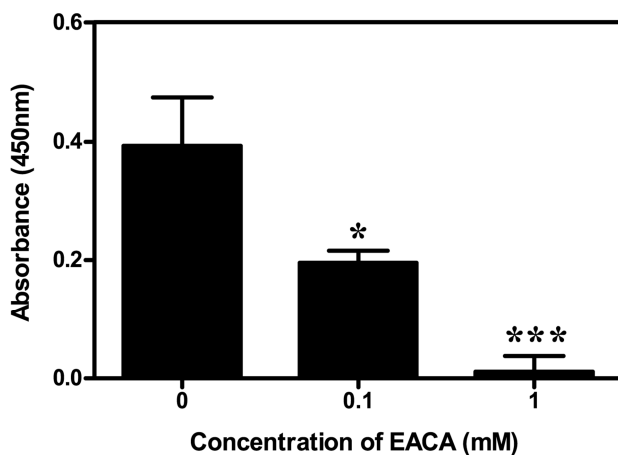## 5.4. Lp(a) Binding to rASP is Probably Mediated by Lysine Residues

Fig. 4. *The inhibition of EACA for binding of Lp(a) to rASP.*

Aliquots of 100 ng Lp(a) were combined with different concentrations of EACA, and the mixtures were bound to immobilized rASP. Lp(a) binding to a BSA-coated surface was utilized as a negative control, which should be eliminated.. The results are presented as means ± SD, each carried out in triplicates. *P<0.05, **P<0.01, ***P<0.001.
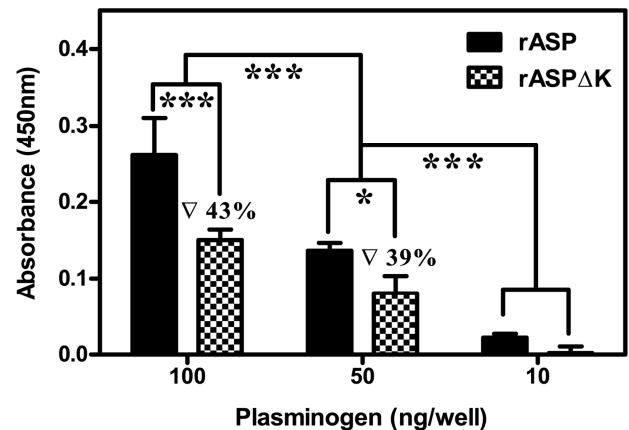

Fig. 5. *Plasminogen binding to recombinant proteins.*

Plg, used in increasing amounts, was bound to immobilized recombinant proteins. Plg binding to a BSA-coated surface was utilized as a negative control, which should be eliminated. The results are presented as means ± SD, each carried out in triplicates. The statistical significance was analyzed by two-way ANOVA. *P<0.05, **P<0.01, ***P<0.001.

To further determine whether lysine residues and lysine-binding sites were relevant for rASP binding to Lp(a), we investigated the effect of EACA. This lysine analog

inhibited Lp(a) binding to rASP, and the effect was dose-dependent (Fig. 4). In a concentration of 0.1 mM, EACA reduced Lp(a) attachment to rASP by 50.2%, and 1 mM EACA suppressed the binding of Lp(a) to rASP completely. Thus, the linkage between Lp(a) and rASP is mediated by lysine residues and lysine-binding sites.

Taken together, Lp(a) associates with the C-terminal lysine residue of ASP via Apo(a), and this interaction is involved in the LBS of Apo(a).

5.5. Plasminogen Binds to Recombinant Proteins

Binding of Plg to the two recombinant proteins was assayed by ELISA. Different amounts of Plg were incubated with immobilized recombinant proteins. The results revealed that Plg bound to rASP and rASPΔK, and the binding was also dose-dependent. However, Plg binding to rASPΔK was approximately 40% lower as compared with that to rASP (Fig. 5).

5.6. Lp(a) and Plg Compete for rASP Binding

Since Plg and Lp(a) share high similarity, and they both bind to ASP, we next investigated whether the two human proteins compete for ASP binding. So the impact of Lp(a) on Plg binding to rASP was examined. Used in increasing amounts, Lp(a) decreased the level of binding of Plg to rASP, which was a dose-dependent process. When no Lp(a) was added, the association of Plg with rASP was reduced by 14.7% and 30.3% in comparison with the treatments, in which Lp(a) was used at 50 ng and 100 ng, respectively (Fig. 6). The results indicated that Lp(a) might inhibit the binding of Plg to rASP.
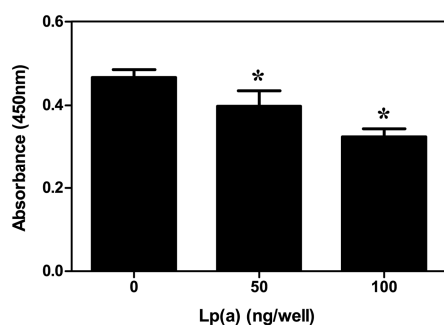

Fig. 6. *Lp(a) and Plg compete for rASP binding.*

Aliquots of 100 ng Plg were combined with different amounts of Lp(a), and the mixtures were bound to immobilized rASP. Plg binding to a BSA-coated surface was used as a negative control, which should be eliminated. The results are presented as means ± SD, each carried out in triplicates. *$P<0.05$, **$P<0.01$, ***$P<0.001$.

6. Discussion

Host zymogen plasminogen (Plg) can be recruited and activated to serine protease Pm on the cell surface of multiple species of bacteria via their Plg receptors (PlgRs), and this event emerges as a central theme in pathogen infection [36,37]. Meanwhile, Lp(a) might play a significant role,

acting as a potent component of the host defense system due to its anti-fibrinolytic activity [30].

NTHi cells recruit Plg on the bacterium surface, by using its Plg receptors. As one of the Plg receptors of NTHi, ASP plays an important role in the process [2]. Our results have shown that ASP aslo binds to Lp(a), and the interaction is mediated by lysine-binding sites (LBS) in Apo(a). Meanwhile, binding of Plg to rASP decreased correspondingly with the increasing amounts of Lp(a), indicating that Lp(a) inhibits the association of Plg with rASP. We speculate that the mechanism involved in the competition between Lp(a) and Plg is similar to that of LBS [38].

Various PlgRs have been shown to have the ability to bind to Plg since they contain carboxyl-terminal lysine residues [36]. The C-terminal lysine residue of PlgRs proteins seems to exert an important role in its Plg and Lp(a) binding function [3,19,39]. The binding of Plg and Lp(a) to rASP and rASPΔK confirmed this assumption. However, the C-terminal lysine residue of ASP is the only site for Lp(a) binding.

Results of an *in vivo* study performed with the utilization of Apo(a) transgenic mice exhibited the ability of Apo(a) to inhibit neutrophil recruitment and its potential function as a cell specific suppressor of the inflammatory process [40]. Actually, we have demonstrated that Lp(a) inhibits the adhesion of Plg to *S. aureus* (CMCC26003) and decreases the fibrinolytic activity of Plg mediated by urokinase on streptococcal cells *in vitro* [30]. Our preliminary study also indicated that Lp(a) bound to NTHi cells via ELISA (data not shown), and we plan to conduct further research on the binding mechanism and the effect on Plg binding to NTHi cells.

7. Conclusion

In our investigation, we demonstrated for the first time that human plasma Lp(a) competes for the Plg binding to the NTHi Plg receptor, and the specific interaction is involved in LBS of Apo(a) and ASP C-terminal lysine residue. The findings of the present study reveal the potent anti-infective immune effects of Lp(a) in the host defense system.

Acknowledgments

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