Interaction of Recombinant Nontypeable Haemophilus influenzae Protein E with Plasma Lipoprotein(a) Postprint

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Abstract

Due to the high homology between apolipoprotein(a) [Apo(a)] in lipoprotein(a) [Lp(a)] and Plg, this study aimed to demonstrate whether Lp(a) would bind to recombinantly expressed PE (rPE). Methods: rPE and rPEΔKK (with two lysine residues deleted at the C-terminus) were prokaryotically expressed and purified; Lp(a) was isolated from human plasma by density gradient centrifugation combined with anion exchange chromatography; the interaction between rPE and Lp(a) was studied through ELISA, pull-down, Western blot, and other methods. Results: rPE bound to Lp(a) but not to LDL, and the binding capacity of rPEΔKK to Lp(a) was significantly lower than that of rPE; the lysine analogue 6-aminocaproic acid (EACA) could effectively inhibit the binding of rPE to Lp(a); Lp(a) had a weak inhibitory effect on the binding of rPE to Plg. Conclusion: rPE can bind to Lp(a), wherein the C-terminal lysine residues of rPE and the lysine binding sites (LBS) of Apo(a) are the main binding sites for the interaction between rPE and Lp(a).

Full Text

The Interaction Between Recombinant Protein E Derived from Nontypeable Haemophilus influenzae and Plasma Lipoprotein(a)

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Abstract

Objective: Protein E (PE) is a plasminogen (Plg) receptor on the surface of nontypeable Haemophilus influenzae (NTHi) that contains two lysine residues at its C-terminus. NTHi can bind Plg via surface-exposed PE and exploit the host fibrinolytic system to invade deep tissues. Based on the high homology between apolipoprotein(a) [Apo(a)] in lipoprotein(a) [Lp(a)] and Plg, this study aimed to investigate whether Lp(a) could bind to recombinant PE (rPE).

Methods: Recombinant PE and a mutant lacking the C-terminal lysine residues (rPEΔKK) were expressed in a prokaryotic system and purified. Lp(a) was isolated from human plasma by density gradient ultracentrifugation combined with anion-exchange chromatography. The interaction between rPE and Lp(a) was examined using ELISA, pull-down assays, and Western blotting.

Results: rPE bound to Lp(a) but not to LDL, and the binding capacity of rPEΔKK to Lp(a) was significantly lower than that of wild-type rPE. The lysine analogue -aminocaproic acid (EACA) effectively inhibited the binding of rPE to Lp(a). Lp(a) exhibited only weak inhibitory effects on the rPE-Plg interaction.

Conclusion: rPE can bind to Lp(a), with the C-terminal lysine residues of rPE and the lysine-binding sites (LBS) of Apo(a) serving as the primary binding sites for this interaction.

Keywords: Nontypeable Haemophilus influenzae; Protein E; Lipoprotein(a); Plasminogen; Lysine-binding sites

Introduction

Nontypeable Haemophilus influenzae (NTHi) is a conditional pathogen that colonizes the human respiratory mucosa and causes sinusitis and acute otitis media, particularly in children. It is also a major etiological agent of chronic obstructive pulmonary disease (COPD) [1]. Like many other pathogenic bacteria, NTHi can adhere plasminogen to its surface through specific receptors and subsequently exploit the host fibrinolytic system to degrade extracellular matrix components, thereby facilitating deep tissue invasion [2].

Plasminogen is a single-chain glycoprotein that constitutes a key component of the fibrinolytic system. It contains five kringle domains enriched with lysine-

binding sites (LBS) that mediate recognition of plasminogen receptors, particularly those harboring C-terminal lysine residues. This interaction can be inhibited by lysine analogues such as -aminocaproic acid (EACA) [3, 4]. Known plasminogen receptors on the NTHi surface include aspartase, Protein E, Protein F, and P4 protein [5-8]. Among these, Protein E (PE) plays a crucial role in NTHi pathogenesis by binding plasminogen to degrade extracellular matrix, adhering to host epithelial cells to trigger inflammatory responses, and binding vitronectin to evade innate immunity [9].

Lipoprotein(a) [Lp(a)] is a plasma lipoprotein found in humans and certain primates whose physiological function remains poorly understood. Lp(a) consists of one molecule of apolipoprotein(a) [Apo(a)] covalently linked to one molecule of low-density lipoprotein (LDL) via a disulfide bond [10]. Apo(a) shares high cDNA sequence homology with plasminogen, and both proteins contain kringle domains enriched with LBS. Based on this structural similarity, our laboratory previously proposed the "anti-infection hypothesis" of Lp(a), suggesting that Lp(a) can bind to bacterial plasminogen receptors and competitively inhibit plasminogen binding, thereby exerting anti-infective effects [11, 12].

In this study, we expressed and purified recombinant PE (rPE) and a C-terminal lysine deletion mutant (rPEΔKK), isolated Lp(a) from human plasma, and investigated the interaction between rPE and Lp(a) using ELISA, pull-down assays, and Western blotting to provide experimental evidence for the anti-infective role of Lp(a).

Materials and Methods

1.1 Materials

Bacterial Strains and Vectors: NTHi ATCC49247 was purchased from the National Center for Clinical Laboratories. *Escherichia coli* BL21 and JM109 were obtained from BBI Life Sciences. The pASK-IBA37Plus plasmid was purchased from IBA GmbH.

Reagents: 2×Ultra-Pfu PCR Master Mix was from Nanjing Bolden; ClonExpress II cloning kit from Nanjing Vazyme; HiTrap Q Sepharose™ Fast Flow column and TALON Superflow™ resin from GE Healthcare; Pierce® BCA Protein Assay Kit and PageRuler™ Prestained Protein Ladder from Thermo Fisher Scientific; human plasminogen, mouse anti-human plasminogen monoclonal antibody, and donkey anti-sheep IgG-HRP from R&D Systems; LDL and sheep anti-human Apo(a) polyclonal antibody from Alfa Aesar; KBr, EACA, and sheep anti-human LDL polyclonal antibody from Sigma-Aldrich; mouse anti-His-tag monoclonal antibody from TransGen Biotech; and Precision Plus Protein™ standards from Bio-Rad.

Instruments: Beckman Optima™ L-100XP ultracentrifuge with SW40Ti rotor; ÄKTA avant 25 automated chromatography system; BIO-RAD Mini

Trans-Blot® transfer cell; BIO-RAD PowerPac™ HC power supply; Hoefer TE70X semi-dry transfer unit; BioTek Multi-Detection Microplate Reader; Gene G:BOX Chemi XT4 gel imaging system; and PALL Cascada™ I ultrapure water system.

1.2 Bacterial Culture

NTHi ATCC49247 was streaked onto sBHI solid medium (BHI supplemented with 4 g/mL -NAD and 10 g/mL hemin) and incubated at 37°C with 5% CO for 24 h for activation. Liquid cultures were grown in sBHI broth at 37°C with shaking at 200 rpm. *E. coli* strains were cultured in LB medium.

1.3 Gene Cloning

Primer Design: The *pe* gene sequence was retrieved from NCBI. Primers were designed using the online tool from Vazyme based on the pASK-IBA37Plus vector and the ClonExpress II one-step cloning protocol (primer sequences are listed in Table 1).

Table 1. List of primers

Primer	Sequence (5' -3')	Restriction e
rPE F	CGAGACCGCGGTCCCGAATTCAAGGCTAAACAAAATGATGTGAAGC	EcoR
rPE R	CCCCTGCAGGTCGACCTCGAGTTATTTTTTATCAACTGAAAATGCTTC	XhoI
rPEΔKK F	CGAGACCGCGGTCCCGAATTCAAGGCTAAACAAAATGATGTGAAGC	EcoR
rPEΔKK R	CCCCTGCAGGTCGACCTCGAGTTAATCAACTGAAAATGCTTCACCA	XhoI

Plasmid Construction: Genomic DNA was extracted from NTHi and verified by electrophoresis. The *pe* and *peΔkk* fragments were amplified by PCR under the following conditions: 94°C for 3 min; 30 cycles of 94°C for 30 s, 54°C for 30 s, and 72°C for 40 s; followed by 72°C for 10 min. The pASK-IBA37Plus plasmid was double-digested and ligated with the PCR products using the ClonExpress II kit. The ligation mixtures were transformed into *E. coli* JM109 competent cells. Positive clones were identified by colony PCR and confirmed by sequencing. Plasmids with correct sequences were extracted and transformed into *E. coli* BL21 for protein expression.

1.4 Expression and Purification of rPE and rPEΔKK

Engineered *E. coli* BL21 strains harboring pASK-IBA37Plus-*pe* or pASK-IBA37Plus-*peΔkk* were induced for expression of N-terminal His-tagged rPE and rPEΔKK following the IBA manual. Proteins were purified using TALON Superflow™ resin, and concentrations were determined by the BCA method [13].

1.5 Isolation and Identification of Human Plasma Lp(a)

1.5.1 Lp(a) Isolation: Human blood was collected aseptically from healthy donors using EDTA-K as anticoagulant, yielding approximately 25 mL of plasma. Solid KBr was added to adjust the plasma density to 1.21 g/mL, and 1 mmol/L EDTA was included as antioxidant. Density solutions of 1.1, 1.063, and 1.006 g/mL (containing 1 mmol/L EDTA) were prepared and layered sequentially (4.5, 3, 3, and 1 mL) in ultracentrifuge tubes. Samples were centrifuged at 40,000 rpm for 3 h at 10°C. The fraction at the 1.063 g/mL interface was collected and dialyzed at 10°C for 24 h.

A 1 mL Q Sepharose™ Fast Flow column was equilibrated with Wash buffer (150 mmol/L NaCl, 20 mmol/L Tris, 1 mmol/L EDTA, pH 7.4) until the A nm reached baseline at a flow rate of 0.5 mL/min. The dialyzed sample was filtered through a 0.22 m membrane and loaded onto the column. Unbound proteins were washed off with Wash buffer until A nm returned to baseline, and bound proteins were eluted with Elution buffer (300 mmol/L NaCl, 20 mmol/L Tris, 1 mmol/L EDTA, pH 7.4). Eluted fractions with A nm above baseline were collected, filtered (0.22 m), and protein concentration was measured by the BCA method.

1.5.2 Purity Assessment and Western Blot Identification: LDL from Alfa Aesar served as a control. Purity of Lp(a) was assessed by SDS-PAGE (6% separating gel, 4% stacking gel). For Western blot detection of Apo(a), proteins were transferred to NC membrane at 400 mA for 2.5 h, blocked with 5% skim milk in TBST for 1.5 h, and incubated with sheep anti-human Apo(a) polyclonal antibody (1:20,000 in TBST) followed by donkey anti-sheep IgG-HRP (1:5,000). For ApoB-100 detection, sheep anti-human ApoB-100 polyclonal antibody (1:20,000) was used as the primary antibody.

1.6 Binding Assays for rPE and Lp(a)

1.6.1 ELISA: Microplates were coated with 100 L/well of rPE or rPEΔKK (20 g/mL) for 1.5 h at room temperature, washed three times with 200 L/well TBST, and blocked with 1% BSA (200 L/well) for 1.5 h. After washing, Lp(a) (500, 50, or 5 ng/well) was added (100 L/well) and incubated for 1.5 h. Bound Lp(a) was detected using sheep anti-human Apo(a) antibody (1:4,000) and donkey anti-sheep IgG-HRP (1:1,000). Color was developed with TMB substrate (100 L/well) for 5 min, stopped with 100 L/well stop solution, and absorbance was measured at 450 nm.

1.6.2 Pull-Down and Western Blot: TALON Superflow™ resin (100 g) was incubated with rPE, rPEΔKK, or PBS (negative control) in separate columns. After washing with 8 column volumes (CV) of Wash buffer, 2 mL of plasma was added and passed through the resin three times. Unbound proteins were removed with 14 CV of Wash buffer, and bound proteins were eluted with 6 CV of Elution buffer. Eluates were precipitated with 10% TCA for electrophoresis. Western blot for Lp(a) detection followed the protocol in Section 1.5.2. For

rPE/rPEΔKK detection, proteins were transferred semi-dry at 45 mA for 30 min, and membranes were probed with mouse anti-His-tag antibody (1:1,000) and sheep anti-mouse IgG-HRP (1:2,000).

1.7 Mechanistic Studies of rPE-Lp(a) Binding

1.7.1 rPE-LDL Binding: ELISA was performed as described in Section 1.6.1 using LDL (500, 50, or 5 ng/well) with sheep anti-human LDL antibody (1:10,000) and donkey anti-sheep IgG-HRP (1:1,000).

1.7.2 EACA Inhibition: ELISA was performed with Lp(a) (500 ng/well) and EACA at 0, 0.2, 2, or 20 mmol/L.

1.7.3 rPE-Plg Binding: ELISA was performed with Plg (500, 50, or 5 ng/well) using mouse anti-human Plg monoclonal antibody (1:2,000) and sheep anti-mouse IgG-HRP (1:3,000).

1.7.4 Lp(a) Inhibition of rPE-Plg Binding: ELISA was performed with Plg (300 ng/well) and Lp(a) at 0, 50, 500, or 1,000 ng/well using antibodies described in Section 1.7.3.

Results

2.1 Gene Cloning and Protein Expression

The amplified *pe* and *peΔkk* gene fragments matched the expected sizes. After ligation, transformation, and colony PCR, sequencing confirmed that the nucleotide sequences were identical to the reference sequences. Following induction and purification, highly pure rPE and rPEΔKK were obtained with molecular weights of approximately 16 kDa, consistent with theoretical values.

2.2 Lp(a) Isolation and Identification

Density gradient centrifugation separated plasma lipoproteins based on particle size and density, with LDL and Lp(a) primarily floating at the 1.063 g/mL interface. At this stage, SDS-PAGE revealed minor contamination with human serum albumin (HSA). Subsequent purification using Q Sepharose™ Fast Flow anion-exchange chromatography yielded highly pure Lp(a), as evidenced by two distinct bands on SDS-PAGE [Figure 1: see original paper]. Western blotting with anti-Apo(a) and anti-ApoB-100 antibodies confirmed these bands corresponded to Apo(a) and ApoB-100 [Figure 1: see original paper]. The final Lp(a) concentration was 169 g/mL by BCA assay.

2.3 rPE Binding to Lp(a)

ELISA Results: Both rPE and rPEΔKK bound to Lp(a) in a concentration-dependent manner, with binding increasing as Lp(a) concentration rose from

0 to 500 ng/well [Figure 2: see original paper]. However, rPEΔKK showed significantly reduced binding capacity compared to rPE, with a 58.42% decrease at 50 ng/well Lp(a).

Pull-Down Results: Western blot analysis confirmed that rPE and rPEΔKK both captured Lp(a) from plasma, but binding was markedly weaker for rPEΔKK [Figure 3: see original paper], consistent with ELISA findings.

2.4 Binding Mechanism

rPE-LDL Interaction: ELISA demonstrated no detectable binding between rPE and LDL [Figure 4: see original paper].

EACA Inhibition: EACA inhibited rPE-Lp(a) binding in a dose-dependent manner, reducing binding by 9.09%, 68.34%, and 91.19% at concentrations of 0.2, 2, and 20 mmol/L, respectively [Figure 5: see original paper].

rPE-Plg Interaction: Both rPE and rPEΔKK bound Plg in a concentration-dependent manner, though the reduction in binding for rPEΔKK was modest (15% decrease at 500 ng/well Plg) [Figure 6: see original paper].

Lp(a) Inhibition: Lp(a) showed only weak inhibition of rPE-Plg binding, reducing the interaction by 10% at 50 ng/well [Figure 7: see original paper].

Discussion

Conventional Lp(a) purification relies on sequential ultracentrifugation combined with size-exclusion chromatography [14, 15]. While this approach enables large-scale preparation, it is time-consuming and cannot effectively separate Lp(a) from LDL. Our method using density gradient centrifugation significantly reduced ultracentrifugation time, and the subsequent Q Sepharose™ Fast Flow ion-exchange chromatography successfully removed contaminating LDL, yielding high-purity Lp(a).

Since its discovery in 1963, Lp(a) research has primarily focused on cardiovascular disease, while its physiological functions remain largely undefined [16]. Based on structural similarities between Apo(a) and Plg, our laboratory proposed the anti-infection hypothesis of Lp(a) and has now demonstrated that rPE binds to Lp(a) but not LDL, indicating that the interaction occurs primarily with Apo(a). The effective inhibition by EACA suggests that rPE binds to the LBS within Apo(a). Both ELISA and pull-down assays showed that rPEΔKK, lacking C-terminal lysine residues, exhibited markedly reduced Lp(a) binding, confirming that these residues constitute the primary binding site on PE. Collectively, these results demonstrate that rPE binds Lp(a) through the C-terminal lysine residues of PE and the LBS of Apo(a).

The modest inhibitory effect of Lp(a) on rPE-Plg binding may be explained by distinct binding sites. Previous work by Barthel et al. [6] identified residues

41-68 in the N-terminus of PE as the primary Plg-binding region. Our finding that rPEΔKK retained most of its Plg-binding activity supports this conclusion and suggests that Lp(a) primarily interacts with the C-terminal lysine residues rather than the N-terminal Plg-binding domain. Consequently, Lp(a) exerts only weak competitive inhibition against Plg binding.

Our laboratory has previously shown that Lp(a) binds to multiple plasminogen receptors on various pathogens, including -enolase and inosine 5' -monophosphate dehydrogenase from *Staphylococcus aureus*, enolase and glyceraldehyde-3-phosphate dehydrogenase from Group A *Streptococcus*, dihydrolipoamide dehydrogenase from *Pseudomonas aeruginosa*, and aspartase and Protein F from NTHi [13, 17-22]. This study extends these findings to PE, another NTHi plasminogen receptor. However, the weak inhibitory effect of Lp(a) on PE-Plg binding suggests that the physiological or pathological significance of this interaction requires further investigation.

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