Binding of Asp-hemolysin from *Aspergillus fumigatus* to Oxidized Low Density Lipoprotein

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Asp-hemolysin is a human low density lipoprotein (LDL) binding protein. We found evidence that Asp-hemolysin binds to oxidized LDL (oxLDL) as well as to LDL in a concentration-dependent manner. This result suggests that Asp-hemolysin is a novel protein, which shares binding abilities to LDL and oxLDL.

Key words Asp-hemolysin; oxidized low density lipoprotein (oxLDL); binding protein

Asp-hemolysin is a hemolytic toxin that is produced by *Aspergillus fumigatus*.¹⁾ The toxin is lethal to animals (mouse, chicken) and lytic for erythrocytes (human, rabbit, and sheep). The gene for Asp-hemolysin has been cloned and the gene sequence has been reported.²⁾ The sequence of the primary Asp-hemolysin gene product, predicted from the cDNA sequence, consists of 131 amino acid residues and has a molecular mass of 14275.

We previously reported that the low density lipoprotein (LDL) apoprotein, apolipoprotein B (apoB), inhibits the hemolytic activity of Asp-hemolysin.³⁾ [¹²⁵I] LDL is bound to the immobilized Asp-hemolysin with high affinity ($K_d = 8.9 \times 10^{-9}$ M).⁴⁾ We recently reported that oxidized LDL (oxLDL) inhibits the hemolytic activity of Asp-hemolysin, and that the effect is greater than the effect of LDL.⁵⁾ In this paper, we report that Asp-hemolysin binds to oxLDL. To our knowledge, Asp-hemolysin is a novel protein, which shares binding abilities to LDL and oxLDL.

MATERIALS AND METHODS

Purification and Modification of Asp-hemolysin The isolation and purification of Asp-hemolysin were performed basically in the same way as previously described.^{1,3)} The homogeneity of the purified Asp-hemolysin was examined by SDS-PAGE⁶⁾ and reverse HPLC analysis. The modification of Asp-hemolysin using sulfo-N-hydroxysulfosuccinimide-acetate (sulfo-NHS-acetate) (Pierce) was performed according to the manufacturer's instructions. Briefly, Asp-hemolysin (2 mg) was incubated with 1 mg of sulfo-NHS-acetate in 1 ml of 20 mM phosphate buffered saline (PBS, pH 7.4) at 21 °C for 1 h, and dialyzed against PBS to separate excess reagent away from Asp-hemolysin.

Preparation and Oxidation of LDL LDL ($d = 1.019$ – 1.063 g/ml) was isolated from normolipidemic human plasma by sequential ultracentrifugation.⁷⁾ OxLDL was pre-

pared by incubating LDL (200 μ g of protein/ml) with 5 μ M CuSO₄ in PBS without EDTA for 2 h at 37 °C. Oxidation was arrested by refrigeration and the addition of 100 μ M EDTA and 20 μ M butylated hydroxytoluene (BHT).⁸⁾ Control incubation was done without CuSO₄ and with EDTA and BHT added prior to the incubation. The lipid peroxide formation of oxLDL was estimated as the thiobarbituric acid reactive substance (TBARS).⁹⁾ Tetraethoxypropane was used as the standard and the results were expressed as nanomoles of malondialdehyde (MDA) equivalents. The TBARS contents of LDL and oxLDL were 1.02 ± 0.11 , 60.71 ± 6.75 (mean $\pm$ S.D.) nmol MDA/mg protein, respectively. The electrophoretic mobility of the oxLDL preparation was identical using one-dimensional agarose electrophoresis and 1.7 times that of LDL.

Purification of IgG and Preparation of Fab fragment

The IgG fraction was purified from goat anti-human apoB antiserum (CHEMICON) by affinity chromatography using the HiTrap Protein G column (Pharmacia). Fab fragments of the IgG fraction were obtained by digestion with papain.¹⁰⁾ The digested mixture was applied to a Protein G column to remove the Fc fragments and any undigested IgG. The purity of the IgG and Fab was monitored by SDS-PAGE.

Binding Analysis The binding analysis of Asp-hemolysin to oxLDL was performed by immunoblotting. Asp-hemolysin or modified Asp-hemolysin (2 μ g) were incubated with oxLDL (0–40 μ g as protein) in 50 μ l of 0.15 M NaCl–20 mM Tris–HCl buffer (pH 7.4) at 37 °C for 5 h. After incubation, the reaction mixture was subjected to nondenaturing PAGE (4% gel) for separating the bound and free Asp-hemolysin. Gels were either stained with coomassie brilliant blue or transferred to Immobilon P (Millipore). The membrane was blocked with 5% skim milk for 2 h, and then shaken with either 1:3000 rabbit anti-Asp-hemolysin antibody or anti-LDL antibody (NBT) for 12 h. Immunodetection using the goat anti-rabbit IgG Fc antibody and rabbit peroxidase anti-peroxidase complex was performed with the enhanced chemiluminescence (ECL) western blot detection kit (Amersham).

In addition, oxLDL (4 μ g as protein) was preincubated with anti-apoB Fab fragments (200 μ g) at 21 °C for 5 h, and then the binding analysis of the treated oxLDL and Asp-hemolysin was also performed.

Other Assay Proteins were determined by the procedure of Lowry *et al.*¹¹⁾ with BSA as the standard.

RESULT AND DISCUSSION

To determine whether Asp-hemolysin binds to oxLDL, we attempted to observe the binding of Asp-hemolysin to oxLDL using immunoblot analysis after nondenaturing PAGE of the Asp-hemolysin and oxLDL mixture (Fig. 1). Fig. 1A shows the Asp-hemolysin and oxLDL migrating bands detected with coomassie brilliant blue staining. In the immunoblot analysis, Asp-hemolysin incubated with oxLDL was detected as the Asp-hemolysin-oxLDL complex run in the same position as oxLDL alone. The intensity of the Asp-hemolysin-oxLDL complex bands increased at higher oxLDL concentrations, whereas the intensity of the free Asp-hemolysin bands decreased as the concentration of oxLDL

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Fig. 1. Dose Dependent Binding of Asp-hemolysin to oxLDL

A. Asp-hemolysin (AH: Lane 1) and oxLDL (Lane 2) were electrophoresed on a 4% nondenaturing PAGE. Proteins were stained with coomassie brilliant blue. B. Asp-hemolysin was incubated with various concentration of oxLDL (Lane 1, 0 μ g; Lane 2, 0.8 μ g; Lane 3, 4 μ g; Lane 4, 8 μ g; Lane 5, 40 μ g). The mixtures were applied to a 4% nondenaturing PAGE and immunoblotted with anti-Asp-hemolysin antibody and detected by ECL. C. Asp-hemolysin was incubated with oxLDL. The mixture was applied to a 4% nondenaturing PAGE and immunoblotted with anti-LDL antibody and detected by ECL.

increased (Fig. 1B). It is possible that the oxLDL promote the aggregation of Asp-hemolysin monomers; hence smear bands with higher molecular weight may be just a polymer form of Asp-hemolysin. Nevertheless, the result in Fig. 1C clearly shows that the products with high molecular weight do contain the oxLDL. These results indicated that Asp-hemolysin binds to oxLDL in a concentration-dependent manner.

The macrophage scavenger receptor and lactoferrin are known as oxLDL binding proteins. The work of Doi *et al.* raised the possibility that a charged collagen structure containing a lysine cluster of the scavenger receptor specifically interacts with oxLDL.¹²⁾ Kajikawa *et al.* reported that a region rich in lysine residues near the N-terminus of lactoferrin, which resembles the ligand-binding site of the scavenger receptor, may be responsible for the oxLDL binding ability.¹³⁾ Since it was suspected that positive-charge amino acids in the Asp-hemolysin molecule may provide the binding to oxLDL, we performed a binding analysis using Asp-hemolysin treated with sulfo-NHS-acetate to block the primary amine (lysine ϵ) groups. As shown in Fig. 2B, the binding ability of the modified Asp-hemolysin to oxLDL completely disappeared. This result suggests that lysine ϵ amino groups of Asp-hemolysin are necessary for the binding of Asp-hemolysin to oxLDL. Furthermore, to define whether the binding of Asp-hemolysin to oxLDL is due to the apoB component of oxLDL, the interaction between Asp-hemolysin and oxLDL treated with the Fab fragment of anti-apoB IgG was investigated. As shown in Fig. 2C, the binding of Asp-hemolysin to oxLDL treated with the anti-apoB Fab fragment was almost abolished. This result suggests that apoB of oxLDL is involved in the binding of Asp-hemolysin to oxLDL.

Our recent studies have provided evidence that Asp-hemolysin binds to LDL, known as a ligand for the LDL recep-

Fig. 2. Effect of Various Condition on Binding of Asp-hemolysin to oxLDL

A. Asp-hemolysin (AH: Lane 1), modified Asp-hemolysin (modified AH: Lane 2), and oxLDL (Lane 3) were electrophoresed on a 4% nondenaturing PAGE. Proteins were stained with coomassie brilliant blue. B. Asp-hemolysin (Lane 1) or modified Asp-hemolysin (Lane 2) were incubated with oxLDL (10 μ g as protein) at 37°C for 5 h. The mixtures were applied to a 4% nondenaturing PAGE and immunoblotted with anti-Asp-hemolysin antibody and detected by ECL. C. OxLDL (4 μ g as protein) was preincubated with anti-apoB Fab fragments (200 μ g) at 21°C for 5 h. The binding analysis of Asp-hemolysin to oxLDL (Lane 1) or the treated oxLDL (Lane 2) was performed as described above.

tor. In this paper, we revealed additional evidence that Asp-hemolysin binds to oxLDL as well as to LDL. Furthermore, we discussed the possibility of the positive charged amino acid residues involved in the binding activity. In addition to sulfo-NHS-acetate modification, site-directed mutagenesis may provide more convincing result, since the cDNA of Asp-hemolysin has been cloned and determined.²⁾ A key event in the pathogenesis of atherosclerosis is thought to be the formation of macrophage-derived foam cells by scavenger receptor uptake of oxLDL. Further study on the mechanism of Asp-hemolysin-oxLDL interaction may provide important information on the prevention and treatment of atherosclerosis.

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