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Oxidized low density lipoprotein inhibits the hemolytic activity of Asp-hemolysin from *Aspergillus fumigatus*

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Abstract

We have examined the effect of chemically modified human low density lipoproteins (LDLs), acetylated LDL and oxidized LDL, on the hemolytic activity of Asp-hemolysin. Oxidized LDL, but not acetylated LDL, inhibited the hemolytic activity of this toxin. The inhibitory effects of oxidized LDL increased with the time of Cu^{2+}-induced LDL oxidation. Similar inhibition was observed in the filtrate which was separated from the incubation mixture of Asp-hemolysin with oxidized LDL (for 2 h of oxidation) following ultrafiltration through a membrane with a molecular mass cutoff of 100000. However, at longer LDL oxidation times, the inhibition by the filtrates was less than the control mixture without ultrafiltration. We suggest that the inhibition by oxidized LDL was due to the binding of oxidized LDL to Asp-hemolysin at shorter LDL oxidation times.

Keywords: Asp-hemolysin; Hemolytic activity; Low-density lipoprotein; Oxidized low-density lipoprotein

1. Introduction

Asp-hemolysin is a cytolytic toxin that is produced by *Aspergillus fumigatus* [1]. This toxin is lethal to mice amd chickens, and lytic for erythrocytes of humans, rabbits and sheep. The gene for Asp-hemolysin has been cloned and the gene sequence 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 recently reported that the low density lipoprotein (LDL) apoprotein, apolipoprotein B, inhibits the hemolytic activity of Asp-hemolysin [3]. ^{125}I-Labeled LDL is bound to the immobilized Asp-hemolysin with high affinity ($K_d = 8.9 \times 10^{-9}$ M) [4].

To better understand the interaction between LDL and Asp-hemolysin, we have examined the effect of two chemically modified lipoproteins, acetylated LDL and oxidized LDL, on the hemolytic activity of Asp-hemolysin.

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2. Materials and methods

2.1. Asp-hemolysin

The isolation and purification of Asp-hemolysin were done as previously described [1,3]. The homogeneity of the purified Asp-hemolysin was examined by sodium dodecylsulfate-polyacrylamide gel electrophoresis [5].

The hemolysin assay is based on the hemolytic activity of the toxin towards human erythrocytes, as previously described [3]. Measurement of the optical absorbance was carried out at 541 nm on the hemoglobin released from erythrocytes incubated at 21°C for 30 min with 20 μl of appropriately diluted toxin in phosphate buffered saline (PBS), pH 7.4.

2.2. LDL isolation and modification

Human LDL was prepared from human plasma by potassium bromide stepwise density gradient centrifugation [6]. After centrifugation, the fractions with a density of 1.019–1.063 g ml^{-1} were recovered as LDL.

Chemical modification of LDL by acetylation was done using the method of Basu et al. [7]. LDL was oxidized by incubation (200 μg protein ml^{-1}) with 5 μM $CuSO_4$ in PBS without EDTA for periods of up to 24 h at 37°C.

Oxidation was arrested by refrigeration and the addition of 100 μM EDTA and 20 μM butylated hydroxytoluene. Control incubation was done without $CuSO_4$, and with EDTA and butylated hydroxytoluene added prior to the incubation [8].

Lipid peroxide formation of oxidized LDL was estimated as the thiobarbituric acid reactive substance. Tetraethoxypropane was used as a standard and results were expressed as nmol of malondialdehyde equivalents. The thiobarbituric acid reactive substance contents of LDL, acetylated LDL and oxidized LDL (24 h of oxidation) were 1.27 ± 0.13, 1.57 ± 0.43 and 58.45 ± 5.60 (mean ± S.D.) nmol malondialdehyde mg $protein^{-1}$, respectively. The electrophoretic mobility of the acetylated LDL and oxidized LDL (24 h of oxidation) preparations were identical and 4.5 times that of LDL. The lysophosphatidylcholine accumulating during oxidation was removed by incubating with 10 mg of fatty acid-free bovine serum albumin per ml for 24 h at 37°C and reisolating the LDL by preparative ultracentrifugation ($d < 1.210$) [9].

2.3. Hemolytic activity after incubation with modified lipoproteins

Modified lipoproteins (10 μl) were incubated for 2 h at 21°C with Asp-hemolysin (10 μg 10 μl^{-1}) containing 20 μg ml^{-1} phenylmethylsulfonyl fluoride. PBS (600 μl) and 400 μl of 2% (v/v) human erythrocyte suspension were added to the mixture and incubated. The control included 10 μg Asp-hemolysin and PBS. After incubation at 21°C for 30 min, the hemolytic activity of the final incubation mixture was then measured.

In addition, the incubation mixture of Asp-hemolysin with oxidized LDL was separated by ultrafiltration using a filter with a molecular cutoff of 100 000 treated with 1% bovine serum albumin, and the hemolytic activity of the filtrate was also measured.

2.4. Hemolytic activity of Asp-hemolysin after incubation with lysophosphatidylcholine

Lysophosphatidylcholine dissolved in chloroform/methanol (2:1, v/v) was coated on the wall of a silanized glass tube during evaporation of the solvent under a stream of nitrogen gas. The lysophosphatidylcholine was hydrated with PBS or 10 mM phosphate buffer (pH 7.6), vortexed vigorously, and then sonicated in a bath sonicator for 30 min before use.

2.5. Analytical procedures

The protein content of the lipoproteins was assayed using the method of Lowry et al. [10] with bovine serum albumin as the standard; 0.05% sodium deoxycholate was added to minimize turbidity [8]. The thiobarbituric acid reactive substance contents were determined using a lipoperoxide test kit according to the method of Yagi [11]. Increases in the electrophoretic mobility of oxidized LDL and acetylated LDL were determined by one-dimensional agarose electrophoresis. Lysophosphatidylcholine contents were determined using a phospholipid test kit according to the method of Takayama et al. [12].

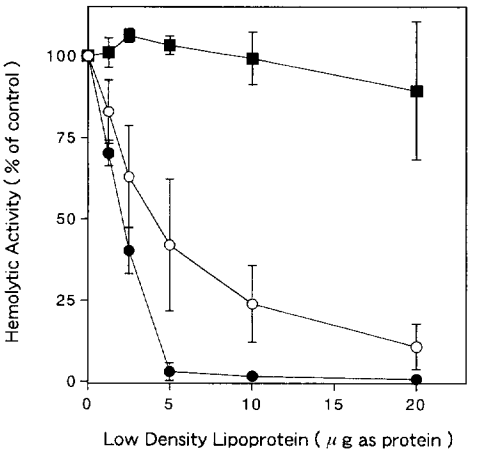

Fig. 1. Effect of modified lipoproteins on the hemolytic activity of Asp-hemolysin. The values are mean ± S.D. of three separate experiments. ○, Low density lipoprotein; ●, oxidized low density lipoprotein; ■, acetylated low density lipoprotein.

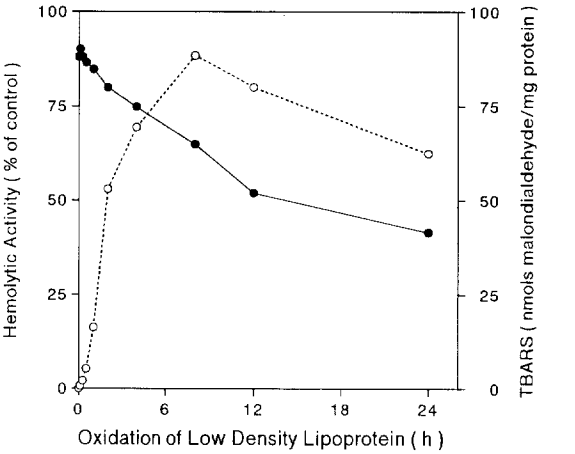

Fig. 2. Dependence of inhibition by oxidized low density lipoprotein on the time of Cu^{2+}-induced oxidation. ●, Hemolytic activity; ○, thiobarbituric acid reactive substances (TBARS).

3. Results

3.1. Effect of modified lipoproteins on the hemolytic activity of Asp-hemolysin

To test the inhibitory effect of each chemically modified LDL on the hemolytic activity of Asp-hemolysin, various amounts of lipoproteins were incubated with the toxin (Fig. 1). When Asp-hemolysin was incubated with oxidized LDL (24 h of oxidation), we observed a dose-dependent inhibition of hemolytic activity. Complete inhibition was achieved at a dose of 10 μg oxidized LDL (as protein). As previously reported, LDL also inhibited the activity of Asp-hemolysin, but the effect was much less pronounced than the effect by oxidized LDL. A 50% inhibition dose was estimated to be 5 μg LDL, and the inhibition reached only 90% at 20 μg. In contrast, acetylated LDL had no effect on the hemolytic activity.

3.2. Dependence of inhibition by oxidized LDL on the oxidation time by Cu^{2+}

We compared the effect of progressive LDL oxidation on the degree of inhibition of the hemolytic activity of Asp-hemolysin. For this purpose, LDL was oxidized with 5 μM $CuSO_4$ at the indicated time points (Fig. 2). When LDL (200 μg of protein ml^{-1}) was incubated at 37°C, time-dependent increases were found in the thiobarbituric acid reactive substance (Fig. 2, open circles) and in the electrophoretic mobility relative to LDL (data not shown). The increase in thiobarbituric acid reactive substance was the greatest between 4 and 12 h.

To test the effects of the various oxidized samples of LDL on the hemolytic activity of Asp-hemolysin, each oxidized LDL (2 μg of protein ml^{-1}) was incubated with Asp-hemolysin. As the LDL oxidation progressed, the hemolytic activity of Asp-hemolysin

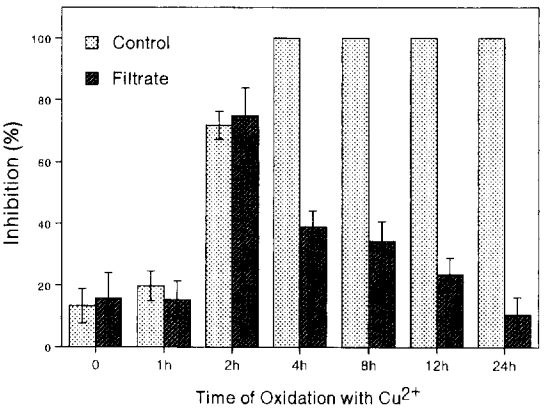

Fig. 3. Effect of ultrafiltration on the inhibition by oxidized low density lipoprotein. The values are mean ± S.D. of three separate experiments.

steadily decreased (Fig. 2, closed circles). Oxidized LDL (24 h) gave 60% inhibition of hemolytic activity.

3.3. *Effect of ultrafiltration on the inhibition by oxidized LDL*

To determine whether the inhibition was due to the effect of oxidized LDL binding to Asp-hemolysin, the incubation mixture of each oxidized LDL (5 μg) with Asp-hemolysin was separated by ultrafiltration (molecular cutoff = 100 000) and the hemolytic activity of the filtrate was measured. As a control, the hemolytic activity of unfiltrated incubation mixture was also measured.

Fig. 3 shows that the inhibition by oxidized LDL in the control experiment increased with the time of oxidation and reached 100%. A similar inhibition was observed in the ultrafiltrate of the mixture of Asp-hemolysin with oxidized LDL within 2 h, but the inhibition declined over the next 24 h. The filtrate of oxidized LDL (24 h) inhibited the activity by only 10%.

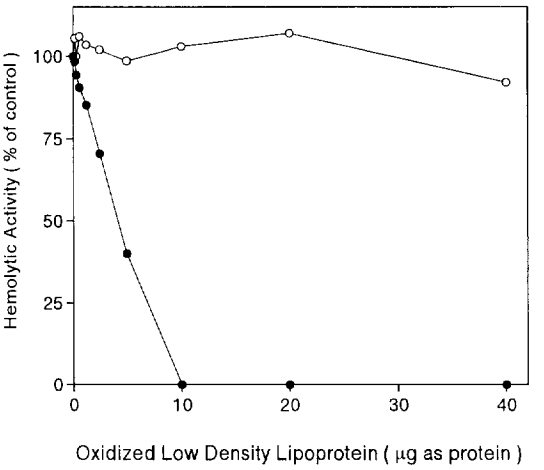

Fig. 4. Effect of bovine serum albumin-treated oxidized low density lipoprotein on the hemolytic activity of Asp-hemolysin. ●, Oxidized low density lipoprotein; ○, bovine serum albumin-treated oxidized low density lipoprotein.

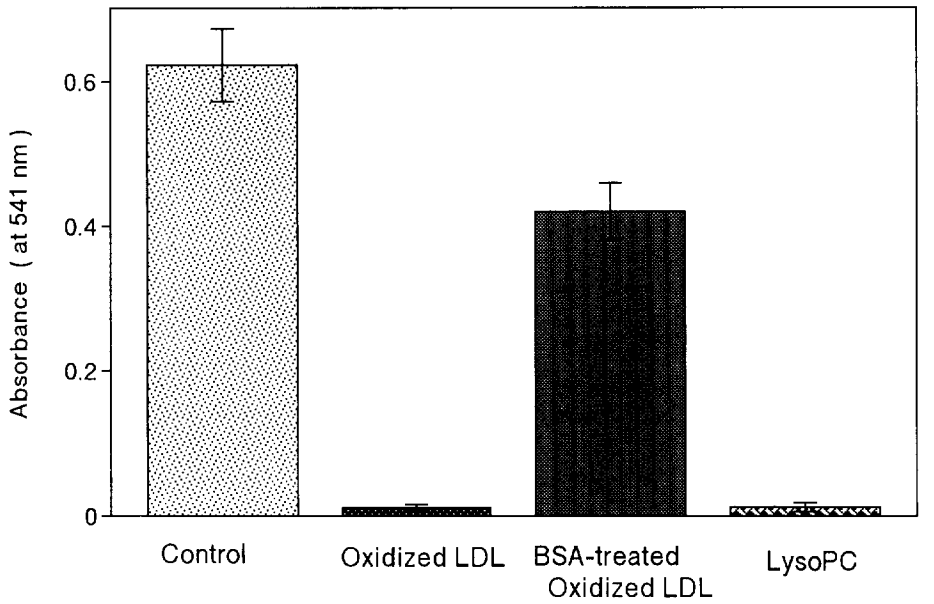

Fig. 5. Effect of preincubation of erythrocytes with oxidized low density lipoprotein on the hemolytic activity of Asp-hemolysin. The erythrocyte suspension (200 μl) was incubated with 10 μg (50 μl) oxidized low density lipoprotein (oxidized LDL), bovine serum albumin-treated oxidized low density lipoprotein (BSA-treated oxidized LDL) or 2 μg lysophosphatidylcholine (lysoPC) suspension in PBS for 1 h at 21°C. As a control, the same volume of PBS instead of each sample was used. Each incubation mixture was washed 3 times with PBS, and then Asp-hemolysin (10 μg) was added. The values are means ± S.D. of three separate experiments.

3.4. Effect of bovine serum albumin-treated oxidized LDL on the hemolytic activity of Asp-hemolysin

To test whether lysophosphatidylcholine in oxidized LDL (24 h) played a role in the inhibition of hemolysis by oxidized LDL, oxidized LDL (24 h) was pretreated with fatty acid-free bovine serum albumin to remove the lysophosphatidylcholine and reisolated by preparative ultracentrifugation. As shown in Fig. 4, 10 μg oxidized LDL (24 h) completely inhibited the hemolytic activity of Asp-hemolysin, whereas the bovine serum albumin-treated oxidized LDL (24 h) had no effect.

In addition, preincubation of the erythrocytes with oxidized LDL (24 h) resulted in the inhibition of hemolysis by Asp-hemolysin and this effect was eliminated by prior treatment of the oxidized LDL (24 h) with bovine serum albumin (Fig. 5). Preincubation of the erythrocytes with 2 μg lysophosphatidylcholine suspension in PBS also resulted in the inhibition of hemolysis by Asp-hemolysin. However, incubation of the erythrocytes with 5 μg lysophosphatidylcholine in PBS caused hemolysis without Asp-hemolysin.

4. Discussion

The data presented here demonstrate that oxidized LDL inhibits the hemolytic activity of Asp-hemolysin, and the effect is greater than the inhibitory effect of LDL. Furthermore, we demonstrated that the inhibition by oxidized LDL increases with Cu^{2+}-induced oxidation time. To our knowledge, this is the first report of a microbial hemolysin whose activity is inhibited by oxidized LDL.

The data in Fig. 3 strongly suggest that the inhibition by oxidized LDL was due to the binding of oxidized LDL to Asp-hemolysin at short LDL oxidation times. Moreover, these data suggest that the affinity for the binding of oxidized LDL (2 h) to Asp-hemolysin may be higher than the affinity for the binding of LDL ($K_d = 8.9 \times 10^{-9}$ M) [4]. However, at longer LDL oxidation times, the inhibition of the incubation mixture of Asp-hemolysin with oxidized LDL by the ultrafiltrates was less than the control mixture without ultrafiltration. Indeed, an additional mechanism has been shown to operate during the inhibition of Asp-hemolysin by oxidized LDL (24 h), as discussed below.

The consequences of oxidation include the peroxidation of polyunsaturated fatty acids in the LDL phospholipids followed by the conversion of phosphatidylcholine to lysophosphatidylcholine [13]. Lysophosphatidylcholine is known to be a hemolytic agent. However, Sambrano et al. reported that oxidized LDL containing lysophosphatidylcholine caused erythrocyte crenation, but not hemolysis [9]. It is conceivable that the inhibition of hemolytic activity of Asp-hemolysin by oxidized LDL (24 h) is due to this structural membrane change in the erythrocytes. There are two lines of evidence that support this hypothesis. First, Asp-hemolysin had no effect on the erythrocytes preincubated with oxidized LDL (24 h) or lysophosphatidylcholine suspension (2 μg). Second, and more important, the ability of oxidized LDL (24 h) was abolished by the removal of lysophosphatidylcholine.

Cell culture studies have identified many cell types capable of oxidizing LDL including endothelial cells, smooth muscle cells, monocytes, macrophages and neutrophils. If oxidized LDL exists in the foci of infection, the interaction between Asp-hemolysin and oxidized LDL may play an important role in the pathogenesis of infection by *A. fumigatus*.

In summary, we have shown that oxidized LDL dramatically inhibits the hemolytic activity of Asp-hemolysin. Our results imply that oxidized LDL binds to Asp-hemolysin at short LDL oxidation times.

References

[1] Yokota, K., Shimada, H., Kamaguchi, A. and Sakaguchi, O. (1977) Studies on the toxin of *Aspergillus fumigatus*. VII. Purification and some properties of hemolytic toxin (Asp-hemolysin) from culture filtrates and mycelia. Microbiol. Immunol. 21, 11–22.

[2] Ebina, K., Sakagami, H., Yokota, K. and Kondo, H. (1994) Cloning and nucleotide sequence of cDNA encoding Asp-hemolysin from *Aspergillus fumigatus*. Biochim. Biophys. Acta 1219, 148–150.

[3] Fukuchi, Y., Kumagai, T., Ebina, K. and Yokota, K. (1996) Apolipoprotein B inhibits the hemolytic activity of Asp-hemolysin from *Aspergillus fumigatus*. Biol. Pharm. Bull. 19, 547–550.

[4] Fukuchi, Y., Kudo, Y., Kumagai, T., Ebina, K. and Yokota,

K. (1996) Binding assay of low density lipoprotein to Asphemolysin from *Aspergillus fumigatus*. Biol. Pharm. Bull. 19, 1380–1381.

[5] Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

[6] Havel, R.J., Eder, H.A. and Bragdon, J.H. (1955) The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. J. Clin. Invest. 34, 1345–1353.

[7] Basu, S.K., Goldstein, J.L., Anderson, R.G.W. and Brown, M.S. (1976) Degradation of cationized low density lipoprotein and regulation of cholesterol metabolism in homozygous familial hypercholesterolemia fibroblasts. Proc. Natl. Acad. Sci. USA 73, 3178–3182.

[8] Steinbrecher, U.P. (1987) Oxidation of human low density lipoprotein results in derivatization of lysine residues of apolipoprotein B by lipid peroxide decomposition products. J. Biol. Chem. 262, 3603–3608.

[9] Sambrano, G.R., Parthasarathy, A. and Steinberg, D. (1994) Recognition of oxidatively damaged erythrocytes by a macrophage receptor with specificity for oxidized low density lipoprotein. Proc. Natl. Acad. Sci. USA 91, 3265–3269.

[10] Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265–275.

[11] Yagi, K. (1976) A simple fluorometric assay for lipoperoxide in blood plasma. Biochem. Med. 15, 212–216.

[12] Takayama, M., Itoh, S., Nagasaki, T. and Tanimizu, I. (1977) A new enzymic method for determination of serum colin-containing phospholipids. Clin. Chim. Acta 79, 93–98.

[13] Steinbrecher, U.P., Parthasarathy, S., Leake, D.S., Witztum, J.L. and Steinberg, D. (1984) Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. Proc. Natl. Acad. Sci. USA 81, 3883–3887.