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The lysine cluster in the collagen-like domain of the scavenger receptor provides for its ligand binding and ligand specificity

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Abstract Scavenger receptors bind modified low-density lipoproteins (LDL) on a collagen-like domain which possesses a lysine cluster at the carboxy end. We previously constructed a receptor model peptide containing the lysine cluster. In the present study, we evaluated the ligand specificity of the receptor model peptide. It selectively bound modified-LDLs, and not LDL. The binding of acetylated-LDL (Ac-LDL) was inhibited by dextran sulfate, fucoidan, and sulfatides in a manner similar to that of the natural receptor. Both polyguanylic and polyinosinic acids inhibit the Ac-LDL binding whereas polycytidylic acid did not. These results indicate that the lysine cluster in the collagen-like domain has important roles in both ligand binding and ligand specificity.

Key words: Scavenger receptor; Collagen-like domain; Inhibitor; Binding assay; Lysine cluster

1. Introduction

The scavenger receptor has broad binding specificity and recognizes a variety of macromolecules. It binds modified low-density lipoproteins (LDL) and internalizes them into the cell. The excessive uptake of modified LDL causes the conversion of macrophages into foam cells [1–5]. These cells contain excess cholesterol esters and form fatty streaks and atherosclerotic lesions. The receptor also binds β-amyloid peptides and may cause the onset of Alzheimer's disease [6]. Moreover, it binds advanced glycation end products (AGE), and AGE binding may be involved in the aging process and in diabetic complications [7]. Thus the scavenger receptor participates in the onset of several diseases. Furthermore, the receptor binds fucoidan, sulfatides, dextran sulfate, maleylated BSA, polyinosinic acid (poly I), and polyguanylic acid (poly G) [8]. On the other hand, it does not bind LDL, BSA, polycytidylic acid (poly C), and polyadenylic acid (poly A). In this way, the receptor has broad ligand-binding specificity but retains the ability to distinguish certain macromolecules.

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Abbreviations: LDL, low-density lipoprotein; Ac-LDL, acetylated low-density lipoprotein; Ox-LDL, oxidized low-density lipoprotein; AGE, advanced glycation end product; DMEM, Dulbecco's modified Eagle medium; PBS, phosphate-buffered saline; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; EDTA, ethylenediaminetetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; h, 4-hydroxyproline; Ahx, aminohexanoic acid; βAla, β-alanine

The type I bovine macrophage scavenger receptor consists of 453 amino acids, as deduced from its nucleotide sequences [9–13], and exists in a trimerized form. It is divided into six domains: an N-terminal cytoplasmic (50 a.a.), a membrane-spanning (26 a.a.), a spacer (32 a.a.), an α-helical coiled-coil (163 a.a.), a collagen-like (72 a.a.), and a C-terminal type-specific (110 a.a.) domains. Analyses of deletion mutants from the C-terminus of the type I receptor showed that the collagen-like domain was essential for ligand binding and that the α-helical coiled-coil domain was important for maintaining a trimeric structure. Analyses of point mutants in the collagen-like domain indicated that four Lys residues, at 327, 334, 337, and 340, which form a lysine cluster, were necessary for ligand binding [14]. We constructed a receptor model peptide derived from Gly^{323} to Lys^{340}, including the lysine cluster of the bovine scavenger receptor, and showed that it formed a collagen structure and bound Ac-LDL [15,16].

In this study, in order to further evaluate the role of the lysine cluster, we analyzed the ligand binding specificity of the receptor model peptide and compared it with that of the natural scavenger receptor. Here we show that the receptor model peptide has ligand binding specificity similar to that of the natural scavenger receptor, indicating that the lysine cluster is involved in not only ligand binding but also in ligand specificity.

2. Materials and methods

2.1. Materials

Dulbecco's modified Eagle medium (DMEM) and fetal calf serum (FCS) were purchased from Gibco (Grand Island, NY). $Na^{125}I$ was purchased from DuPont New England Nuclear (Boston, MA). Streptavidin-Dynabeads M-280 were purchased from DYNAL A.S. (Oslo, Norway). Female ddY mice were obtained from Nihon SLC Co. (Shizuoka, Japan). All other reagents were purchased from Sigma (St. Louis, MO).

2.2. Receptor model peptide and $(GPK)_6$ peptide

The triple stranded receptor model peptide was prepared as described previously [15,16]. The triple stranded $(Gly–Pro–Lys)_6$, $(GPK)_6$ peptide, was prepared by the same method.

Streptavidin-Dynabeads M-280 (0.5 ml) were washed with 5 mM phosphate buffer (pH 7.4) containing 150 mM NaCl. The beads were then mixed with the biotinylated peptide (2.5 nmol) dissolved in the same buffer. After 30 min at room temperature, the peptide resin was washed 3 times with the same buffer, and was suspended in 0.5 ml of the same buffer. About 100 pmol of peptide were coupled to the beads (100 μl). Prior to the binding assay, the peptide resin (0.5 ml) was resuspended in 0.25 ml of DMEM.

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2.3. Lipoproteins

LDL was isolated from normolipidemic human plasma, containing 0.1% EDTA, 0.02% sodium azide, and 0.5 mg/ml benzamidine, at a density of 1.019–1.063 g/ml by sequential ultracentrifugation [17]. The lipoprotein-deficient serum (LPDS) was also isolated at a density greater than 1.21. LDL was acetylated by repeated additions of acetic anhydride as described [18], or was oxidized by exposure to $CuSO_4$ as described [19]. Ac-LDL was radioiodinated at a specific activity of 100–300 cpm/ng protein with $Na^{125}I$, using the iodine monochloride method [20].

2.4. Macrophages

Mouse peritoneal macrophages were prepared according to the method of Cohn and Morse [21]. Peritoneal macrophages were harvested in phosphate-buffered saline without Ca^{2+} and Mg^{2+} (PBS[−]) from non-stimulated female ddY mice (25–30 g). The cells were centrifuged at $800\times g$ for 5 min, washed once with PBS[−], and suspended at 2×10^6 cells/ml in DMEM containing 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cell (1 ml) suspension was plated in 12-well plastic dishes. After an incubation at 37°C for 2 h, the non-adherent cells were removed by washing twice with DMEM. Adherent cell monolayers were cultured with 1 ml of DMEM containing 10% FCS, and were used for the binding assay on the next day.

2.5. Ac-LDL binding assay

The peptide resin suspension (8 μl in DMEM) was mixed with 15 μg/ml ^{125}I-Ac-LDL and a drug in DMEM containing 10 mM HEPES-Na (pH 7.4) and 10% LPDS, in a total volume of 50 μl, and was incubated for 2 h at 4°C. Non-specific binding was estimated by using beads lacking the biotinylated peptide. After the incubation, the peptide resin was washed 3 times with ice-cold 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mg/ml BSA. Bound ^{125}I-Ac-LDL was eluted with 50 mM NaCl, 10 mM HEPES-Na (pH 7.4), and 4 mg/ml dextran sulfate for 1.5 h at 4°C, and the radioactivity of the eluted ^{125}I-Ac-LDL was counted in a gamma counter.

The cells were pre-incubated in DMEM containing 10 mM HEPES-Na (pH 7.4) and 10% LPDS for 30 min at 4°C. The medium was then changed to 1 ml of DMEM containing 10 mM HEPES-Na (pH 7.4), 10% LPDS, 10 μg/ml ^{125}I-Ac-LDL, and a drug, and the cells were further incubated for 2 h at 4°C. Non-specific binding was estimated in the presence of a 40-fold excess of unlabeled Ac-LDL. After the incubation, the cells were washed once rapidly and twice for 10 min with ice-cold 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, and 2 mg/ml BSA, and were washed once with ice-cold 50 mM Tris-HCl (pH 7.4), and 150 mM NaCl. Bound ^{125}I-Ac-LDL was eluted with 50 mM NaCl, 10 mM HEPES-Na (pH 7.4), and 4 mg/ml dextran sulfate for 1.5 h at 4°C, and the radioactivity of the eluted ^{125}I-Ac-LDL was counted in a gamma counter. The amount of the released ^{125}I-Ac-LDL by dextran sulfate represents the amount of the surface-bound ^{125}I-Ac-LDL. After the elution of the bound ^{125}I-Ac-LDL, the cells were dissolved in 0.1 N NaOH, and the protein concentration of the cell lysate was determined by the method of Lowry et al. [22].

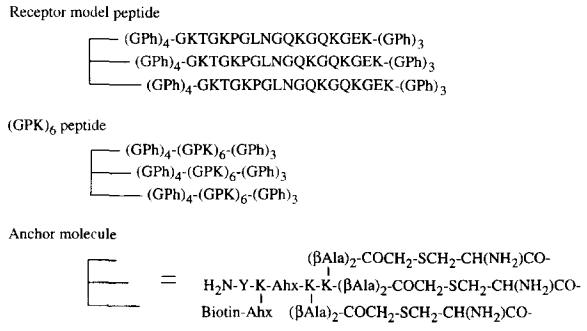

Fig. 1. Structures of the receptor model peptide, the $(GPK)_6$ peptide, and the anchor molecule.

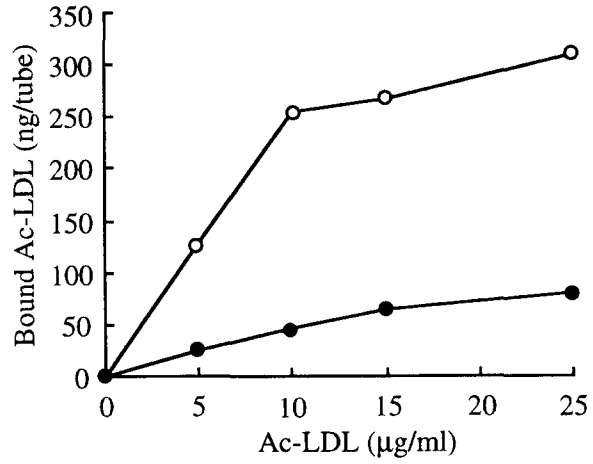

Fig. 2. Activities of ^{125}I-Ac-LDL binding to the receptor model peptide (●) and the $(GPK)_6$ peptide (○). The resin, immobilized with either the receptor model peptide or the $(GPK)_6$ peptide, was incubated with various amounts of ^{125}I-Ac-LDL at 4°C for 2 h. The amount of ^{125}I-Ac-LDL bound to the peptides was measured as described in Section 2. Each point represents the mean of duplicate determinations.

3. Results

3.1. Ac-LDL binding to the receptor model peptide

Fig. 1 shows the structures of receptor model peptide mimicking the lysine cluster in the collagen-like domain of the bovine macrophage scavenger receptor. The three collagenous peptides, in which a peptide encompassing residues 323 to 340 of the bovine macrophage scavenger receptor is surrounded by four and three repeats of a Gly–Pro–Hyp triad, are cross-linked at their amino termini. Both the cross-linking and the addition of the Gly–Pro–Hyp triad facilitate the formation of a collagen structure and increase the stability. Biotin was attached to the anchor molecule of the three collagenous peptides to immobilize the receptor model peptide to the support. Since the Lys residues are involved in ligand binding, a tris-*N*-terminal cross-linked $(Gly\text{-}Pro\text{-}Lys)_6$, $(GPK)_6$ peptide, was also prepared as a control peptide. The Lys residues appear at every Y position of the Gly–X–Y sequence, which is characteristic of a collagenous sequence. This peptide also exhibited a collagen structure from the CD spectrum, with a midpoint of thermal transition (T_m) of 42°C (data not shown).

In order to analyze the Ac-LDL binding, the two peptides immobilized on the Dynabeads were incubated with 5–25 μg/ml of ^{125}I-Ac-LDL for 2 h at 4°C. The radioactivity of the ^{125}I-Ac-LDL bound to the peptides was measured. Binding of Ac-LDL to both the receptor model peptide and the $(GPK)_6$ peptide was dose dependent (Fig. 2). The amount of Ac-LDL bound to the $(GPK)_6$ peptide was 4 times greater than that bound to the receptor model peptide. Scatchard analyses show apparent dissociation constants (K_d) of 31 μg/ml for the receptor model peptide and 6.8 μg/ml for the $(GPK)_6$ peptide, respectively. With the same experiment, the mouse peritoneal macrophages has an apparent K_d of 3.2 μg/ml (data not shown). Therefore the binding activitiy of the receptor model peptide is 10-fold lower than that of the macrophages. We used a concentration of 15 μg/ml of ^{125}I-AcLDL in the following experiments.

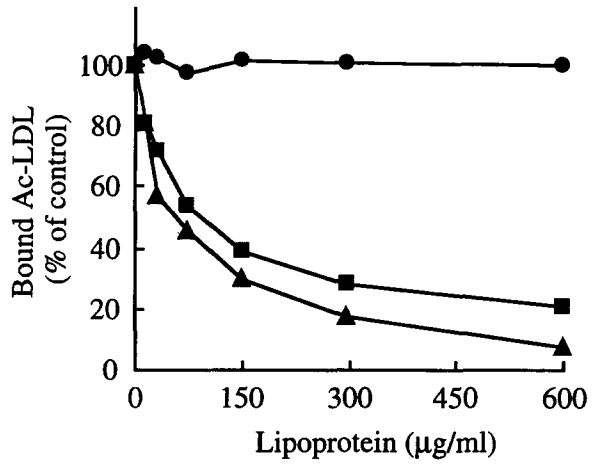

Fig. 3. Competition of ^{125}I-Ac-LDL binding to the receptor model peptide by LDL, Ac-LDL, and Ox-LDL. The resin immobilized with the receptor model peptide was incubated with 15 μg/ml of ^{125}I-Ac-LDL at 4°C for 2 h. The unlabeled LDL (●), Ac-LDL (▲), and Ox-LDL (■) were added at the indicated concentrations. The amount of ^{125}I-Ac-LDL bound to the peptide was measured as described in Section 2. In the absence of the competitors, the amount of ^{125}I-Ac-LDL bound was 30 ng/tube. Each point represents the mean of two experiments.

3.2. Binding of LDL, Ac-LDL, and Ox-LDL

The scavenger receptor binds modified-LDLs, such as Ac-LDL and Ox-LDL, and not LDL [12]. A competitive inhibition assay was performed to examine the effects of modified lipoproteins on ^{125}I-Ac-LDL binding to the receptor model peptide. Binding of ^{125}I-Ac-LDL to the receptor model peptide was inhibited competitively by the addition of an excess of unlabeled Ac-LDL or Ox-LDL (Fig. 3). The inhibitory activity of Ac-LDL was higher than that of Ox-LDL. The addition of 600 μg/ml Ac-LDL reduced the bound ^{125}I-AcLDL to 7.7% of the control, while the addition of 600 μg/ml Ox-LDL reduced it to 20.8%. On the other hand, the addition of excess of unlabeled LDL at the concentration of 600 μg/ml had no effect. Thus, the receptor model peptide binds modified-LDLs, and not LDL.

3.3. Binding specificity

Many compounds are known to inhibit Ac-LDL binding to the natural scavenger receptor [8]. To analyze the binding specificity of the receptor model peptide, we tested fucoidan, sulfatides, dextran sulfate, poly I, and poly G as inhibitor molecules, and poly C as a control molecule. The inhibition mode of the receptor model peptide was compared to those of the scavenger receptor of mouse peritoneal macrophages and the $(GPK)_6$ peptide. The macrophages have several receptors to bind modified-LDLs; however, 70% of Ac-LDL binding is mediated by the scavenger receptor (types I and II) [23]. Therefore, we consider here that the Ac-LDL binding by the macrophages is mediated by the scavenger receptor.

Fig. 4 shows the percent inhibition of ^{125}I-Ac-LDL binding by the compounds described above. Dextran sulfate, fucoidan, and sulfatides inhibited Ac-LDL binding to the natural receptor expressed on mouse peritoneal macrophages in a dose-dependent manner (Fig. 4A). Dextran sulfate showed the strongest inhibition of Ac-LDL binding (IC_{50}, 0.15 μg/ml), while the inhibition by fucoidan was a little weaker (IC_{50}, 0.35 μg/ml). Sulfatides inhibited Ac-LDL binding with the IC_{50} value of 3.5 μg/ml, although the inhibition was 10–20 times weaker than those of dextran sulfate and fucoidan. Similar inhibition tendencies by dextran sulfate, fucoidan, and sulfatides were observed with the receptor model peptide (IC_{50}, 0.7, 0.5 and 8 μg/ml, respectively), although the inhibitions were 2-fold weaker (Fig. 4B). Sulfatides showed more than 10-fold weaker inhibitory activity with both receptors than dextran sulfate and fucoidan. Thus, the receptor model peptide has a similar ligand binding specificity to the natural receptor. On the other hand, when the $(GPK)_6$ peptide was used, dextran sulfate, fucoidan and sulfatides showed almost same inhibition (IC_{50}, 2.5–7 μg/ml) (Fig. 4C), indicating the absence of ligand binding specificity by the $(GPK)_6$ peptide.

Poly G and poly I are known to be effective inhibitors of Ac-LDL binding to the scavenger receptor, but poly C is ineffective. We obtained the same results using the natural receptor, as shown in Fig. 5A. The IC_{50} values of both poly G and poly I were 0.15 μg/ml. Poly C did not show any inhibitory activity. When the receptor model peptide was tested,

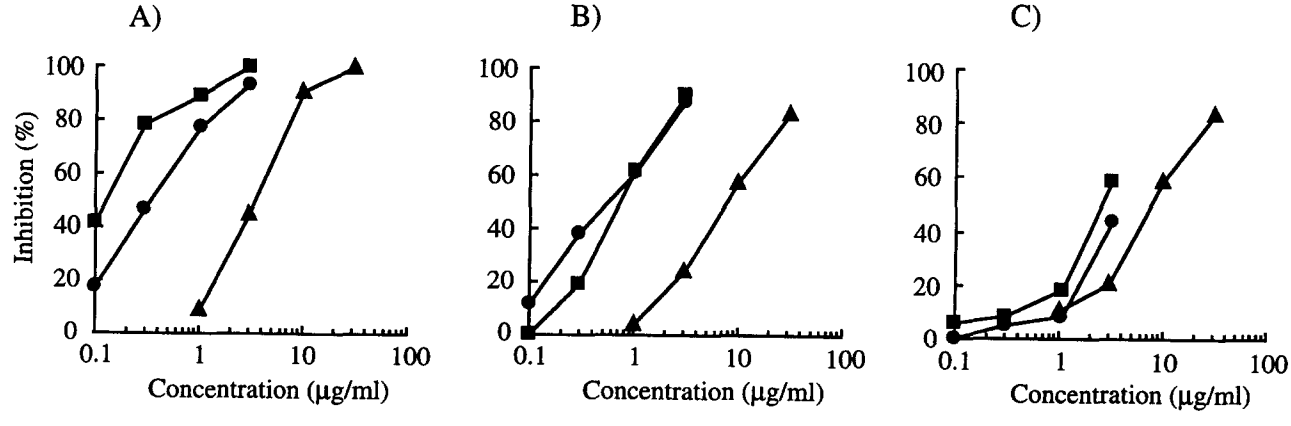

Fig. 4. Inhibition of ^{125}I-Ac-LDL binding to the natural receptor of mouse peritoneal macrophages (A), the receptor model peptide (B), and the $(GPK)_6$ peptide (C), by fucoidan, sulfatides, and dextran sulfate. Mouse peritoneal macrophages were incubated with 10 μg/ml of ^{125}I-Ac-LDL at 4°C for 2 h. The resin, immobilized with either the receptor model peptide or the $(GPK)_6$ peptide, was incubated with 15 μg/ml of ^{125}I-Ac-LDL at 4°C for 2 h. Fucoidan (●), sulfatides (▲), and dextran sulfate (■) were added at the indicated concentrations. The amount of ^{125}I-Ac-LDL bound to the macrophages or the peptides was measured as described in Section 2. In the absence of the inhibitors, the amounts of ^{125}I-Ac-LDL bound were 192 ng/mg cell protein (A), 51 ng/tube (B), and 430 ng/tube (C), respectively. Each point represents the mean of duplicate determinations (A) or two experiments (B), (C).

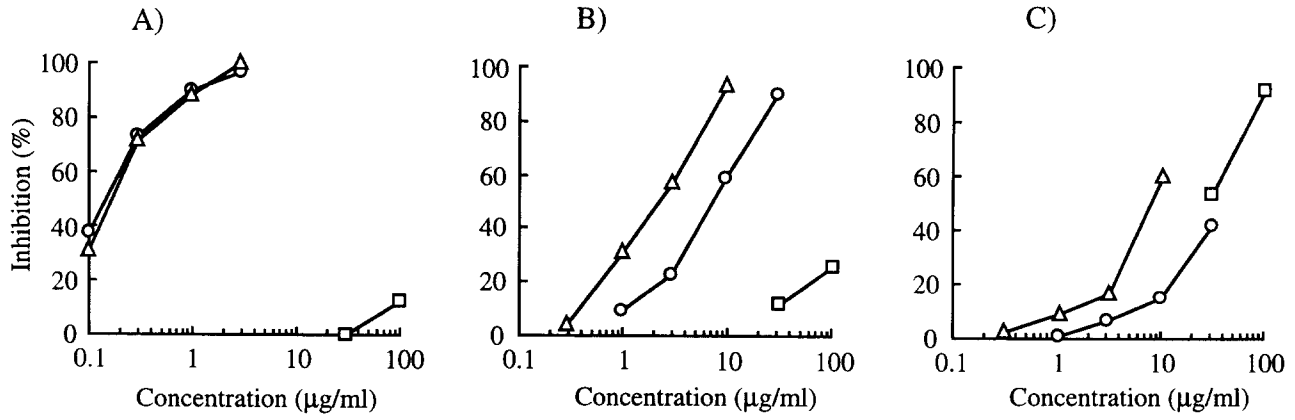

Fig. 5. Inhibition of ^{125}I-Ac-LDL binding to the natural receptor of mouse peritoneal macrophages (A), the receptor model peptide (B), and the $(GPK)_6$ peptide (C) by poly I, poly G, and poly C. Mouse peritoneal macrophages were incubated with 10 μg/ml of ^{125}I-Ac-LDL at 4°C for 2 h. The resin, immobilized with either the receptor model peptide or the $(GPK)_6$ peptide, was incubated with 15 μg/ml of ^{125}I-Ac-LDL at 4°C for 2 h. Poly I (○), poly G (△), and poly C (□) were added at the indicated concentrations. The amount of ^{125}I-Ac-LDL bound to macrophages or the peptides was measured as described in Section 2. In the absence of the inhibitors, the amounts of ^{125}I-Ac-LDL bound were 192 ng/mg cell protein (A), 51 ng/tube (B), and 430 ng/tube (C), respectively. Each point represents the mean of duplicate determinations (A) or two experiments (B), (C).

the inhibitory activities of poly G and poly I were 20- and 40-fold reduced with the IC_{50} values of 2 and 7 μg/ml, respectively (Fig. 5B). Inhibition by poly C was also observed, but to a much lower extent. This shows that the receptor model peptide still has binding specificity, although it was reduced. On the other hand, the $(GPK)_6$ peptide did not discriminate between poly G, poly I, and poly C (Fig. 5C). They showed the same inhibitory activities with IC_{50} values of 8–30 μg/ml. In comparison with the receptor model peptide, the inhibitory activities of poly G and poly I were 3-fold lower, while that of poly C was greatly enhanced. As a consequence, poly G, poly I, and poly C showed the same levels of inhibition.

4. Discussion

In the present study, we analyzed the ligand binding specificity of the receptor model peptide from Gly^{323} to Lys^{340}, including the lysine cluster, of the bovine scavenger receptor. The receptor model peptide specifically bound modified-LDLs, and this binding was inhibited by Ac-LDL and Ox-LDL. On the other hand, LDL did not have any inhibitory activity, even with an excess of ligand (Fig. 3). These characteristics coincide with those of the natural receptor [12]. Comparing the binding of Ac-LDL and Ox-LDL, Ac-LDL binds more strongly than Ox-LDL, implying that the binding sites for Ac-LDL and Ox-LDL might be somewhat different. The binding characteristics of Ac-LDL and Ox-LDL to the natural receptor are also different, since the substitutions of the Lys residues with Ala residues in the lysine cluster reduced the Ac-LDL binding more than the Ox-LDL binding [14]. This suggests that the binding site for Ox-LDL may shift toward the middle of the collagen-like domain. The receptor model peptide is derived from the lysine cluster of the collagen-like domain, and therefore the binding of Ox-LDL to the receptor model peptide was weaker than that of Ac-LDL.

Inhibition of Ac-LDL binding to the receptor model peptide by dextran sulfate, fucoidan, and sulfatides showed tendencies similar to those of the natural receptor, althought 2-fold higher amounts of inhibitors were needed for 50% inhibitions (Fig. 4A,B). This indicates that the affinities of the inhibitors to the receptor model peptide were twice as affected as that of Ac-LDL. The inhibitions by poly G and poly I were 20–40-fold weaker, whereas the inhibition by poly C was detectable, but slight (Fig. 5A,B). Thus, the receptor model peptide has ligand specificity, although the ligand discrimination is weaker than that of the natural receptor.

The $(GPK)_6$ peptide showed 4-fold higher affinity for Ac-LDL than the receptor model peptide (Fig. 2). The binding affinity may be influenced by the number of Lys residues. For example, the $(GPK)_6$ peptide has six Lys residues, while the receptor model peptide has five. The increasing number of positive charges enhances the binding of Ac-LDL. However, the $(GPK)_6$ peptide did not possess ligand binding specificity (Fig. 4CFig. 5C). This indicates that other amino acids, in addition to the Lys residues, participate in the ligand binding specificity. In the bovine scavenger receptor, three Lys residues are at the Y positions and two Lys residues are at the X positions of the Gly–X–Y sequences, with a three amino acid interval in the lysine cluster of the collagen-like domain [10,11]. The positions of the Lys residues might be important for ligand binding specificity. The amino acid sequences within the lysine cluster are well conserved among the bovine, human, mouse, and rabbit scavenger receptors [10,11,14,24].

These results further suggest that the receptor model peptide has a similar binding specificity to that of the natural scavenger receptor, and that the target site for Ac-LDL is the lysine cluster. Therefore, the receptor model peptide should be a valuable tool to study the structure–function relationship and the ligand binding specificity of the scavenger receptor. The scavenger receptor may be a possible target for the development of drugs for atherosclerosis, Alzheimer's disease, and diabetic complications. For example, blocking the excessive uptake of modified lipoproteins through the macrophage scavenger receptor is expected to suppress foam cell formation, and treatments using scavenger receptor inhibitors may be able to prevent atherosclerosis. One of the applications of this receptor model peptide will be to establish a cell-free assay system to search for scavenger receptor inhibitors, as well as a means to perform high-through-put screening.

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