Biochimica et Biophysica Acta, 984 (1989) 273–280
Elsevier

BBAMEM 74548

Scavenger receptor-mediated recognition of maleylated albumin and its relation to subsequent endocytic degradation

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(Received 30 November 1988)
(Revised manuscript received 20 April 1989)

Key words: Scavenger receptor; Maleylated bovine serum albumin; Acetylated LDL; Liver metabolism; Macrophage; Endocytosis; Receptor-mediated endocytosis; (Rat)

Rat sinusoidal liver cells take up maleylated bovine serum albumin (maleyl-BSA) and its demaleylated form (demaleyl-BSA) by scavenger receptor-mediated endocytosis. Cellular binding of maleyl-BSA and demaleyl-BSA and its quantitative relation to subsequent intracellular degradation were investigated. The binding affinities of these ligands were almost equal whereas the number of binding sites for maleyl-BSA was more than twice as large than that for demaleyl-BSA. However, no difference was observed in their endocytic degradation. The amounts of maleyl-BSA degraded were proportional to those bound to the cell surface up to a certain level. However, a further increase in cell-bound ligands did not affect the degradation of maleyl-BSA. Several polyanions such as fucoidin and dextran sulfate of $M_r = 5000$ inhibited the binding of maleyl-BSA but did not affect its degradation. In contrast, acetylated or oxidized low density lipoprotein had virtually no effect on cellular binding of maleyl-BSA but exhibited profound effects on its intracellular degradation. Similar results were obtained with rat peritoneal macrophages. Based on these data, we would propose that two binding sites are involved in the receptor-mediated ligand recognition; one is coupled to subsequent endocytic degradation, and the other serves as a binding site for polyanionic compounds.

Introduction

Receptor-mediated endocytosis of chemically modified LDL by macrophages or macrophage-derived cells has recently been a subject of intensive studies because in vitro intracellular accumulation of cholesteryl esters via this system results in formation of foam cells, cells characteristically found in atherosclerotic lesions [1,2]. The scavenger receptor, a term used to imply the membrane receptor involved in this phenomenon, was demonstrated to recognize several ligands such as acetyl-LDL, MDA-LDL, oxidized and other biologically modified LDL [3–11]. The receptor was partially purified from murine macrophage tumor cells [12], rat liver [13] and recently purified from bovine lung [14]. However, less is known about its ligand specificity. For instance, it remains unclear why the receptor exhibits somewhat broad ligand specificity for a diverse array of these polyanionic compounds. In this context, maleyl-BSA was found to act as a ligand in addition to these modified lipoproteins [1]. This finding was further verified by ligand blotting analyses using the purified receptor [12–14]. However, some differences did occur in the recognition by the receptor; the binding of maleyl-BSA and its endocytic degradation by human monocyte macrophages was inhibited by MDA-LDL at low ligand concentrations, but not at high ligand concentrations [15]. Furthermore, maleyl-BSA was shown to possess a chemotactic activity for human monocyte macrophages while MDA-LDL or acetyl-LDL did not [16]. Thus, albeit very similar to each other, it is likely that some difference does occur between MDA-LDL (acetyl-LDL) and maleyl-BSA in their interaction with macrophages.

Several lines of evidence indicate that the structure change or domain structure induced on a ligand molecule by chemical modification might be important for the receptor-mediated recognition [17–19], rather than a simple increase in net negative charge. However, this

Abbreviations: BSA, bovine serum albumin; maleyl-BSA, bovine serum albumin chemically modified with maleic anhydride; demaleyl-BSA, demaleylated preparation of maleyl-BSA; LDL, low density lipoprotein; acetyl-LDL, acetylated LDL; MDA-LDL, malondialdehyde-modified LDL; TNBS, trinitrobenzenesulfonic acid; TCA, trichloroacetic acid.

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notion does not explain another feature of the scavenger receptor; the ligand binding to the receptor is inhibited by several polyanionic compounds such as poly(inosinic acid), fucoidin and dextran sulfate [17–21], although these linear polymers obviously lack the domain structure. Thus, one of the critical questions as to the ligand specificity would be how recognition of a ligand domain(s) by the receptor does correlate with the polyanion sensitive nature of the receptor. Two ligands for the receptor which only differ in their net negative charges would be desirable to answer this question.

In the present study, by taking advantage of the fact that a demaleylated preparation of maleyl-BSA (demaleyl-BSA) retained the ligand activity [22], the binding parameters of maleyl-BSA and demaleyl-BSA were compared and their correlation to subsequent intracellular degradation was determined using sinusoidal liver cells, main scavenger cells in vivo [19,22–24], and peritoneal macrophages as well. Results support the 'two binding sites' model for the scavenger receptor-mediated recognition of maleyl-BSA.

Materials and Methods

Chemicals. BSA (Fraction V, Sigma) was further purified by Sephacryl S-200 chromatography and the monomeric fraction was used for ligand preparation. Maleic anhydride and TNBS were from Wako Chemical Co. (Osaka, Japan). [^{14}C]Maleic anhydride (25 mCi/mmol) and Na^{125}I were from Du Pont-New England Nuclear. Other chemicals were the best grade available from commercial sources.

Preparation of maleyl-BSA and demaleyl-BSA. This was performed according to the method reported in Ref. 26. Briefly, to 2 ml BSA solution (10 mg/ml) in 0.1 M potassium pyrophosphate buffer (pH 8.5) were added various amounts (1–80 μl) of 1 M maleic anhydride in dioxane. After incubation on ice for 5 min (maintaining the pH at 9.0 with NaOH), 2 ml of ice-cold 0.5 M sodium phosphate buffer (pH 7.4) was added to each reaction tube, followed by extensive dialysis at 4°C against 0.15 M NaCl and 20 mM sodium phosphate buffer (pH 7.4). To prepare demaleyl-BSA, maleyl-BSA with a modification of > 90% was incubated at 37°C in 0.1 M sodium acetate (pH 3.5) for 12 h to 4 days. Time aliquots were dialyzed against 20 mM sodium phosphate buffer and 0.15 M NaCl (pH 7.4). Stoichiometric incorporation of maleic anhydride into BSA was determined in the same way as described above using [^{14}C]maleic anhydride (1550 dpm/nmol) and expressed as mol maleic anhydride incorporated into mol BSA (see Table I). BSA was assumed to contain 60 mol of free amino groups (59 ϵ-amino acids and one α-amino terminus) per mol of BSA [27]. Protein was measured as described [28]. When determined by TNBS methods [29], the extents of lysine modification of maleyl-BSA were 23.5, 31.2, 48.9, 64.5, 73.3, 79.8, and 91.5%, and those for demaleyl-BSA were 43.9, 23.7, and 4.7%.

Preparation of other ligands and iodination. LDL (d = 1.019–1.063 g/ml) was isolated from normolipidemic human blood by sequential ultracentrifugation [20]. MDA-LDL and acetyl-LDL were prepared as described [3,20]. To prepare oxidized LDL, LDL (0.2 mg/ml) was incubated with 5 μM $CuSO_4$ in EDTA-free phosphate-buffered saline for 20 h at 37°C, followed by addition of 0.1 mM EDTA [30]. The extents of lysine modification of acetyl-LDL, MDA-LDL and oxidized LDL were 91.5, 86.2 and > 80%, respectively. Ligands were labeled with ^{125}I as described previously [20].

Binding assay. Sinusoidal liver cells were prepared from male Wistar rats [31] and suspended in Eagle's minimum essential medium containing 3% BSA buffered with 20 mM 2-(4-hydroxyethyl)-1-piperazineethanesulfonic acid to pH 7.4 (buffer A) [20]. Rat peritoneal macrophages were harvested in phosphate-buffered saline and resuspended in buffer A as described [32]. These cell suspensions were used for binding and uptake assays. A reaction mixture containing, in a total volume of 0.1 ml of buffer A, sinusoidal cells ($1.6 \cdot 10^6$) or macrophages ($1.1 \cdot 10^6$), and ^{125}I-maleyl-BSA (740 cpm/ng) or ^{125}I-demaleyl-BSA (1540 cpm/ng) was incubated for 1 h at 0°C. Cells were then washed three times with 1.0 ml of ice-cold buffer A by brief centrifugation and the cell-associated radioactivity (total binding) was determined. Nonspecific binding was measured by parallel incubation with 1.2 mg/ml of same ligand

TABLE I

Binding properties of maleyl-BSA and demaleyl-BSA to sinusoidal liver cells

Cells were incubated at 0°C for 1 h with increasing concentrations (0–30 μg/ml) of ^{125}I-labeled maleyl-BSA and demaleyl-BSA in the presence or absence of 1.2 mg/ml of unlabeled same ligand. The cell-associated radioactivity was determined and a dose-dependent curve for specific binding was constructed with each sample as shown in Fig. 1. Apparent K_d and B_{max} values were determined by the Scatchard analyses.

Preparations	Lysine modified [a] (%)	[^{14}C]Maleic anhydride incorporated [b] (mol/mol BSA)	K_d ($\times 10^7$ M)	B_{max} (pmol/10^6 cells)
Maleyl-BSA	91.5	53.1	2.3 ± 0.2	1.47 ± 0.24
	79.8	46.3	2.4 ± 0.1	1.28 ± 0.06
	73.3	42.5	2.3 ± 0.2	1.23 ± 0.25
	64.5	37.4	2.1 ± 0.3	0.67 ± 0.07
	48.9	28.4	1.8 ± 0.2	0.29 ± 0.06
Demaleyl-BSA	43.9	25.5	1.7 ± 0.4	0.48 ± 0.04
	23.7	13.7	1.4 ± 0.5	0.36 ± 0.02

[a] Determined by the TNBS method [29].

[b] Determined by incorporation of [^{14}C]maleic anhydride into BSA in which 60 amino groups/mol BSA were available [27].

but not labeled, which was < 10% of the total binding under these conditions. Specific cellular binding was determined by subtracting nonspecific binding from total binding as described [20]. Unless otherwise specified, assays were run in triplicate.

Uptake assay. Sinusoidal cells ($3.2 \cdot 10^6$) or macrophages ($2.2 \cdot 10^6$) were incubated for 1 h at 37°C, in a total volume of 0.2 ml of buffer A, with indicated amounts of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA. Cells were centrifuged at 4°C and each supernatant was determined for TCA-soluble radioactivity [33]. To determine specific degradation, cells were incubated in parallel with ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA in the presence of the corresponding unlabeled ligand and the difference in TCA-soluble radioactivity was determined. Unless otherwise specified, the assays were run in triplicate.

Chase experiment. Cells ($2.0 \cdot 10^7$) were incubated at 37°C in a total volume of 2.0 ml of buffer A with 2.7 μg/ml of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA. After 40 min incubation, cells were washed three times with ice-cold buffer A, resuspended in 2.0 ml of buffer A, and subjected to chase experiments by further incubation at 37°C [34]. Time aliquots (0.1 ml) were briefly centrifuged. The pelleted cells were washed and the cell-associated radioactivity was determined. The supernatant was measured for TCA-soluble and TCA-precipitable radioactivity.

Results

Correlation of surface binding of maleyl-BSA and demaleyl-BSA with their subsequent intracellular degradation

To know the correlation between the extent of maleylation and the ligand activity, several preparations of maleyl-BSA and demaleyl-BSA were determined for their binding parameters by binding experiments using sinusoidal liver cells. The total binding was replaced by > 90% by an excess unlabeled ligand. The specific binding exhibited a saturation pattern with each ^{125}I-labeled sample (Fig. 1). Apparent K_d and B_{max} values of each ligand were determined and summarized in Table I. No ligand activity was found in maleyl-BSA preparations with a lysine modification of 23.5 and 31.2%. Specific binding of maleyl-BSA occurred upon a modification of 48.9% and further modification up to 91.5% resulted in a 5-fold increase in B_{max} values, but did not affect the apparent K_d values. When maleyl-BSA (91.5%) was subjected to demaleylation to 43.9% or to 23.7%, its B_{max} value was reduced to 25–33%, whereas demaleylation per se did not virtually affect the apparent K_d values.

We next compared the initial cellular binding with subsequent intracellular degradation. For this purpose, maleyl-BSA (91.5% in Fig. 1A and Table I) and demaleyl-BSA (43.9% in Fig. 1B and Table I) were used. When the specific binding of ^{125}I-maleyl-BSA to sinusoidal liver cells was plotted, it was more than twice as high as that of ^{125}I-demaleyl-BSA at the ligand concentrations tested (Fig. 2A). Since the cell surface-bound maleyl-BSA is known to undergo endocytic degradation, the degradation of these ligands was determined under comparative conditions. As observed with the binding experiments (Fig. 1), the degradation of both ligands was effectively (> 90%) inhibited by an excess unlabeled ligand and their specific degradation exhibited hyperbolic curves as a function of the ligand concentrations. However, despite the significant difference in amounts of binding between these two ligands, the amounts of ^{125}I-maleyl-BSA degraded were almost equal to those of ^{125}I-demaleyl-BSA up to a ligand concentration of approx. 10 μg/ml. This was a somewhat unexpected finding, because it is generally believed that amounts of cell-associated ligand obtained

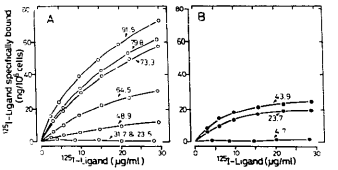

Fig. 1. The binding of maleyl-BSA and demaleyl-BSA to sinusoidal liver cells. Sinusoidal cells were incubated on ice for 1 h with increasing amounts of ^{125}I-maleyl-BSA (A) or ^{125}I-demaleyl-BSA (B) with or without 1.2 mg/ml of the same ligand not labeled. Cells were washed and the specific binding of ^{125}I-ligand was determined. The numbers indicate the extent of lysine modification for maleyl-BSA and demaleyl-BSA preparations.

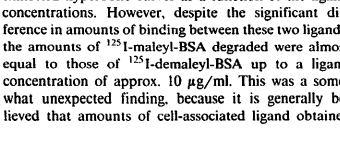

Fig. 2. Specific binding of maleyl-BSA and demaleyl-BSA to sinusoidal liver cells and their subsequent endocytic degradation. (A) Sinusoidal cells were incubated on ice for 1 h with ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA under conditions identical to those for Fig. 1. Cells were washed to determine specific binding of ^{125}I-maleyl-BSA (○) and ^{125}I-demaleyl-BSA (●). (B) Under identical conditions, cells were incubated at 37°C for 1 h and the amounts of specific degradation of ^{125}I-maleyl-BSA (○) and ^{125}I-demaleyl-BSA (●) were determined. Extents of lysine modification of maleyl-BSA and demaleyl-BSA were 91.5 and 43.9%, respectively (see Table I).

by incubation at 0°C represent those bound to outer surface of the plasma membranes and, furthermore, ligands bound to surface membranes function as a crucial determinant for subsequent endocytic degradation (the amounts of ligands bound to the cells are quantitatively proportional to those of subsequent degradation). If this rule was valid for the maleyl-BSA and demaleyl-BSA used in the present study, the amounts of degradation of ^{125}I-maleyl-BSA would be twice that of ^{125}I-demaleyl-BSA. However, this assumption does not apply to the present case, suggesting that the other mechanism might be operative.

As a further test, from both maleyl-BSA and demaleyl-BSA preparations (Fig. 1), the surface binding and intracellular degradation were compared at the fixed ligand concentrations. Fig. 3A shows cellular binding and subsequent degradation as a function of lysine modification. The specific binding of maleyl-BSA became observable with maleylation of > 48.9% and increased sharply up to a maleylation of 79.8%. Although exhibiting a similar threshold pattern, the intracellular degradation of maleyl-BSA reached a plateau with a modification of approx. 65%. Demaleylation of maleyl-BSA (91.5%) to 43.9% or 23.7% reduced the specific binding by > 70%. However, demaleyl-BSA with lysine modification of < 5% showed no ligand activity (Fig. 1 and Table I).

In Fig. 3B, the amounts of maleyl-BSA and demaleyl-BSA degraded for 1 h at 37°C were replotted from Fig. 3A as a function of those bound to the surface membranes. The intracellular degradation of ^{125}I-maleyl-BSA and ^{125}I-demaleyl-BSA reached a maximal level when the amount of cell surface-bound ligands approached 7 ng/10^6 cells. Upon further increase in cell surface-bound maleyl-BSA, a corresponding increase in endocytic degradation did not occur, rather, the amount of ligand degraded remained constant (Fig. 3B). Thus, the binding sites of these cells for maleyl-BSA or demaleyl-BSA which are coupled to subsequent intracellular degradation might be limited in number. In other words, a significant portion of maleyl-BSA bound to the binding sites may not undergo endocytic degradation.

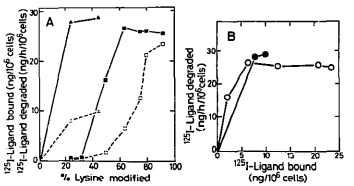

Fig. 3. Correlation of surface binding with subsequent intracellular degradation. (A) The amounts of ^{125}I-maleyl-BSA and ^{125}I-demaleyl-BSA bound to surface membranes and those degraded by sinusoidal cells were plotted against the extent of lysine modification of each sample. Each reaction mixture contained, in 0.2 ml of buffer A, 3.2·10^6 cells and 2.0 μg/ml of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA whose lysine modification was specified in Table I. After 1 h-incubation at 0°C (for cellular binding) or at 37°C (for degradation), specific binding and specific degradation were determined as described in Materials and Methods. Cellular binding of ^{125}I-maleyl-BSA (□) and ^{125}I-demaleyl-BSA (△); intracellular degradation of ^{125}I-maleyl-BSA (■) and ^{125}I-demaleyl-BSA (▲). (B) Specific degradation of ^{125}I-maleyl-BSA (○) or ^{125}I-demaleyl-BSA (●) obtained from (A) was replotted against its specific cellular binding.

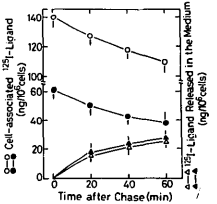

Fig. 4. Post-binding fates of maleyl-BSA and demaleyl-BSA. Sinusoidal cells were incubated at 37°C for 40 min with the same concentration (2.7 μg/ml) of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA. After washing three times with ice-cold buffer A, cells were chased at 37°C and time aliquots were taken to determine the cell-associated radioactivity of ^{125}I-maleyl-BSA (○) and ^{125}I-demaleyl-BSA (●). The radioactivity released in the medium (> 95% was TCA-soluble) was also determined for maleyl-BSA (△) and demaleyl-BSA (▲). Bars show the range.

To test this possibility, the post-binding fate of maleyl-BSA was compared with that of demaleyl-BSA by chase experiments of cell surface-bound ^{125}I-ligands. The cells were incubated at 37°C for 40 min with the same concentration of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA and washed with ligand-free medium. Upon chase experiments, the cell-associated ligands were released into the medium as TCA-soluble forms (> 95%), indicating that cell-associated ligands delivered to the lysosomes where endocytosed ligands underwent degradation (Fig. 4). The amounts of cell-associated ligands for maleyl-BSA were 2.3-fold higher than that for demaleyl-BSA. However, the amount of ^{125}I-ligands degraded by the cells was virtually the same for maleyl-BSA and demaleyl-BSA. Thus, despite these two ligands having the same endocytic fate, major portions of cell-associated ^{125}I-maleyl-BSA might not be coupled to endocytic degradation.

To determine whether the same results obtained with sinusoidal liver cells would be obtained with other cells, similar experiments were performed with rat peritoneal macrophages. The specific binding versus ligand concentration revealed that the number of binding sites for

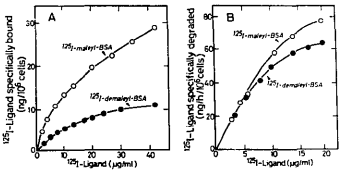

Fig. 5. Specific binding of maleyl-BSA and demaleyl-BSA to peritoneal macrophages and their subsequent endocytic degradation. (A) Macrophages were incubated on ice for 1 h with increasing amounts of ^{125}I-maleyl-BSA or ^{125}I-demaleyl-BSA with or without 1.2 mg/ml of unlabeled same ligand. Cells were then washed to determine specific binding of ^{125}I-maleyl-BSA (○) and ^{125}I-demaleyl-BSA (●). (B) Cells were incubated in the same way at 37°C for 1 h and the amounts of specific intracellular degradation of ^{125}I-maleyl-BSA (○) and ^{125}I-demaleyl-BSA (●) were determined. The extents of lysine modification of maleyl-BSA and demaleyl-BSA were 91.5 and 43.9%, respectively (see Table I).

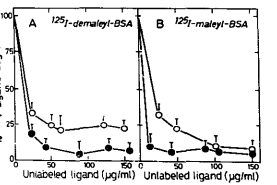

Fig. 6. Effect of maleyl-BSA and demaleyl-BSA on their intracellular degradation by peritoneal macrophages. Macrophages were incubated at 37°C for 1 h with 3.7 μg/ml of ^{125}I-maleyl-BSA (A) or ^{125}I-demaleyl-BSA (B) at indicated concentrations of maleyl-BSA (●) and demaleyl-BSA (○). Cells were then centrifuged and the TCA-soluble radioactivity in the supernatant was determined. The 100% values for degradation of ^{125}I-maleyl-BSA and ^{125}I-demaleyl-BSA were 22.3 and 19.4 ng/10^6 cells per h.

^{125}I-maleyl-BSA was about 3-times as high as that of ^{125}I-demaleyl-BSA (Fig. 5A). However, no virtual difference was observed in their subsequent degradation (Fig. 5B); the results are consistent with those in Fig. 2. Moreover, both maleyl-BSA and demaleyl-BSA competed effectively for the binding of ^{125}I-maleyl-BSA or demaleyl-BSA to the cells, suggesting that these ligands were taken up by macrophages via a common endocytic pathway (Fig. 6).

Effect of several polyanions on cellular binding and endocytic degradation of maleyl-BSA

The scavenger receptor-mediated binding or degradation are effectively inhibited by several polyanionic compounds [17–21], suggesting the presence of a polyanion-binding site on or near the receptor. We therefore determined whether the polyanion-binding sites might functionally relate to the binding sites for maleyl-BSA with sinusoidal liver cells. Effects of the polyanionic compounds on the cellular binding of ^{125}I-maleyl-BSA were compared with those on the endocytic degradation. As shown in Fig. 7A, the binding of ^{125}I-maleyl-BSA was effectively inhibited by several polyanions such as fucoidin, polyinosinic acid, and dextran sulfate (5 and 500 kDa), whereas its intracellular degradation was not prevented by fucoidin and dextran sulfate (5 kDa). Polyinosinic acid and dextran sulfate (500 kDa) showed 40% and 80% inhibition, respectively. Effect of poly(L-glutamate) mimicked fucoidin (data not shown). This finding indicates that all these polyanions play as effective competitors for ^{125}I-maleyl-BSA binding to the

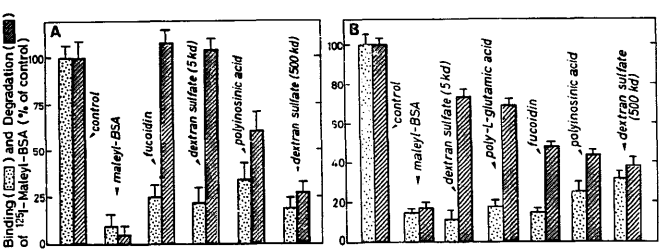

Fig. 7. Effect of several polyanionic compounds on cellular binding of maleyl-BSA and its intracellular degradation. Sinusoidal cells (A) or peritoneal macrophages (B) were incubated with 3.9 μg/ml of ^{125}I-maleyl-BSA in the presence of 50 μg/ml of the indicated polyanionic compound. After incubation for 1 h at 0°C (for binding) or at 37°C (for degradation), the amounts of cell-bound ligands (▨) and those degraded (▩) were determined. As control experiments, effects of maleyl-BSA on these processes were examined at 0.1 mg/ml of unlabeled maleyl-BSA. The 100% values for binding and degradation were 21.5 ng/10^6 cells and 17.2 ng/10^6 cells per h for (A), and 7.4 ng/10^6 cells and 27.5 ng/10^6 macrophages per h for (B). The numbers in parentheses denote molecular mass.

cells, but do not always affect its intracellular degradation. The similar results were obtained with peritoneal macrophages (Fig. 7B). Dextran sulfate (5 kDa), poly(L-glutamate) and fucoidin showed an effective inhibition for the binding of maleyl-BSA (> 85%), but inhibitory effects on its endocytic degradation were partial; 30% for dextran sulfate (5 kDa) and poly(L-glutamate) and 50% for fucoidin (Fig. 7B). Thus, the surface binding sites for these polyanions are similar, in part, to the binding sites for maleyl-BSA in that they are not coupled to subsequent lysosomal degradation.

Effect of acetyl-LDL and oxidized LDL on cellular binding and endocytic degradation of maleyl-BSA and demaleyl-BSA

To determine how binding sites for acetyl-LDL or oxidized LDL might correlate with polyanion binding sites or binding sites for maleyl-BSA, acetyl-LDL and oxidized LDL were examined for their effect on the binding and subsequent degradation of ^{125}I-maleyl-BSA and ^{125}I-demaleyl-BSA. As Fig. 8A shows, the binding of ^{125}I-demaleyl-BSA to sinusoidal cells was competed for by acetyl-LDL up to 60% of the total binding, whereas the binding of ^{125}I-maleyl-BSA was less affected by acetyl-LDL. In contrast, however, acetyl-LDL effectively prevented the degradation of both ^{125}I-maleyl-BSA and ^{125}I-demaleyl-BSA (Fig. 8B). Although 100% inhibition was not achieved, effects of acetyl-LDL on the degradation of these ligands were dose-dependent and, more important, the degradation of ^{125}I-demaleyl-BSA was inhibited to a greater degree by acetyl-

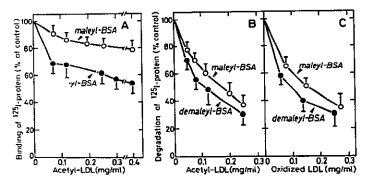

Fig. 8. Effect of acetyl-LDL and oxidized-LDL on binding and degradation of maleyl-BSA and demaleyl-BSA by sinusoidal cells. (A) Cells were incubated on ice for 1 h with 3.9 μg/ml of ^{125}I-maleyl-BSA (○) or 2.3 μg/ml of ^{125}I-demaleyl-BSA (●) in the presence of acetyl-LDL, and the cell-associated radioactivity was determined. The 100% values were 21.5 ng/10^6 cells for maleyl-BSA and 9.5 ng/10^6 cells for demaleyl-BSA. (B) Sinusoidal cells were incubated at 37°C for 1 h with 3.9 μg/ml of ^{125}I-maleyl-BSA (○) or 2.3 μg/ml of ^{125}I-demaleyl-BSA (●) in the presence of acetyl-LDL. Cells were washed and amounts of ligand degraded were determined. The 100% values were 17.2 ng/10^6 cells per h for maleyl-BSA and 13.2 ng/10^6 cells per h for demaleyl-BSA. (C) Experiments were performed in the same way as B except that acetyl-LDL was replaced by oxidized LDL. Bars show the deviation.

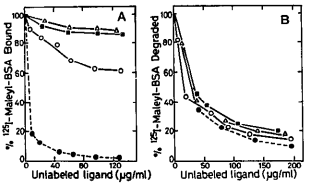

Fig. 9. Effect of acetyl-LDL, MDA-LDL, and oxidized LDL on binding (A) and degradation (B) of ^{125}I-maleyl-BSAby peritoneal macrophages. (A) Cells were incubated on ice for 1 h with 3.7 μg/ml of ^{125}I-maleyl-BSA in the presence of acetyl-LDL (○), MDA-LDL (■), oxidized LDL (△) and maleyl-BSA (●). The cells were washed and the cell-associated radioactivity was determined. The 100% value was 7.4 ng/10^6 cells. (B) Cells were incubated at 37°C for 1 h with 3.7 μg/ml of ^{125}I-maleyl-BSA in the presence of an unlabeled ligand. The ligands used were identical to those used in (A). The amounts of endocytic degradation of ^{125}I-maleyl-BSA were determined by an increase in TCA-soluble radioactivity released into the medium. The 100% value represents amounts of ligand degraded in the absence of any competing compounds which corresponds to 23.6 ng/10^6 cells per h.

LDL than that of ^{125}I-maleyl-BSA. A similar effect was seen with oxidized LDL (Fig. 8C) and MDA-LDL (data not shown), suggesting that oxidized LDL and MDA-LDL might be endocytosed via a receptor identical or similar to acetyl-LDL. Thus, these results indicated that maleyl-BSA and demaleyl-BSA are endocytosed via the scavenger receptor, and a larger portion of the binding sites for maleyl-BSA might serve as polyanion binding sites.

This notion was further supported by experiments using peritoneal macrophages. As Fig. 9A shows, the cellular binding of ^{125}I-maleyl-BSA was partially inhibited by acetyl-LDL, and both MDA-LDL and oxidized LDL exhibited much weaker effects, whereas these ligands were equally effective in competing with ^{125}I-maleyl-BSA for its degradation by macrophages. Thus, the same or similar mechanism might occur to the recognition of maleyl-BSA and subsequent endocytic degradation in macrophages.

Discussion

From the data presented here a possible scheme for the interaction of maleyl-BSA with the scavenger receptor of rat sinusoidal liver cells and peritoneal macrophages is illustrated in Fig. 10. Two sites (site A and site B) are present on the outer surface membranes of these cells. These two sites are functionally distinct from each other. Ligands bound to site A are internalized and delivered to lysosomes for degradation. However, ligands bound to site B do not undergo subsequent intracellular degradation, although the binding to site B is specific in

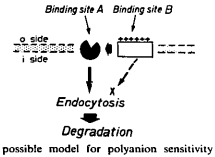

Fig. 10. A possible model for polyanion sensitivity of scavenger receptor. See details in the text.

the sense that these outer surface-bound ligands are replaceable by the same ligands. According to this 'two binding sites' model, maleyl-BSA binds to both site A and site B whereas demaleyl-BSA only binds to site A. Other lipoprotein ligands such as acetyl-LDL, MDA-LDL and oxidized LDL are virtually recognized by the cells via binding to site A. On the other hand, polyanions such as dextran sulfate (5 kDa), poly(L-glutamate) and fucoidin exhibited a high affinity for site B (Fig. 7). However, other polyanions such as poly(inosinic acid) and dextran sulfate (500 kDa) inhibited the cellular binding of ^{125}I-maleyl-BSA as well as its intracellular degradation (Fig. 7). Although its mechanism remains speculative, one possible explanation would be that when site B is occupied by these polyanions, it might exert some inhibitory effect on site A, thus decreasing intracellular degradation occurred to ligands bound to site A.

Another mechanism by which a polyanion interact with macrophages is that the complex formation of LDL with dextran sulfate or proteoglycan would induce the formation of the domain structure on a LDL molecule, thus facilitating the binding affinity for site A through which LDL was endocytosed in a piggy back mechanism [21,35]. The previous finding that endocytic degradation of ^{125}I-acetyl-LDL by mouse peritoneal macrophages was effectively inhibited by poly(inosinic acid) while the degradation of ^{125}I-LDL-dextran sulfate complexes was totally unaffected by this polyanion [21] would be in part explained by this mechanism.

Our data on effects of fucoidin differed from those of Haberland et al. [15]. In their experiments with human monocyte macrophages, fucoidin had a significant inhibitory effect on the cellular binding and endocytic degradation of ^{125}I-maleyl-BSA. In the present study with sinusoidal liver cells, fucoidin showed the same inhibitory effect on the binding of ^{125}I-maleyl-BSA, but little or a very weak effect on its endocytic degradation of maleyl-BSA (Fig. 7A). To examine whether this discrepancy was due to a difference in cell types used, we did the same experiments with rat resident peritoneal macrophages. Dextran sulfate (5 kDa) and poly(L-glutamate) affected the binding of ^{125}I-maleyl-BSA, but had little effect on its endocytic degradation (Fig. 7B). Although some difference was observed with peritoneal macrophages in that fucoidin showed a partial but significant inhibitory effect on the degradation of ^{125}I-maleyl-BSA (Fig. 7B), these data taken together suggest that a common mechanism may be operative in the recognition of maleyl-BSA by the scavenger receptor of macrophages.

In receptor-mediated endocytosis, the binding of a ligand to its cell surface receptor is efficiently coupled to subsequent internalization of the ligand [36]. On a quantitative basis, the amounts of ligands bound to cell surface receptors (specific binding) function as a primary determinant for the subsequent endocytic events such as internalization and intralysosomal degradation of the ligands. In this context, the present results seem to be exceptional: intracellular degradation of maleyl-BSA increased proportionally as did specific binding to the surface membranes up to a certain level. However, further increases in cellular binding above this level failed to increase subsequent intracellular degradation (see Figs. 2 and 3). It is possible that a difference in cellular binding and subsequent intracellular degradation is due to the difference in susceptibility of these ligands to lysosomal proteinases. However, both maleyl-BSA and demaleyl-BSA were degraded to a similar extent when incubated with cell lysates at 37°C (data not shown).

Haberland et al. [15] proposed that in addition to the scavenger receptor, another membrane receptor (maleyl-BSA receptor) might be involved in recognition of maleyl-BSA by human monocytes. This notion may well explain the larger number of binding sites for maleyl-BSA than those for MDA-LDL or acetyl-LDL, and the presence of the chemotactic activity of maleyl-BSA but not in acetyl-LDL or MDA-LDL [16]. Consistent data were recently provided by Murata et al. [37]. The lipoprotein lipase secretion by murine macrophages was stimulated almost 3-fold by maleyl-BSA and 1.4-fold by acetyl-LDL, and this stimulatory effect was inhibited by dextran sulfate but not by casein, a ligand believed to be specific for the maleyl-BSA receptor [15]. They suggested therefore that the stimulatory effect by maleyl-BSA was not due to the maleyl-BSA receptor but due to the scavenger receptor [37].

However, the purified receptor from rat liver [13] and bovine lung [14] were shown to bind both acetyl-LDL and maleyl-BSA, and the binding was inhibited by polyanions. Thus, it is much more likely that binding site for acetyl-LDL (or maleyl-BSA) and binding site for the polyanions may occur on the same molecule, proposing a high molecular weight complex with multiple ligand binding sites as its structural model [13]. Although our 'two binding sites' model neither specifies which of these models is functioning in vivo, nor gives logical explanations to all the observations so far reported, the present suggestion on two binding sites for

maleyl-BSA and their correlation to subsequent endocytic events might help reveal the functional aspect of the scavenger receptor-mediated endocytosis.

Acknowledgements

This work was supported in part by Grant-in-Aid for Scientific Research (No. 63870014) from the Ministry of Education, Science and Culture of Japan and a grant from Sagawa Science Foundation.

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