A Simple Method for the Assessment of Macrophage Scavenger Receptor–Ligand Interaction: Adherence of Erythrocytes Coated with Oxidized Low Density Lipoprotein and Modified Albumin to Macrophages

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A simple method is described for the assessment of the binding of macrophage scavenger receptor and its ligands such as oxidized low density lipoprotein (ox-LDL) and maleylated bovine serum albumin (m-BSA). In this method, the binding of ox-LDL or m-BSA to macrophages is observed as the adherence of erythrocytes precoated with ox-LDL or m-BSA under phase-contrast microscopy. Erythrocytes were coated with native LDL or ox-LDL simply by incubating mouse erythrocytes with LDL. The ox-LDL-coated erythrocytes attached to the monolayer of mouse peritoneal macrophages without phagocytosis at 37°C, while native LDL-coated erythrocytes did not. The extent of the adherence of the ox-LDL-coated erythrocytes to the macrophages was conveniently expressed as the proportion of the macrophages binding the coated erythrocytes. An optimal concentration of ox-LDL for erythrocyte coating giving maximum erythrocyte adherence to macrophages was 10 µg/ml as protein at an erythrocyte concentration of 2%. The binding of the ox-LDL-coated erythrocytes to the macrophages could be inhibited by ligands for scavenger receptor, and reflected the extent of LDL oxidation. Mouse erythrocytes were successfully coated with m-BSA using chromium(III) ion as adsorbent. The m-BSA-coated erythrocytes attached to the macrophages, while native BSA-coated erythrocytes did not. The adherence was inhibitable with ligands for scavenger receptor, and reflected the extent of maleylation. The method would be useful particularly for measurement of the ligand-binding activity of the receptor, and the receptor-binding activity of the ligands.

Keywords macrophage; scavenger receptor; oxidized low density lipoprotein; atherosclerosis; maleylated bovine serum albumin; receptor-binding assay

Macrophages poorly take up low density lipoprotein (LDL), but avidly take up chemically-modified LDL such as acetylated LDL,¹⁾ malonaldehyde-modified LDL,²⁾ and oxidized LDL (ox-LDL).³⁾ The excessive uptake of the modified LDL results in accumulation of cholesterol ester within the cells and converts the cells into lipid-laden foam cells *in vitro*.^{1–3)} The conversion of macrophages into foam cells by the uptake of modified LDL, particularly that of ox-LDL which can occur *in vivo*,^{4–6)} has been considered to be a possible event involved in the formation of early atherosclerotic lesions,^{7–9)} where cholesterol ester-rich foam cells originating from macrophages are found.^{8,10)}

Uptake of the modified LDL by the macrophages is mediated by a specific receptor, termed a scavenger receptor, distinct from that for native LDL.^{7–9)} Thus, the initial step in the process of foam cell formation is binding of modified LDL to the scavenger receptor on macrophages. The scavenger receptor recognizes and binds modified LDL but not native LDL.^{7,8)} It also recognizes polyanionic macromolecules such as maleylated or aldehyde-modified proteins, dextran sulfate, fucoidan and polyinosinic acid.^{1,7,8,11)}

Measurement of the interaction between the scavenger receptor and its ligand is important in functional studies of the scavenger receptor and its ligands. Binding or/and uptake of modified LDL by macrophages is usually performed using radioiodinated, modified LDL following the method used for native LDL.¹²⁾ Fluorescently labeled, modified LDL is also used for this purpose.¹²⁾ In these method, facilities for radioisotope-handling or fluorescence microscopy are required. If a simpler method for the assessment of the binding of scavenger receptor to its

ligands were available, it would be useful for functional studies of the scavenger receptor and the ligands, including detection and measurement of receptor and ligand activity.

In this report, we present a simple method for the assessment of the binding of ox-LDL and modified proteins to the scavenger receptor, where the ligand binding is reflected in adherence of erythrocytes, precoated with ox-LDL and modified proteins, to macrophages observed under microscopy.

MATERIALS AND METHODS

Materials Chromium(III) chloride was obtained from Nakarai Chemicals Ltd., Kyoto. Maleic anhydride, formaldehyde solution (37%), trinitrobenzene sulfonic acid (TNBS), thiobarbituric acid (TBA), and luminol were purchased from Wako Pure Chemical Industries, Osaka. Dextran sulfate, fucoidan, polyinosinic acid (poly I), polycytidylic acid (poly C), bovine serum albumin (BSA) (globulin-free), and cytochrome c (horse heart) were obtained from Sigma Chemical Co., St. Louis, MO. Butylated hydroxytoluene (BHT), tetramethoxypropane, Hanks' balanced salt solution, and *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) were from Nikki Universal Co., Tokyo, Tokyo Kasei Kogyo Co., Tokyo, Nissui Pharmaceutical Co., Tokyo, and Dojindo Laboratories, Kumamoto, respectively. RPMI1640 medium and fetal bovine serum were purchased from Gibco Laboratoreis, Grand Island, NY. *N*-Succinimidyl [2,3-³H]propionate was the product of American Radiolabeled Chemicals Inc., St. Louis, MO. Phosphatidylcholine hydroperoxide (PCOOH) was given from Dr. Teruo Miyazawa (Tohoku University, Sendai).

Isolation and Oxidation of LDL LDL was isolated according to the preparative ultracentrifugation method of Hatch and Lee¹³⁾ with minor modifications as described previously¹⁴⁾ using pooled frozen human plasma. The LDL fraction obtained was dialyzed against 10 mM sodium phosphate buffered-saline (pH 7.4) at 4 °C, and subjected to Cu^{2+} -catalyzed oxidation according to the method of Steinbrecher *et al.*¹⁵⁾ Briefly, the dialyzed LDL was diluted to 2.0 mg/ml (as protein) with the same buffer, mixed with a solution of CuSO_4 in the same buffer, and incubated at 37 °C. Unless otherwise stated, the final concentration of CuSO_4 was 5 μM , and the incubation time was 24 h. The reaction was terminated by addition of BHT (final concentration, 50 μM). The mixture was dialyzed against isotonic saline through which nitrogen gas had been bubbled, and stored at 4 °C under nitrogen gas. LDL only treated with 50 μM BHT, followed by dialysis against the nitrogen gas-treated saline was used as native LDL. The protein content of LDL was determined by the Lowry method¹⁶⁾ using BSA as a standard.

Analysis of ox-LDL The extent of the lipid oxidation of LDL was assessed by measurement of PCOOH and TBA-reactive substances (TBARS).

PCOOH measurement was carried out according to the high performance liquid chromatography-chemiluminescence (HPLC-CL) method of Miyazawa *et al.*¹⁷⁾ The LDL lipids for the HPLC analysis were extracted by the method of Bligh and Dyer.¹⁸⁾ The lipid extract in chloroform corresponding to 20 μg LDL (as protein) was injected. HPLC was performed using a column (4.6 mm i.d. $\times$ 25 cm) of YMC A-012 S-5 120A SIL (Yamamura Chemical Laboratories, Ltd., Kyoto), and the chromatograph used chloroform-methanol (1:9, v/v) as mobile phase at a flow rate of 1.1 ml/min. The chemiluminescence reagent, comprising of 10 μg /ml of cytochrome c and 1 μg /ml of luminol in 50 mM borate buffer (pH 9.3), was mixed with the eluate at a flow rate of 1.0 ml/min and the generated chemiluminescence was monitored with a 825-CL chemiluminescence detector (Japan Spectroscopic Co., Ltd., Tokyo). The PCOOH content of the sample was determined from the peak height of standard PCOOH.

TBARS was monitored according to the fluorometric method of Yagi¹⁹⁾ using tetramethoxypropane as a standard, except that protein precipitation with phosphotungstic acid was omitted. Results were expressed as the amount of the red pigment produced by the reaction with TBA.²⁰⁾

Maleylation and Formaldehyde Modification of BSA Maleylation of BSA was conducted according to the method of Butler and Hartley.²¹⁾ Briefly, to a well-stirred solution of BSA (30 μg /ml) in 0.1 M carbonate buffer (pH 9.0) in an ice bath was added solid maleic anhydride little by little, maintaining the pH of the solution between 8.5–9.2 with 1 N NaOH. After 3 h, the reaction mixture was thoroughly dialyzed against 0.01 M carbonate buffer (pH 9.0) at 4 °C and stored at –20 °C. The stored samples were dialyzed against 10 mM phosphate buffered saline (pH 7.4) prior to use. Protein concentration was determined according to the method of Lowry *et al.*¹⁶⁾ The extent of maleylation was estimated by measuring the free amino groups of the protein by the TNBS method according to

Habeeb.²²⁾ Native BSA was used as a standard. By varying the amount of maleic anhydride for the modification, maleylated BSA (m-BSA) with various degrees of modification was obtained (approx. 40–95% modification of total amino groups). Modification of BSA with formaldehyde was carried out according to Horiuchi *et al.*¹¹⁾ as described,²³⁾ and a single preparation of formaldehyde-modified BSA (f-BSA), in which approximately 50% of the total amino groups were modified, was obtained. Protein concentration and the number of modified amino groups were determined as described above.

Erythrocytes Erythrocytes were obtained from ddY mice. Mouse blood was collected by cardiac puncture using acid citrate-dextrose preservative solution, and erythrocytes were centrifuged, washed three times with Dulbecco's phosphate-buffered saline (DPBS) without Ca^{2+} and Mg^{2+} (Ca^{2+} -, Mg^{2+} -free DPBS) after removal of the buffy coats and plasma, and resuspended in isotonic saline to make a 2.5% cell suspension.

Coating Erythrocytes with ox-LDL One vol. of a 2.5% erythrocyte suspension in saline prepared as above was mixed with 0.25 vol. of a solution of native LDL or ox-LDL in saline (5–500 μg protein/ml giving final concentrations of 1–100 μg protein/ml) and incubated at 37 °C for 15 min with occasional mixing. The mixture was then diluted with an equal volume of ice-cold Ca^{2+} -, Mg^{2+} -free DPBS, and centrifuged (170 $\times g$, 15 min) at 4 °C. The cell pellet was washed twice with the same buffer at 4 °C by centrifugation (170 $\times g$, 15 min), and resuspended in RPMI1640 medium supplemented with 20 mM HEPES (pH 7.2), 50 unit/ml penicillin and 50 μg /ml streptomycin (RPMI-HEPES medium) to make a 2% cell suspension. The cell suspension was prepared on the day of use.

Coating Erythrocytes with Modified-BSA One vol. of a 2.5% erythrocyte suspension in saline prepared as above was mixed with 0.125 vol. of a solution of native BSA, m-BSA, or f-BSA (2 mg/ml) in saline or 10 mM phosphate-buffered saline (pH 7.4), and then with 0.125 vol. of a solution of $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ (1 mg/ml) that had been prepared by diluting a stock solution of 1% $\text{CrCl}_3 \cdot 6\text{H}_2\text{O}$ in saline (pH 5.0) with 0.2 M sodium acetate buffer (pH 5.5). The mixture was incubated at 37 °C for 15 min with occasional mixing, diluted with an equal volume of ice-cold Ca^{2+} -, Mg^{2+} -free DPBS, and centrifuged (170 $\times g$, 15 min) at 4 °C. The cell pellet was washed twice with the same buffer at 4 °C and resuspended in RPMI-HEPES medium containing 1% BSA to make a 2% cell suspension. The cell suspension was then allowed to stand at 4 °C for 1 h before use to ensure blocking of adherent sites on the Cr^{3+} -treated erythrocytes with excess BSA.

Quantitation of ox-LDL and Modified BSA Adsorbed on Erythrocytes The amount of ox-LDL and modified BSA adsorbed on erythrocytes was estimated using ^3H -labeled ox-LDL and modified BSA. ^3H -labeled ox-LDL and modified BSA were prepared by mixing the solutions of ox-LDL (2 mg/ml) and modified BSA (m-BSA preparation with 50% modification of total amino groups, and f-BSA, 20 mg/ml) in 0.1 M borate buffer (pH 8.5) with *N*-succinimidyl [2,3- ^3H]propionate (0.2 mCi, 80 Ci/mmol) at

4°C overnight, followed by extensive dialysis against Ca^{2+} -, Mg^{2+} -free DPBS. The specific activity was 10000–12000 dpm/ μg protein for ox-LDL and 1000–1300 dpm/ μg protein for modified BSA. Native LDL and native BSA were similarly labeled with ^3H .

Mouse erythrocytes were coated with the ^3H -labeled ox-LDL, modified BSA, and their native counterparts according to the procedure described above, and the coated cells (approx. 7×10^7 cells) were solubilized with 0.5 ml of 10% sodium dodecylsulfate. The solubilized solution was bleached by treatment with 30% H_2O_2 (200 μl) at 37°C for 1 h, followed by the addition of 10% ascorbic acid (100 μl). Radioactivity of the bleached solution was measured using a liquid scintillation counter. Results are expressed as the mean $\pm$ S.D. of triplicate assays.

Macrophages Macrophages were obtained from the peritoneal cavity of 7–10-week old ddY male mice with or without prior injection of 2–3 ml of 3% thioglycolate medium (Difco Laboratories, Detroit, MI) 4 d before harvesting. The thioglycolate-induced peritoneal exudate cells or uninduced resident peritoneal cells obtained were washed twice with Hanks' balanced salt solution by centrifugation ($80 \times g$, 10 min) at 4°C. The cells were then resuspended in RPMI-HEPES medium at 0.85×10^6 cells/ml for the induced cells and 2×10^6 cells/ml for the resident cells, and 0.2 ml of each cell suspension was loaded onto a round coverglass (18-mm diameter, 0.15–0.18 mm thick). After incubation at 37°C for 1 h, nonadherent cells were removed by washing three times with Ca^{2+} -, Mg^{2+} -free DPBS, and adherent cell monolayers of the induced macrophages were immediately used. Those of the resident macrophages were further incubated at 37°C for 15–20 h in a CO_2 incubator (5% CO_2 –95% air) in RPMI1640 medium supplemented with 10% heat-inactivated fetal bovine serum to ensure cell spreading on the coverglass, rinsed with Ca^{2+} -, Mg^{2+} -free DPBS, and used.

Binding and Phagocytosis of ox-LDL- and Modified BSA-Coated Erythrocytes by Macrophages A 2% cell suspension of ox-LDL- or modified BSA-coated erythrocytes (200 μl) in RPMI-HEPES medium prepared above was loaded on the macrophage monolayer. After incubation at 37°C for 1 h in a CO_2 incubator, nonadhering erythrocytes were removed by gentle washing with DPBS and the cells were fixed with 1.25% glutaraldehyde in Ca^{2+} -, Mg^{2+} -free DPBS. The number of macrophages binding two or more erythrocytes on their surface was scored for random fields of the coverglass under phase-contrast microscopy. For observation of phagocytosis, incubation of the macrophage monolayer with erythrocytes was extended to 3 h. After removal of nonadhering erythrocytes by washing, the macrophage monolayer was treated with 0.83% NH_4Cl /17 mM Tris-HCl (pH 7.6) for 5 min to lyse the surface-bound erythrocytes. The macrophage monolayer was then fixed with methanol, dried and stained with 3% Giemsa's solution (E. Merk, Darmstadt) for 20 min. The number of macrophages ingesting one or more erythrocytes was scored. At least 200 macrophages were examined for binding and phagocytosis of the erythrocytes and the percentage of macrophages binding two or more erythrocytes and

the percentage of macrophages ingesting one or more erythrocytes were determined. Results are expressed as the mean $\pm$ S.D. of the triplicate experiments.

RESULTS

Adherence of ox-LDL-Coated Erythrocytes to Thioglycolate-Induced Mouse Peritoneal Macrophages ox-LDL was prepared as described¹⁵⁾ by incubation of LDL in the presence of $5 \mu\text{M}$ Cu^{2+} at 37°C for 24 h. The ox-LDL was designated ox24h-LDL. To measure the binding ability of ox24h-LDL to macrophages, as the adherence of erythrocytes precoated with ox24h-LDL to macrophages, we

Fig. 1. Relationship between the Extent of LDL Adsorption to Erythrocytes and the Extent of Recognition of the LDL-Coated Erythrocytes by Thioglycolate-Induced Macrophages

A: Extent of LDL adsorption to erythrocytes as a function of LDL concentration. Erythrocytes were coated with the indicated concentrations of native LDL (○) and ox24h-LDL (●) labeled with ^3H using *N*-succinimidyl [2,3- ^3H]propionate, and the amounts of the spontaneously adsorbed LDL were measured as described in Materials and Methods. B: Extent of macrophage recognition of the LDL-coated erythrocytes as a function of the LDL concentration used in coating the erythrocytes. Erythrocytes were coated with the indicated concentrations of native LDL and ox24h-LDL, and the extent of the binding of the native LDL (○) and ox24h-LDL-coated erythrocytes (●) by thioglycolate-induced mouse peritoneal macrophages was measured as described in Materials and Methods. C: Extent of macrophage recognition of the LDL-coated erythrocytes as a function of the amount of LDL adsorbed on the erythrocytes. Data of A and B were combined. ○, native LDL-coated erythrocytes; ●, ox24h-LDL-coated erythrocytes.

first attempted to coat erythrocytes with ox24h-LDL. The ox24-LDL as well as native LDL was radiolabeled with *N*-succinimidyl [2,3-³H]propionate and various concentrations of ³H-labeled LDL were incubated with mouse erythrocytes at 37 °C for 15 min. The extent of LDL adsorption to erythrocytes was determined by the radioactivity associated with the cells. As shown in Fig. 1A, both ox24h-LDL and native LDL were found to spontaneously adhere to the erythrocytes under these conditions. The adsorption of both ox24h-LDL and native LDL was dependent on the concentration of LDL. At 10 µg protein of LDL/ml, 2.5 ng protein of ox24h-LDL and 1 ng protein of native LDL were adsorbed to 10⁶ erythrocytes and at 100 µg/ml, 5.5 ng of ox24h-LDL and 3.2 ng of native LDL were adsorbed. The ox24h-LDL was somewhat more adherent to erythrocytes than native LDL.

Erythrocytes were similarly coated with the unlabeled ox24-LDL and native LDL, and then examined using the monolayer of thioglycolate-induced mouse peritoneal macrophages to see whether the ox24h-LDL-coated erythrocytes were recognized by macrophages. Erythrocytes coated with various concentrations of native LDL (native-LDL-erythrocytes) and ox24h-LDL (ox24h-

LDL-erythrocytes) were loaded on the macrophage monolayers and incubated at 37 °C for 1 h in the absence of serum. After removal of nonadherent erythrocytes by washing, the macrophage monolayers were observed under phase-contrast microscopy. The extent of the macrophage recognition of the LDL-coated erythrocytes was expressed as the percentage of macrophages binding two or more erythrocytes; the data are expressed as a function of the LDL concentration used in coating the erythrocytes (Fig. 1B). While very few macrophages bound native LDL-erythrocytes at LDL concentrations up to 100 µg/ml, more than 60% of macrophages bound ox24h-LDL-erythrocytes at LDL concentrations above 5 µg/ml. Figure 2 shows a representative picture of the macrophages, in which nearly no native-LDL-erythrocytes attached to the macrophages (A), and many ox24h-LDL-erythrocytes attached to the macrophages forming erythrocyte rosettes (B).

The data from Figs. 1A and B were combined and the extent of macrophage recognition of the LDL-coated erythrocytes was plotted as a function of the extent of LDL adsorption to erythrocytes (Fig. 1C). Native LDL-erythrocytes were never recognized by macrophages even though the amount of LDL adsorbed to erythrocytes increased. The extent of recognition of ox24h-LDL-erythrocytes increased as the amount of LDL adsorbed to erythrocytes increased and reached a maximum at 2.5 ng protein/10⁶ erythrocytes, corresponding to the LDL concentration of 10 µg/ml used in the coating, and then slightly declined. It is evident that the difference in the recognition of ox24h-LDL-erythrocytes and native LDL-erythrocytes by macrophages is not due to the difference in the adhesiveness of ox24h-LDL and native LDL to erythrocytes, but to a qualitative difference in the properties of native LDL and ox24h-LDL particles.

Whether the ox24h-LDL-erythrocytes were ingested by macrophages was then examined. When erythrocytes coated with 100 µg/ml of native LDL or ox24h-LDL were incubated with the monolayer of thioglycollate-induced mouse peritoneal macrophages at 37 °C for 3 h in the absence of serum, very few macrophages ingested either type of LDL-coated erythrocytes (data not shown).

It has been postulated that macrophages recognize ox-LDL by the degree of LDL oxidation.¹⁵⁾ Whether the extent of the binding of ox-LDL-coated erythrocytes to macrophages reflects the degree of LDL oxidation was examined. LDL was incubated for various times up to 24 h in the presence or absence of CuSO₄ (1 or 5 µM), and the resultant LDL preparations were analyzed for lipid oxidation (PCOOH and TBARS formation) and their capacity to bind to macrophages when coated on erythrocytes (Fig. 3). At 1 µM CuSO₄, PCOOH reached a maximum at 8 h and was unchanged at 24 h while TBARS increased gradually up to 24 h (A). At 5 µM CuSO₄, the PCOOH level reached a maximum at 4 h then dropped to the control level at 24 h while TBARS similarly reached a maximum at 4 h but then declined only slightly thereafter (A). The level of PCOOH and TBARS of control LDL did not increase. The binding potency of the erythrocytes coated with LDL oxidized at each concentration of CuSO₄ increased depending on the time of

Fig. 2. Microscopic Observation of Thioglycollate-Induced Macrophages Binding Native LDL-Coated Erythrocytes (A) and ox24h-LDL-Coated Erythrocytes (B)

Erythrocytes were coated with 100 µg/ml of native LDL or ox24h-LDL, and the binding of the coated erythrocytes by thioglycollate-induced mouse peritoneal macrophages was observed under phase-contrast microscopy as described in Materials and Methods.

Fig. 3. Correlation between the Extent of LDL Oxidation and the Extent of Recognition of the ox-LDL-Coated Erythrocytes by Thioglycolate-Induced Macrophages

A: PCOOH and TBARS generation of LDL variously oxidized with Cu^{2+} . LDL (2 mg protein/ml) in 10 mM sodium phosphate-buffered saline (pH 7.4) was incubated without (○) or with $1 \mu\text{M}$ (▲) and $5 \mu\text{M}$ (●) CuSO_4 at 37°C for the indicated time, and PCOOH and TBARS generated were determined as described in Materials and Methods. B: Macrophage recognition of the erythrocytes coated with LDL variously oxidized with Cu^{2+} . LDL was incubated similarly to A and the extent of the recognition of the erythrocytes coated with $100 \mu\text{g}/\text{ml}$ of the oxidized LDL by thioglycolate-induced mouse peritoneal macrophages was determined as described in Materials and Methods.

LDL oxidation and the concentration of CuSO_4 used (B). Erythrocytes coated with control LDL did not show any increase in binding to macrophages (B). The increases in the levels of PCOOH and TBARS appear to precede the increase in the binding capacity of erythrocytes coated with the oxidized LDL (compare A and B). These results indicate that the binding capacity of erythrocytes coated with oxidized LDL to macrophages reflects the degree of LDL oxidation.

To clarify that the binding of ox-LDL-erythrocytes to macrophages is scavenger receptor-specific, the effect of free ligands for the scavenger receptor on the binding of ox-LDL-erythrocytes to macrophages was tested. Macrophage monolayers were preincubated with various concentrations of native LDL, ox24h-LDL, and LDL oxidized with $5 \mu\text{M}$ Cu^{2+} at 37°C for 48 h (ox48h-LDL) for 2 h. After washing the monolayer, the binding of ox24h-LDL-erythrocytes was measured. As shown in Fig. 4, pretreatment of macrophages with ox24h-LDL or ox48h-LDL dose dependently inhibited the subsequent binding of ox24h-LDL-erythrocytes, while native LDL was ineffective. The inhibitory activity of ox48h-LDL was more potent than that of ox24h-LDL (Fig. 4). Similarly, other ligands for the scavenger receptor such as m-BSA, dextran sulfate, and poly I^{7,8)} completely (m-BSA) or

Fig. 4. Inhibition of the Adherence of ox24h-LDL-Coated Erythrocytes to Thioglycolate-Induced Macrophages by Free ox-LDL

Monolayers of thioglycolate-induced mouse peritoneal macrophages were preincubated with the indicated concentrations of native LDL (○), ox24h-LDL (●) or ox48h-LDL (▲) in RPMI-HEPES medium ($200 \mu\text{l}$ per coverglass) at 37°C for 2 h. After washing the monolayer with DPBS, the extent of the adherence of the erythrocytes coated with $100 \mu\text{g}/\text{ml}$ ox24h-LDL was examined as described in Materials and Methods.

TABLE I. Inhibition of the Adherence of ox24h-LDL-Coated Erythrocytes to Thioglycolate-Induced Macrophages by the Ligands for Scavenger Receptor

Pretreatment of macrophages with	Concn. (mg/ml)	Macrophages binding ox24h-LDL-erythrocytes (% of total macrophages)
None		58.8 ± 4.1
Native BSA	10	51.0 ± 3.5
m-BSA ^{a)}	10	2.4 ± 0.5
Dextran sulfate	0.2	27.6 ± 1.8
Poly I	0.2	31.1 ± 4.9
Poly C	0.2	49.7 ± 3.1

Monolayers of thioglycolate-induced mouse peritoneal macrophages were preincubated with or without the indicated concentrations of ligands and nonligands for the scavenger receptor in RPMI-HEPES medium ($200 \mu\text{l}$ per coverglass) at 37°C for 2 h. After washing the monolayers once with DPBS, the extent of the adherence of erythrocytes coated with $10 \mu\text{g}/\text{ml}$ of ox24h-LDL was examined as described in Materials and Methods. The same concentrations of ligands and nonligands used in the preincubation of the monolayer were also contained in the respective ox24h-LDL-erythrocyte suspensions. ^{a)} Preparation with 95% modification of total amino groups.

partially (dextran sulfate and poly I) inhibited the binding of ox24h-LDL-erythrocytes to macrophages, while nonligands (BSA and poly C)^{7,8)} had little effect (Table I). These results indicate that ox-LDL-erythrocytes are specifically recognized by the scavenger receptor.

Adherence of m-BSA- and f-BSA-Coated Erythrocytes to Thioglycolate-Induced Mouse Peritoneal Macrophages
We further attempted to measure the binding of protein ligands for the scavenger receptor to macrophages as the adherence of erythrocytes precoated with the protein. Both m-BSA^{7,8)} and f-BSA¹¹⁾ were used as ligands. To coat the mouse erythrocytes with these proteins, conventional methods of coating erythrocytes with soluble protein antigens by tannic acid or chromium chloride in passive hemagglutinating tests^{24,25)} were examined. Coating the erythrocytes with modified BSA using tanned erythrocytes gave a high level of nonspecific binding of erythrocytes to macrophages and was of no use for our purpose. Then, erythrocytes were incubated with modified BSA at $0.2 \text{ mg}/\text{ml}$ at 37°C for 15 min in the presence

Fig. 5. Binding and Phagocytosis by Thioglycolate-Induced Macrophages of the Erythrocytes Coated with BSA Maleylated to Various Degrees

Erythrocytes were coated with 200 μ g/ml BSA modified with maleic anhydride to various degrees, and the binding (●) and phagocytosis (○) by thioglycolate-induced mouse peritoneal macrophages were measured as described in Materials and Methods.

of chromium chloride as an adsorbent. The amounts of native BSA, m-BSA (a preparation in which 50% of total amino groups maleylated), and f-BSA (50% of total amino groups modified) bound to erythrocytes were 31.6 ± 4.3 , 51.0 ± 4.6 , and 65.2 ± 7.1 ng/ 10^6 cells, respectively, as determined using native and modified BSA labeled with *N*-succinimidyl [2,3-³H]propionate.

By means of this coating method, erythrocytes were coated with m-BSA preparations with various degrees of maleylation, and their binding and phagocytosis by thioglycolate-induced mouse peritoneal macrophages were measured. Very few macrophages bound erythrocytes coated with native BSA, but many macrophages bound m-BSA-coated erythrocytes (m-BSA-erythrocytes) (Fig. 5). The binding increased with the increasing degree of maleylation of BSA (Fig. 5). Erythrocytes coated with m-BSA which had been 50% modified were recognized by $29.1 \pm 5.0\%$ of macrophages, and those coated with m-BSA which had been 80% modified were recognized by $76.6 \pm 2.6\%$ of macrophages. Phagocytosis of these erythrocytes did not take place (Fig. 5). The binding of erythrocytes coated with m-BSA which had been 50% modified to macrophages was inhibited in a dose-dependent fashion by m-BSA preparations which had been 50 and 80% modified (Fig. 6A). The inhibitory activity of m-BSA which had been 80% modified was more potent than that of m-BSA which had been 50% modified (Fig. 6A). These results indicate that binding of m-BSA-erythrocytes to macrophages is mediated by the scavenger receptor.

Mouse erythrocytes coated with f-BSA which had been 50% modified (f-BSA-erythrocytes) were also recognized by the macrophages ($29.8 \pm 1.5\%$), while native BSA-erythrocytes were only slightly recognized ($11.7 \pm 2.8\%$). The recognition was decreased when the macrophages were

Fig. 6. Inhibition of the Adherence of Modified BSA-Coated Erythrocytes to Thioglycolate-Induced Macrophages by Free Modified BSA

A: Inhibition of the adherence of erythrocytes coated with m-BSA by free m-BSA. Adherence of the erythrocytes coated with m-BSA (preparation with 50% modification of total amino groups) to the monolayers of thioglycolate-induced mouse peritoneal macrophages at 37°C for 1 h was measured in the presence of the indicated concentrations of free BSA with 50% (●) and 80% (▲) modification of the total amino groups. The concentration of total free BSA during the binding assay was adjusted to 10 mg/ml with native BSA. B: Inhibition of the adherence of erythrocytes coated with f-BSA (50% modification of the total amino groups) to the macrophages was similarly performed as in A in the presence of free f-BSA (50% modification).

TABLE II. Inhibition of the Adherence of ox24h-LDL-Coated Erythrocytes to Resident Macrophages by the Ligands for the Scavenger Receptor

Pretreatment of macrophages with	Concn. (mg/ml)	Macrophages binding ox24h-LDL-erythrocytes (% of total macrophages)
Expt. A		
None		42.0 ± 5.6
ox24h-LDL	0.1	6.7 ± 3.6
Expt. B		
None		41.1 ± 1.3
Native BSA	10	47.8 ± 7.8
m-BSA ^{a)}	10	3.7 ± 0.7
Dextran sulfate	0.2	1.7 ± 0.6
Fucoidan	0.2	13.8 ± 3.6
Poly I	0.2	2.8 ± 1.0
Poly C	0.2	27.8 ± 7.6

Expt. A: Monolayers of resident mouse peritoneal macrophages were preincubated with or without 0.1 mg/ml of ox24h-LDL in RPMI-HEPES medium (200 μ l per coverglass) at 37°C for 1 h. After washing the monolayers with DPBS, the extent of the adherence of erythrocytes coated with 10 μ g/ml of ox24h-LDL was examined as described in Materials and Methods except that the macrophage incubation with ox24h-LDL-erythrocytes was for 30 min. Expt. B: Using resident peritoneal macrophages, the experiment was carried out as described in the legend to Table I except that macrophage preincubation was for 1 h, and macrophage incubation with ox24h-LDL-erythrocytes was for 30 min. ^{a)} Preparation with 95% modification of total amino groups.

pretreated with free f-BSA (Fig. 6B), indicating that binding of f-BSA-erythrocytes to macrophages is mediated by the scavenger receptor.

Adherence of ox-LDL-Coated Erythrocytes to Resident Mouse Peritoneal Macrophages Whether this method for

the assay of ox-LDL binding to macrophages described above is applicable to other types of macrophage was examined using resident mouse peritoneal macrophages. Mouse erythrocytes were incubated with 10 or 100 $\mu\text{g/ml}$ ox24h-LDL or native LDL and the binding of the LDL-coated erythrocytes to the monolayers of the resident peritoneal macrophages was examined. The ox24h-LDL-erythrocytes bound to the macrophages (bound by $30.4 \pm 2.6\%$ of macrophages) when the erythrocytes were coated with 10 $\mu\text{g/ml}$ of ox24h-LDL. However, erythrocytes coated with 100 $\mu\text{g/ml}$ of ox24h-LDL or with either concentration of native LDL exhibited only slight binding (bound by less than 4% of macrophages). Erythrocytes coated with 10 $\mu\text{g/ml}$ of ox24h-LDL formed a rosette around the macrophages (not shown), as observed in thioglycolate-induced macrophages (Fig. 2). The binding of the ox24h-LDL-erythrocytes to the resident macrophages was inhibited by pretreatment of the macrophages with the ligands for the scavenger receptor (ox-LDL, m-BSA, dextran sulfate, fucoidan, and poly I),⁷⁻⁹⁾ but was unaffected or only slightly inhibited by nonligands (BSA and poly C)^{7,8)} (Table II). These results indicate that ox-LDL-erythrocytes are recognized also by resident mouse peritoneal macrophages through their scavenger receptor.

DISCUSSION

Functional studies on the macrophage scavenger receptor and its ligands require measurement of ligand-binding to the scavenger receptor. The present method, where the ligands are attached to erythrocytes and the adherence of the ligand-coated erythrocytes to macrophages is measured under microscopy, offers a simple method for the assessment of scavenger receptor-ligand interactions.

Erythrocytes were readily coated with native LDL and ox-LDL simply by incubating the cells. Although the mechanism of spontaneous LDL adherence to erythrocytes was not investigated here, we suppose that the lipid portion of the LDL particle may be involved in the interaction with erythrocytes and the binding of ox-LDL adsorbed on erythrocytes to the scavenger receptor is probably through the oxidatively-modified apoprotein portion of the ox-LDL particle, as has been suggested for the ox-LDL-macrophage interaction.²⁶⁾ The adherence of LDL to erythrocytes appeared not to occur in the presence of high concentrations of other proteins since erythrocytes incubated with ox-LDL (10 $\mu\text{g/ml}$) in the presence of BSA (5 mg/ml or higher) were not recognized by thioglycolate-induced macrophages (data not shown). Thus, high concentrations of proteins such as are found in blood may prevent erythrocytes from adhering to LDL.

The binding of ox-LDL-coated erythrocytes to macrophages depended on the concentration of the LDL used in the coating. Coating the erythrocytes with 10 $\mu\text{g/ml}$ of ox24h-LDL resulted in the maximum binding of ox24h-LDL-erythrocytes to thioglycolate-induced and non-induced peritoneal macrophages, but coating with 100 $\mu\text{g/ml}$ of ox24h-LDL resulted in weaker recognition in both types of macrophages. At present, we have no

data explaining why macrophage recognition declines when the ox-LDL coating of the erythrocytes exceeds the optimum. However, one possibility may be that excessive adherence of ox-LDL to erythrocytes may increase the negative charges on the erythrocyte surface because of the negative net charge of ox-LDL,^{3,15,27)} and this may generate repulsive forces between the erythrocytes and macrophages. The ability of noninduced peritoneal macrophages to bind the ox-LDL-coated erythrocytes appeared more dependent on the dose of ox-LDL used for erythrocyte coating than that of thioglycolate-induced counterparts. The difference between the two types of macrophages may be due to their surface properties, including the surface charge and density of the scavenger receptor.

It is evident from these observations that the binding of ligand-coated erythrocytes to macrophages was mediated by binding of the ligands on erythrocytes to the scavenger receptor. Firstly, erythrocytes coated with ox-LDL or modified BSA, that are known to be the ligands for the scavenger receptor, but not with the nonligand native ones, bound to macrophages. Secondly, pretreatment of macrophages with various ligands for the scavenger receptor, but not with nonligands, prevented the subsequent binding of the ligand-coated erythrocytes to the macrophages. For assessment of scavenger receptor-ligand interactions, the present method is convenient in that (i) only small amounts of erythrocytes and modified LDL or proteins are needed; (ii) the whole procedure does not require any special facilities and is quite simple.

Measurement of ligand binding to the scavenger receptor has been performed at a low temperature, near 0°C, to prevent receptor-mediated endocytosis of the ligand that would take place at a physiological temperature.^{1,12)} In the present adhesion study of ox-LDL or modified BSA-coated erythrocytes to macrophages, erythrocytes were not phagocytosed at all within 3 h, in spite of the fact that the assay was performed at 37°C. It is likely that it takes much a longer time for macrophages to phagocytose the ligand-coated large particles of erythrocytes, compared with endocytosis.

The extent of adherence of ox-LDL-erythrocytes and m-BSA-erythrocytes to macrophages appeared to reflect the extent of LDL oxidation and BSA maleylation, respectively. Therefore, this assay method may be used for assessing the binding capacity of modified LDL or proteins. Another method for assessing the activity of any substance as a ligand for the scavenger receptor is to measure its ability to inhibit the binding of a known ligand, such as ox-LDL and m-BSA. This is usually performed by the inhibition of the binding or uptake of radioiodinated ligand LDL or proteins.^{1,2,7)} Since the adherence of ox-LDL-, m-BSA-, and f-BSA-coated erythrocytes to macrophages was prevented by preincubation of macrophages with free ox-LDL, m-BSA, and f-BSA, respectively, in a dose-dependent fashion, it should be possible to assess the ligand activity of unknown samples by this method. This inhibition procedure is useful particularly for determining the ligand activity of samples that are not suitable for erythrocyte coating or have only weak activity.

In conclusion, the method presented here is useful for studying the interaction between the macrophage scavenger receptor and its ligands, particularly for the assessment of the ligand-binding activity of the receptor and the receptor-binding activity of the ligands.

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