Endocytic uptake of advanced glycation end products by mouse liver sinusoidal endothelial cells is mediated by a scavenger receptor distinct from the macrophage scavenger receptor class A

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Previous studies with peritoneal macrophages obtained from macrophage scavenger receptor class A (MSR-A) knock-out mice showed that the endocytic uptake of advanced glycation end products (AGE) by macrophages was mediated mainly by MSR-A. However, it is controversial whether the endocytic uptake of intravenously injected AGE proteins by liver sinusoidal endothelial cells (LECs) is similarly explained by receptor-mediated endocytosis via MSR-A. The present study was conducted to compare the capacity to endocytose AGE proteins in LECs and peritoneal macrophages obtained from MSR-A knock-out and littermate wild-type mice. The endocytic degradation capacity of MSR-A knock-out LECs for AGE-BSA was indistinguishable from that of wild-type LECs, whereas that of MSR-A knock-out peritoneal macrophages for AGE-BSA was decreased to 30% of that in wild-type cells. Similarly, the endocytic degradation of MSR-A knock-out LECs for acetylated low-density lipoprotein (acetyl-LDL) did not differ from that of

wild-type LECs, whereas the endocytic degradation of acetyl-LDL by MSR-A knock-out peritoneal macrophages was less than 20% of that in wild-type cells. Furthermore, formaldehyde-treated serum albumin (f-Alb), a ligand known to undergo scavenger-receptor-mediated endocytosis by LECs, was effectively taken up by MSR-A knock-out LECs at a capacity that did not differ from that of wild-type LECs. Moreover, the endocytic uptake of AGE-BSA by LECs was effectively competed for by unlabelled f-Alb or acetyl-LDL. These results indicate that the scavenger-receptor ligands AGE proteins, acetyl-LDL and f-Alb are endocytosed by LECs through a non-MSR-A pathway.

Key words: acetylated low-density lipoprotein, endocytosis, formaldehyde-treated albumin, galectin-3, peritoneal macrophage.

INTRODUCTION

Prolonged incubation of proteins with glucose leads, through the formation of early-stage products such as Schiff bases and Amadori rearrangement products, to the formation of advanced glycation end products (AGE), which are characterized physico-chemically by fluorescence, a brown colour and cross-linking, and biologically by specific recognition by the AGE receptors. Three types of AGE receptor so far identified are macrophage scavenger receptor class A (MSR-A) [1,2], RAGE (receptor for AGE) [3] and the receptor complex of oligosaccharyltransferase-48, a 80–87 kDa protein substrate for protein kinase C and galectin-3 [4].

Our previous studies showed that more than 90% of intravenously injected AGE-BSA is eliminated within 15 min by sinusoidal liver cells such as sinusoidal endothelial cells (LECs) and Kupffer cells, which was explained by receptor-mediated endocytosis [5]. LECs and Kupffer cells were responsible for 60–65% and 24–28% respectively of the hepatic uptake, whereas the contribution of hepatocytes was less than 20%. Experiments *in vitro* showed that AGE proteins undergo receptor-mediated endocytosis by isolated cultured LECs. In addition to AGE proteins, acetylated low-density lipoprotein (acetyl-LDL), an authentic ligand for MSR-A, is also known to be specifically

endocytosed by LECs of rat [6] and mouse [7]. Other ligands known to be recognized by LECs include chemically modified proteins such as formaldehyde-treated serum albumin (f-Alb) [8], nitroguanidine-treated albumin [9] and acetaldehyde-treated albumin [10], and physiological ligands such as hyaluronan [11], collagen α -chains [12], chondroitin sulphate [13] and N-terminal propeptides of types I and III procollagen [14].

In spite of numerous endocytosis studies with cultured LECs, the molecular nature of the receptors expressed by LECs involving endocytic uptake of these ligands is not well understood, owing largely to a lack of molecular-genetics studies. In contrast, our understanding at a molecular level of the endocytosis receptors in macrophages, such as MSR-A [1], CD36 [15], macrophage receptor with a collagenous structure ('MARCO') [16] and other members of the scavenger receptor family, has markedly increased owing to extensive molecular studies. Ligand cross-competition experiments in our earlier studies with sinusoidal liver cells (a mixture of mainly LECs and Kupffer cells) indicated that the receptor for f-Alb in these cells is distinct from that for acetyl-LDL [17]. In contrast, our study with peritoneal macrophages from MSR-A knock-out mice showed that the endocytic capacity for AGE-BSA by these cells was decreased to approx. 20–30% of those of wild-type littermate mice [18], suggesting a major role of MSR-A in the endocytic uptake of

Abbreviations used: acetyl-LDL, acetylated low-density lipoprotein; AGE, advanced glycation end products; CHO, Chinese hamster ovary; f-Alb, formaldehyde-treated serum albumin; LECs, liver sinusoidal endothelial cells; MSR-A, macrophage scavenger receptor class A; RAGE, receptor for AGE.

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AGE proteins by macrophages. LECs also show a high endocytic capacity for acetyl-LDL and AGE proteins, which explains the rapid plasma clearance of intravenously injected ^{125}I -acetyl-LDL [6] and ^{125}I -AGE-BSA [5]. Two experiments with MSR-A knock-out mice have previously been performed to assess the contribution of MSR-A to the endocytic uptake of acetyl-LDL by LECs. Ling et al. [19] found that the endocytic capacity for acetyl-LDL by LECs obtained from MSR-A knock-out mice was only 30 % of that in the wild-type LECs. A similar result was obtained independently by van Berkel et al. [7], who reported that the endocytic degradation of acetyl-LDL by MSR-A knock-out LECs was decreased to 40 % of that in the wild-type LECs. These results point to the notion that MSR-A has a major role in the endocytic uptake of acetyl-LDL by LECs. However, the finding that the extent of contribution of MSR-A to the endocytic uptake of acetyl-LDL by LECs was smaller than that of peritoneal macrophages strongly suggests that a receptor other than MSR-A might be involved in the endocytic uptake of acetyl-LDL. To obtain a clearer picture of the contribution of MSR-A to the endocytic uptake of AGE proteins as well as acetyl-LDL, the present study was conducted to compare the capacity to endocytose AGE proteins in LECs and peritoneal macrophages obtained from either MSR-A knock-out or wild-type littermate mice. The results obtained are compatible with the notions that (1) MSR-A does not significantly contribute to the endocytic uptake by LECs and (2) the major part of the LECs' endocytic capacity for AGE proteins and acetyl-LDL is mediated by a novel scavenger receptor that also efficiently recognizes f-Alb.

MATERIALS AND METHODS

Chemicals

BSA (fraction V) was purchased from Wako (Osaka, Japan). RPMI 1640 medium, penicillin G and streptomycin were purchased from Life Technologies (New York, NY, U.S.A.). Collagenase was obtained from Wako (Osaka, Japan). Na^{125}I was purchased from Amersham Pharmacia Biotech (Little Chalfont, Bucks., U.K.). All reagents used were of the highest grade available from commercial sources.

Ligand preparation and iodination

LDL ($d = 1.019\text{--}1.063\text{ g/ml}$) was isolated by sequential ultracentrifugation of fresh plasma from normolipidaemic subjects after overnight fasting, and dialysed against 0.15 M NaCl/1 mM EDTA (pH 7.4) [20]. Acetyl-LDL was prepared by chemical modification of LDL with acetic anhydride as described previously [20]. AGE-BSA was prepared as described previously [21]. In brief, 2.0 g of BSA was dissolved in 10 ml of 0.5 M sodium phosphate buffer, pH 7.4, with 3.0 g of D-glucose. Each sample was sterilized by ultrafiltration, incubated at 37 °C for 40 weeks and dialysed against 20 mM sodium phosphate buffer, pH 7.4. f-Alb was prepared by a modification of the method reported [22]. In brief, 0.4 g of BSA was dissolved at room temperature in 5.2 ml of 0.45 M sodium carbonate buffer, pH 10.0, followed by centrifugation to remove insoluble debris. To the supernatant solution was added, dropwise and with stirring, 5.4 ml of formaldehyde solution (37 %, w/v) to give a final concentration of 20 % (w/v). The mixture was incubated at 37 °C for 1 h and dialysed overnight at 4 °C against 0.5 M sodium phosphate buffer, pH 7.4. AGE-BSA and f-Alb were labelled with ^{125}I by Iodogen from Pierce Chemical Co.

(Rockford, IL, U.S.A.) to specific radioactivities of 450 and 850 c.p.m./ng respectively. Acetyl-LDL was labelled with ^{125}I by the method of McFarlane [23] to a specific radioactivity of 510 c.p.m./ng.

Animals

Inactivation of the gene encoding MSR-A was performed as described previously [18], by using homologous recombination of a targeted construct spanning exon 4 of the type AI/type AII MSR gene in A3-1 embryonic stem cells. Descriptions of the construct and the phenotype expression in homozygous knock-out mice were reported previously [18]. Normal littermates were bred as controls.

Isolation and culture of LECs

Isolation of LECs from mouse was performed similarly to the method devised previously for the preparation of rat LECs [24]. The portal vein was cannulated with a polyethylene catheter 0.9 mm in diameter, and the liver was perfused with Gey's balanced salt solution (GBSS) buffer, pH 7.5, without Ca^{2+} for 10 min at a flow of 8 ml/min, and then with GBSS buffer, pH 7.5, containing 0.05 % collagenase and 4 mM CaCl_2 for 3 min at 37 °C. The liver was minced and suspended in ice-cold RPMI 1640 medium. The cell suspension was centrifuged at 100 g for 2 min. The supernatant was then centrifuged at 800 g for 10 min. The cells in the pellet were resuspended in 10 ml of RPMI 1640 medium and layered on top of a two-step Percoll gradient. The gradient consisted of 25 % (v/v) Percoll (top) and 50 % (v/v) Percoll (bottom). The gradient was centrifuged at 800 g for 30 min. The intermediate zone was collected. The enriched LECs suspended in Percoll were washed twice with 50 ml of PBS and suspended in 10 ml of RPMI 1640 medium supplemented with 0.1 mg/ml streptomycin and 100 i.u./ml penicillin (medium A). Aliquots (1 ml) of the cell suspension were seeded into each well (wild-type 2.0×10^6 per well, MSR-A knock-out type 3.0×10^6 per well) of human fibronectin-coated 24-multiwell plates (15.5 mm in diameter; Beckton-Dickinson, Bedford, U.K.). The multiwell plate was incubated for 40 min at 37 °C in a humidified incubator under air/ CO_2 (19:1). Each well was washed 10 times with 1 ml of PBS to remove non-adherent cells, then further incubated for 2 h with 1 ml of medium A and replaced by 1 ml of fresh medium A followed by a 1 h incubation before experiments.

Culture of peritoneal macrophages

Peritoneal macrophages were collected [25] and suspended in RPMI 1640 medium supplemented with 3 % (w/v) BSA, 0.1 mg/ml streptomycin and 100 i.u./ml penicillin (medium B) and then seeded (wild-type, 10^6 per well; MSR-A knock-out, 1.5×10^6 per well) on a 24-well plastic plate (15.5 mm diameter; Corning, Corning, NY, U.S.A.). Adherent macrophages were cultured for 3 h at 37 °C in a humidified incubator under air/ CO_2 (19:1) before the cells were used for experiments.

Cell assays

Unless mentioned otherwise, all cell experiments were performed at 37 °C in a humidified incubator under air/ CO_2 (19:1). The cells in each well (LECs and peritoneal macrophages) were washed with 1 ml of PBS and refurnished with 1.0 ml of medium B. The cells in each well were incubated, in a total volume of

1.0 ml of medium B, with various concentrations of the radio-labelled ligand to be tested, with or without an excess of corresponding unlabelled ligand. After incubation for indicated durations, 0.75 ml of culture medium was taken from each well and mixed with 0.3 ml of 40% trichloroacetic acid in a vortex mixer. To this solution was added 0.2 ml of 0.7 M AgNO₃, followed by centrifugation. The resulting supernatant (0.5 ml) was used to determine trichloroacetic-acid-soluble radioactivity, which was taken as an index of cellular degradation. The remaining cells in each well were washed three times with 1 ml of PBS containing 1% (w/v) BSA and another three times with PBS. The cells were lysed at 37 °C for 40 min with 1.0 ml of 0.1 M NaOH. One portion was used to determine the radioactivity as the cell-associated ligand; the other portion was used to determine cellular proteins with bicinchoninic acid protein assay reagent (Pierce). As reported previously [18], we noticed that LECs and peritoneal macrophages from MSR-A knock-out mice show a lower capacity to adhere to culture dishes. Because a functional difference between MSR-A knock-out cells and control cells should be compared at the same density, the initial cell densities of the MSR-A knock-out LECs and peritoneal macrophages in the present study were 1.5-fold higher than that of the corresponding control cells, so that the same cell density was maintained during the experiment.

Antibodies against MSR-A, galectin-3 and RAGE

Rat monoclonal antibody against mouse MSR-A (2F8) was purchased from BMA Biomedicals (Tokyo, Japan). Galectin-3 has been described as Mac-2 or carbohydrate-binding protein 35 (CBP 35) [26]. Rat monoclonal antibody against mouse galectin-3 (M3/38) that had been originally established as the specific antibody against Mac-2 [27] was purchased from Cedarlane (Hornby, Ontario, Canada). To generate an antibody specific for RAGE, a fragment of mouse RAGE peptide (C¹⁶⁵DGKETLVKEETRRH¹⁷⁹; single-letter amino acid codes) was conjugated with keyhole-limpet haemocyanin. The conjugated peptide (0.5 mg) in 50% (v/v) Freund's complete adjuvant was injected intradermally into a rabbit at 20 skin sites, followed by three booster injections with 0.2 mg of the conjugated peptide in 50% (v/v) Freund's incomplete adjuvant. The serum was taken 10 days after the final immunization. We prepared Chinese hamster ovary (CHO) cells overexpressing human RAGE (CHO-RAGE cells) as RAGE-positive cells. To prepare CHO-RAGE cells, human RAGE cDNA was prepared from a human lung cDNA library (Clontech, Tokyo, Japan) by reverse-transcriptase-mediated PCR and inserted into the mammalian expression vector pCR3.1 (Invitrogen, Groningen, The Netherlands). This vector was transfected to CHO cells by the lipofection method by the protocol recommended by the manufacturer (Lipofectin; Life Technologies, New York, NY, U.S.A.), and cells resistant to 0.4 mg/ml G418 were selected. CHO-RAGE cells thus obtained were examined for its cDNA and protein expression levels by reverse-transcriptase-mediated PCR and immunoblotting.

SDS/PAGE and immunoblotting

Cultured LECs and peritoneal macrophages from wild-type and MSR-A knock-out mouse were prepared as above. Cells were harvested and lysed by scraping in a ice-cold hypotonic buffer [1 mM Tris/1 mM EDTA (pH 7.4)]. Protein concentrations of the cell lysates were kept between 2 and 4 mg/ml. The lysates were dissolved in 125 mM Tris/HCl, pH 6.8, containing 4%

(w/v) SDS, 20% (v/v) glycerol, 0.02% Bromophenol Blue and 750 mM 2-mercaptoethanol, and were heated for 5 min in a boiling-water bath. SDS/PAGE was performed with 4–20% (w/v) polyacrylamide gel from Daiichi Pure Chemicals (Tokyo, Japan). After electrophoretic transfer, the nitrocellulose membrane was treated with the blocking buffer [20 mM Tris/HCl (pH 7.6)/150 mM NaCl/0.3% (v/v) Tween 20/5% (w/v) skimmed milk]. The membrane was further incubated with anti-(MSR-A) antibody, anti-(galectin-3) antibody or anti-RAGE antibody in blocking buffer as a primary antibody, followed by incubation with horseradish-peroxidase-labelled goat anti-rabbit IgG or horseradish-peroxidase-labelled goat anti-rat IgG from Dako (Glostrup, Denmark) in blocking buffer as a secondary antibody. Signals were detected by enhanced-chemiluminescence reagents in accordance with the manufacturer's instructions (Amersham). The molecular mass was estimated with a molecular-mass marker kit (Daiichi Pure Chemicals).

RESULTS

Endocytic uptake of AGE-BSA, acetyl-LDL and f-Alb by mouse LECs

¹²⁵I-AGE-BSA was avidly taken up by cultured LECs. The association (Figure 1A) and subsequent degradation of endocytosed ligand by the cells (Figure 1B) increased in a dose-dependent manner. The cell association of ¹²⁵I-AGE-BSA and its endocytic degradation were effectively competed for by a 50-fold excess of unlabelled AGE-BSA. The specific cell association of ¹²⁵I-AGE-BSA and its specific degradation exhibited a saturation pattern, indicating that AGE-BSA underwent an effective receptor-mediated endocytosis by mouse LECs, as observed previously with rat LECs [5]. LECs also showed a high endocytic capacity for ¹²⁵I-acetyl-LDL (Figures 1C and 1D) and ¹²⁵I-f-Alb (Figures 1E and 1F), indicating the presence of specific binding sites for these ligands in mouse LECs.

Figure 2 shows the results of the time-course experiments. The specific cell association of ¹²⁵I-AGE-BSA to LECs, which represented more than 90% of the total cell association, increased with time (Figure 2A); after cell association, ¹²⁵I-AGE-BSA was endocytosed and degraded effectively by LECs in a time-dependent manner (Figure 2B). Parallel time-course experiments showed that both ¹²⁵I-acetyl-LDL and ¹²⁵I-f-Alb were also effectively endocytosed and degraded by cultured LECs (results not shown).

Endocytic uptake of AGE-BSA by MSR-A knock-out mouse peritoneal macrophages

We previously reported that the endocytic uptake of AGE-BSA by peritoneal macrophages was mediated mainly by MSR-A, because its endocytic uptake by such cells prepared from MSR-A knock-out mice was only 30% of that of wild-type peritoneal macrophages [18]. To confirm this result, the endocytic capacities of MSR-A knock-out macrophages for AGE-BSA and acetyl-LDL were compared with those of wild-type macrophages under the present conditions. The cell association of ¹²⁵I-AGE-BSA and its subsequent endocytic degradation by MSR-A knock-out peritoneal macrophages were 50% and 30% respectively of that of wild-type peritoneal macrophages (Table 1). The endocytic capacity of MSR-A knock-out macrophages for acetyl-LDL was decreased to a more significant extent. The cell association of ¹²⁵I-acetyl-LDL with these knock-out macrophages was approx. 20% of that of the wild-type cells, and the amount of ¹²⁵I-acetyl-LDL degraded by these cells was 18% of that in the wild-type

Figure 1 Dose-dependent effects of endocytic uptake of AGE-BSA, acetyl-LDL and f-Alb by mouse LECs

LECs from mice were prepared as described in the Materials and methods section. The cells were incubated with 1.0 ml of medium B containing increasing amounts of ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL and ^{125}I -f-Alb in the presence (●) or absence (□) of a 50-fold excess of unlabelled ligands. After 3 h of incubation, the amount of cell-associated ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL and ^{125}I -f-Alb (A, C, E respectively) and their degradation (B, D, F respectively) were determined as described in the Materials and methods section. The specific cell association and degradation (●) were plotted after correcting for non-specific cell association and degradation. Results are means $\pm$ S.D. for three separate experiments.

cells (Table 1). These results were highly consistent with those obtained with MSR-A knock-out peritoneal macrophages [18].

Expression of AGE receptors in mouse LECs and peritoneal macrophages

Three AGE receptors demonstrated so far are RAGE, galectin-3 and MSR-A. Before examining the endocytic capacity of LECs from MSR-A knock-out mice, we compared LECs and peritoneal macrophages in their expression of these AGE receptors by immunoblot analyses.

Previous immunohistochemical studies with anti-(MSR-A) antibody (2F8) demonstrated the presence of MSR-A in mouse LECs [5] and peritoneal macrophages [28]. Immunoblot experiments with the same anti-(MSR-A) antibody detected the monomer of MSR-A as a 70 kDa band in both LECs and peritoneal macrophages (Figure 3A). The extent of expression in LECs was slightly weaker than that in peritoneal macrophages.

Previous immunochemical studies demonstrated that galectin-3 was weakly expressed on mouse resident peritoneal macrophages, but was strongly induced by elicitation with thioglycollate [29], and that galectin-3 was also expressed on mouse liver sinusoidal cells such as Kupffer cells and endothelial cells [30]. In immunoblot analyses with anti-(galectin-3) antibody (M3/38), galectin-3 was detected as a band of approx. 32 kDa in both LECs and peritoneal macrophages (Figure 3B). The quantities of galectin-3 in LECs and peritoneal macrophages seemed to be indistinguishable at the same lysate concentrations. Immunoblotting of cellular lysate of LECs from MSR-A knock-out and wild-type mice with M3/38 showed approximately the same extent of expression of galectin-3 on both cells (Figure 3D), indicating

that there was no compensatory up-regulation of galectin-3 in LECs with a deficiency in MSR-A.

Immunohistochemical expression of RAGE in bovine tissues has been characterized extensively by Brett et al. [31]. These authors found that RAGE was concentrated on parenchymal cells, whereas no significant expression was observed with sinusoidal cells such as Kupffer cells and LECs. RAGE was highly positive in alveolar macrophages but negative in circulating monocytes. However, the presence of RAGE in peritoneal macrophages remains unclear. In immunoblot analyses, no positive bands were detected in either LECs (Figure 3C, lane 1) or peritoneal macrophages (Figure 3C, lane 2). Under parallel conditions, immunoblot analyses of lysates of CHO-RAGE cells overexpressing human RAGE demonstrated the positive band at approx. 50 kDa (Figure 3C, lane 3). These immunoblot studies indicate that MSR-A and galectin-3 are expressed on both mouse LECs and peritoneal macrophages, whereas RAGE is poorly expressed in these cells.

Endocytic uptake of AGE-BSA and f-Alb by LECs from MSR-A knock-out mice

We then determined whether mouse LECs behaved in the same way as peritoneal macrophages in their endocytic capacity for AGE-BSA. The endocytic capacities for AGE-BSA, acetyl-LDL and f-Alb were compared between LECs isolated from MSR-A knock-out and wild-type littermate mice. Unexpectedly, the quantities of cell-associated ^{125}I -AGE-BSA and those subsequently degraded by MSR-A knock-out LECs were indistinguishable from those of wild-type LECs (Table 2). A parallel experiment with ^{125}I -acetyl-LDL gave a surprising result.

Figure 2 Time-dependent effects of endocytic uptake of AGE-BSA by mouse LECs

LECs from mice were prepared as described in the Materials and methods section. The cells were incubated with 1.0 ml of medium B containing 5.0 µg/ml ^{125}I -AGE-BSA in the presence (◆) or absence (□) of a 50-fold excess of unlabelled ligands. After the indicated durations of incubation, the amounts of cell-associated ^{125}I -AGE-BSA (A) and its degradation (B) were determined as described in the Materials and methods section. The specific cell association and degradation (●) were plotted after correcting for non-specific cell association and degradation. Results are means ± S.D. for three separate experiments.

Table 1 Endocytic uptake of ^{125}I -AGE-BSA and ^{125}I -acetyl-LDL by peritoneal macrophages from MSR-A knock-out mice

Peritoneal macrophages were prepared from MSR-A knock-out mice as described in the Materials and methods section. The cells were incubated for 3 h at 37 °C with 1.0 ml of medium B containing 5 µg/ml ^{125}I -AGE-BSA or ^{125}I -acetyl-LDL in the presence or absence of 250 µg/ml unlabelled ligands. The extents of cellular association of ^{125}I -AGE-BSA and ^{125}I -acetyl-LDL and their degradation were determined as described in the Materials and methods section. The specific association and degradation of ^{125}I -AGE-BSA and ^{125}I -acetyl-LDL were calculated after correction for non-specific association and degradation. Results are means ± S.D. for three separate experiments.

Compound	Specific association (ng/mg of cell protein)		Specific degradation (ng/mg of cell protein per 3 h)	
	MSR ^{+/+}	MSR ^{-/-}	MSR ^{+/+}	MSR ^{-/-}
^{125}I -AGE-BSA	752 ± 32	356 ± 8	426 ± 18	123 ± 15
^{125}I -acetyl-LDL	1267 ± 118	258 ± 16	478 ± 35	81 ± 15

The cell association of ^{125}I -acetyl-LDL with MSR-A knock-out LECs and its endocytic degradation were at almost the same order of magnitude as those of wild-type LECs (Table 2).

Figure 3 Immunoblot analyses of MSR-A, galectin-3 and RAGE in LECs and peritoneal macrophages

(A–C) Cell lysates from wild-type LECs (20 µg) and cell lysates from wild-type peritoneal macrophages (20 µg) were subjected to SDS/PAGE [4–20% (w/v) gel] and immunoblotted as follows: (A) with anti-(MSR-A) antibody (2F8), lane 1 for LECs and lane 2 for peritoneal macrophages; (B) with anti-(mouse galectin-3) antibody (M3/38), lane 1 for lysate of LECs and lane 2 for lysates from peritoneal macrophages; (C) with anti-(mouse RAGE) antibody, lane 1 for cell lysates of LECs and lane 2 for lysates from peritoneal macrophages. Cell lysates (20 µg) from CHO-RAGE cells were used as a positive control (lane 3). (D) Cell lysates from wild-type LECs (20 µg) and cell lysates from MSR-A knock-out LECs (20 µg) were subjected to SDS/PAGE [4–20% (w/v) gel] and immunoblotted with anti-(mouse galectin-3) antibody (M3/38). Lanes 1 and 2 contained lysate of wild-type LECs and lysates from MSR-A knock-out LECs respectively.

Table 2 Endocytic uptake of ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL and ^{125}I -f-Alb by LECs prepared from MSR-A knock-out mice

LECs from MSR-A knock-out mice were prepared as described in the Materials and methods section. The cells were incubated for 3 h at 37 °C with 1.0 ml of medium B containing 5 µg/ml ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL or ^{125}I -f-Alb in the presence or absence of 250 µg/ml unlabelled ligands. The amounts of cell-associated ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL or ^{125}I -f-Alb and their degradation were determined. The specific association and degradation of ^{125}I -AGE-BSA, ^{125}I -acetyl-LDL and ^{125}I -f-Alb were calculated after correction for non-specific association and degradation. Results are means ± S.D. for three separate experiments.

Compound	Specific association (ng/mg of cell protein)		Specific degradation (ng/mg of cell protein per 3 h)	
	MSR ^{+/+}	MSR ^{-/-}	MSR ^{+/+}	MSR ^{-/-}
^{125}I -AGE-BSA	1089 ± 69	1125 ± 103	1269 ± 105	1308 ± 96
^{125}I -acetyl-LDL	968 ± 63	982 ± 59	886 ± 53	905 ± 47
^{125}I -f-Alb	748 ± 55	813 ± 48	789 ± 49	812 ± 45

Additional experiments showed that LECs from MSR-A knock-out mice were able to endocytose ^{125}I -f-Alb as effectively as LECs from wild-type littermate mice (Table 2). Taken together, these results (Table 1 and 2) indicate that in peritoneal macrophages AGE-BSA and acetyl-LDL are endocytosed almost exclusively through MSR-A, whereas the endocytic uptake of AGE-BSA and acetyl-LDL by LECs takes place through a pathway distinct from MSR-A. Because the endocytic capacity of mouse LECs for f-Alb was not significantly affected by knock-

Figure 4 Effect of acetyl-LDL and f-Alb on endocytic uptake of ¹²⁵I-AGE-BSA by mouse LECs

The cells were incubated with 1.0 ml of medium B containing 5 μ g/ml ¹²⁵I-AGE-BSA for 3 h in the presence or absence of 250 μ g/ml unlabelled competitor. The extents of cellular association of ¹²⁵I-AGE-BSA (A) and degradation (B) were determined as described in the Materials and methods section. Results are means $\pm$ S.D. for three separate experiments and are expressed as percentages of inhibition. The 100% values of association and degradation were 1480 and 1050 ng/mg of cell protein respectively.

out of MSR-A from these cells (Table 2), it is also likely that f-Alb is endocytosed by LECs via a route different from MSR-A.

To characterize a plausible receptor pathway of mouse LECs that was distinct from MSR-A but responsible for the endocytic uptake of AGE-BSA, we tested the effects of several ligands on the endocytic uptake of ¹²⁵I-AGE-BSA by LECs. As Figure 4(A) shows, the cell association of ¹²⁵I-AGE-BSA with these cells and its subsequent endocytic degradation were inhibited by excess unlabelled acetyl-LDL (250 μ g/ml), by 60 % and 80 % respectively. Furthermore, the endocytic degradation of ¹²⁵I-AGE-BSA by LECs was also significantly inhibited, by more than 60 %, by excess unlabelled f-Alb (Figure 4B). These results suggest that f-Alb competed with AGE-BSA for the same or closely related binding sites on LECs. To test this notion further, we examined whether the binding sites for f-Alb were also expressed by peritoneal macrophages. The endocytic degradation of ¹²⁵I-AGE-BSA by mouse peritoneal macrophages was inhibited by acetyl-LDL as effectively as by AGE-BSA, whereas f-Alb had no appreciable effect (Figure 5B). Experiments with ¹²⁵I-f-Alb failed to reveal specific binding to these cells (results not shown). Furthermore, the extent of endocytic degradation of ¹²⁵I-f-Alb by peritoneal macrophages was negligible compared with that of ¹²⁵I-AGE-BSA and ¹²⁵I-acetyl-LDL (Figure 6). These results indicate that f-Alb is not recognized at all by peritoneal macrophages as a ligand for MSR-A. Taken together,

Figure 5 Effect of acetyl-LDL and f-Alb on endocytic uptake of ¹²⁵I-AGE-BSA by mouse peritoneal macrophages

The cells were incubated for 3 h with 1.0 ml of medium B containing 5 μ g/ml ¹²⁵I-AGE-BSA in the presence or absence of 250 μ g/ml unlabelled competitor. The extents of cellular association of ¹²⁵I-AGE-BSA (A) and degradation (B) were determined as described in the Materials and methods section. Results are means $\pm$ S.D. for three separate experiments and are expressed as percentages of inhibition. The 100% values of association and degradation were 820 and 410 ng/mg of cell protein respectively.

Figure 6 Endocytic degradation of ¹²⁵I-AGE-BSA, ¹²⁵I-acetyl-LDL and ¹²⁵I-f-Alb by mouse peritoneal macrophages

Mouse peritoneal macrophages were prepared as described in the Materials and methods section. The cells were incubated with 1.0 ml of medium B containing 5 μ g/ml labelled ligands (¹²⁵I-AGE-BSA, ¹²⁵I-acetyl-LDL or ¹²⁵I-f-Alb) in the presence or absence of 250 μ g/ml unlabelled ligands. After 3 h of incubation, the amounts of labelled ligands that had been degraded by mouse peritoneal macrophages were determined as described in the Materials and methods section. The columns represent specific degradation of labelled ligands, plotted after correcting for non-specific degradation. Results are means $\pm$ S.D. for three separate experiments.

our results indicate that the receptor for f-Alb is hardly expressed in peritoneal macrophages, whereas it is highly expressed in mouse LECs, which mediate the endocytic uptake of AGE-BSA and acetyl-LDL in addition to that of f-Alb.

DISCUSSION

The present study with LECs and peritoneal macrophages from MSR-A knock-out mice showed clearly that the endocytic uptake of AGE-BSA by LECs is mediated by one or more receptors other than MSR-A, whereas MSR-A is responsible mainly for effective endocytic uptake of AGE-BSA by peritoneal macrophages. Because the present results obtained with MSR-A knock-out LECs were in sharp contrast with previous reports with the use of peritoneal macrophages isolated from MSR-A knock-out mice [18,32,33], we thought it better to repeat the experiments with MSR-A knock-out peritoneal macrophages under parallel conditions. The result obtained (Table 1) was consistent not only with our previous report [18], but also with others [7,32,33], confirming the notion that MSR-A has a major role in the endocytic uptake of AGE-BSA as well as acetyl-LDL by peritoneal macrophages.

Our results are inconsistent with previous reports of Ling et al. [19] and van Berkel et al. [7], who used LECs from MSR-A knock-out mice. The capacity of their MSR-A knock-out LECs to endocytose ^{125}I -acetyl-LDL was significantly decreased (30 % and 50 % of control respectively), whereas our MSR-A knock-out LECs did not differ from wild-type LECs in their endocytic uptake of ^{125}I -acetyl-LDL (Table 2). Several possibilities could explain this inconsistency. First, our method of separating LECs from mouse liver is slightly different from that of van Berkel et al. [7] and Ling et al. [19]; the former group [7] used centrifugal elutriation to separate LECs from Kupffer cells, whereas the latter group [19] and the present study employed Percoll gradient centrifugation. Secondly, there are several factors or variables that affect the function of LECs in culture systems. In the system of Ling et al. [19], LECs were grown on fibronectin-coated substrate and allowed to proliferate to confluence by the aid of endothelial growth supplement. After one passage these LECs were used for experiments. However, in our system a primary culture of LECs was established on fibronectin-coated dishes in the presence of only antibiotics and used for experiments. van Berkel and his associates used primary LECs in suspension [7]. In addition to mediating the endocytosis of many chemically modified proteins, MSR-A is known to possess several cellular functions, for example, cell adhesion [28], intracellular signalling [34] and growth-factor secretion [35]. In fact, peritoneal macrophages obtained from MSR-A knock-out mice are reported to decrease their capacity to adhere to culture plates [18]. These alterations in cellular functions induced either directly or indirectly by knock-out of the gene encoding MSR-A might affect their MSR-A-mediated endocytic capacity. In contrast, from a quantitative standpoint, the results of van Berkel et al. [7] and Ling et al. [19] could also be interpreted to indicate that LECs lacking MSR-A could retain 30–50 % of their endocytic capacity for acetyl-LDL. Furthermore, these two groups also showed that the hepatic uptake and plasma clearance of intravenously injected acetyl-LDL in MSR-A knock-out mice did not differ from those of wild-type mice [7,19]. Taken together, these results strongly suggest the presence of alternative receptor(s) responsible for the endocytic uptake of acetyl-LDL by LECs.

It has long been known that f-Alb is taken up by sinusoidal liver cells after intravenous injection [36]. Blomhoff et al. [8] showed that f-Alb was effectively taken up by rat LECs via a receptor-specific mechanism, but endocytic uptake was not

observed in Kupffer cells. Our earlier study with isolated rat sinusoidal liver cells, a mixture of LECs and Kupffer cells, revealed that f-Alb undergoes receptor-mediated endocytosis; cross-competition experiments showed that the ligand is taken up via a route somewhat distinct from acetyl-LDL [21], an authentic ligand for MSR-A. This notion was supported by the present result that deprivation of MSR-A from LECs did not alter their endocytic capacity for ^{125}I -f-Alb. We therefore compared the endocytic route of f-Alb with that of AGE-BSA in LECs. Cultured mouse LECs have a high endocytic capacity for f-Alb (Figures 1E and 1F). The endocytic degradation of ^{125}I -AGE-BSA by LECs was competed for (by 40 %) by excess f-Alb. In contrast with LECs, peritoneal macrophages had no endocytic capacity for f-Alb (Figure 6), and f-Alb had no effect on the endocytic uptake of ^{125}I -AGE-BSA (Figure 5). These results suggest strongly that LECs, but not peritoneal macrophages, express one or more receptors for f-Alb, through which AGE proteins might also mainly be taken up.

On the basis of previous results and the present results discussed above, it is evident that the receptor(s) that mediate the endocytic uptake of AGE proteins, acetyl-LDL and probably f-Alb by LECs differ from MSR-A at least. The receptor(s) involved, however, are not known. RAGE [3] and galectin-3 [4] are categorized as AGE receptors in addition to MSR-A. To test the possibility of whether these receptors are responsible for this phenomenon, a preliminary experiment was performed in the present study. Immunoblot analyses with specific antibodies detected MSR-A in LECs, a finding consistent with the previous study [18]. Galectin-3 was also positively detected in LECs, whereas RAGE was not (see Figure 3). Although a further attempt has to be made to identify the receptor(s) in LECs, it does not seem likely that the efficient endocytic uptake of AGE proteins by LECs is due to a high expression of other known AGE receptors such as galectin-3 and RAGE.

In conclusion, the present study provides evidence that AGE-BSA as well as acetyl-LDL are endocytosed mainly through MSR-A in peritoneal macrophages, whereas the endocytic uptake of these ligands by LECs takes place through a pathway distinct from MSR-A. The receptor for f-Alb, which is expressed exclusively by LECs, might be involved in the endocytic uptake of AGE-BSA and acetyl-LDL.

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