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CD36 Is Not Involved in Scavenger Receptor–Mediated Endocytic Uptake of Glycolaldehyde- and Methylglyoxal-Modified Proteins by Liver Endothelial Cells

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Circulating proteins modified by advanced glycation end-products (AGE) are mainly taken up by liver endothelial cells (LECs) *via* scavenger receptor–mediated endocytosis. Endocytic uptake of chemically modified proteins by macrophages and macrophage-derived cells is mediated by class A scavenger receptor (SR-A) and CD36. In a previous study using SR-A knockout mice, we demonstrated that SR-A is not involved in endocytic uptake of AGE proteins by LECs [Matsumoto *et al.* (2000) *Biochem. J.* 352, 233–240]. The present study was conducted to determine the contribution of CD36 to this process. Glycolaldehyde-modified BSA (GA-BSA) and methylglyoxal-modified BSA (MG-BSA) were used as AGE proteins. ^{125}I-GA-BSA and ^{125}I-MG-BSA underwent endocytic degradation by these cells at 37°C, and this process was inhibited by several ligands for the scavenger receptors. However, this endocytic uptake of ^{125}I-GA-BSA by LECs was not inhibited by a neutralizing anti-CD36 antibody. Similarly, hepatic uptake of ^{111}In-GA-BSA after its intravenous injection was not significantly attenuated by co-administration of the anti-CD36 antibody. These results clarify that CD36 does not play a significant role in elimination of GA-BSA and MG-BSA from the circulation, suggesting that the receptor involved in endocytic uptake of circulating AGE proteins by LEC is not SR-A or CD36.

Key words: advanced glycation end-products, CD36, endocytosis, intermediate aldehydes, liver endothelial cells.

Abbreviations: Ac-LDL, acetylated low-density lipoprotein; AGE, advanced glycation end-products; GA, glycolaldehyde; LDL, low-density lipoprotein; LEC, liver endothelial cell; MG, methylglyoxal; Ox-LDL, oxidized low-density lipoprotein; SR-A, class A scavenger receptor.

Long-term incubation of proteins with glucose leads to formation of advanced glycation end-products (AGE), *via* formation of early products such as Schiff base and Amadori products. AGE proteins are characterized physicochemically by fluorescence, a brown color and intramolecular and intermolecular cross-linking (*1*), and biologically by specific recognition by the AGE receptors (*2*). Recent studies show that several aldehydes, including glycolaldehyde (GA), glyoxal, methylglyoxal (MG) and 3-deoxyglucoson, are generated during the Maillard reaction from glucose, Schiff base or Amadori products (*3*, *4*). The fact that these aldehydes have much stronger chemical reactivity than glucose indicates that they play an important role in generation of AGE structures *in vivo* (*5*). Nagai *et al.* found that in human atherosclerotic lesions, GA-pyridine (a novel structure derived from GA-modified proteins) accumulated in the cytoplasm of foam cells and extracellularly in the central region of atheroma (*6*). In an immunohistochemical study, proteins modified with glyceraldehydes, GA, MG or glyoxal were detected in diabetic serum (*7*).

Previous studies show that AGE proteins accumulate in liver extremely rapidly after they are intravenously administered, due to receptor-mediated endocytosis by nonparenchymal cells, particularly liver endothelial cells (LECs) (*8*, *9*). *In vitro* experiments have shown that AGE proteins undergo scavenger receptor–mediated endocytosis by LECs, which is inhibited by acetylated low-density lipoprotein (Ac-LDL), an authentic ligand for scavenger receptor, and formaldehyde-treated albumin (*10*).

Despite numerous studies of endocytosis by cultured LECs, the molecular nature of the receptors that are expressed by LECs and are involved in endocytic uptake of AGE proteins is not well understood. AGE receptors that have been reported include the receptor for AGE (RAGE) (*11*), oligosaccharyltransferase-48 (OST-48), 80K-H, galectin-3 complex (*12*) and scavenger receptors such as class A scavenger receptor (SR-A) (*13*), CD36 (*14*), SR-BI (*15*) and LOX-1 (*16*). Peritoneal macrophages obtained from SR-A knockout mice have a significantly reduced capacity to degrade Ac-LDL (<30%) and oxidized LDL

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(Ox-LDL) (~50%), compared with wild-type macrophages (*17*). In addition, the capacity of these cells to endocytose AGE-BSA and Ac-LDL is approximately 30 to 50% of that of cells of wild-type littermate mice (*10*), suggesting that SR-A plays a major role in endocytosis of AGE proteins and modified LDL by macrophages. Furthermore, recent experiments using peritoneal macrophages obtained from SR-A/CD36 double knockout mice clearly show that SR-A and CD36 account for 75 to 90% of endocytic degradation of Ac-LDL and Ox-LDL, strongly indicating that SR-A and CD36 are responsible for endocytic uptake of modified LDL by macrophages and macrophage-derived cells (*18*). However, in a previous study, we found that LECs obtained from SR-A knockout mice did not differ from LECs of wild-type mice in their capacity to endocytose AGE-BSA, strongly suggesting that a receptor(s) other than SR-A is involved in the endocytic uptake of AGE proteins by LECs (*10*). Consequently, we examined the role of CD36 in endocytic uptake of AGE proteins by LECs.

CD36 is a multifunctional receptor expressed in several cell types including macrophages (*19*), adipocytes (*20*), LECs (*21*) and platelets (*22*). Recent experiments using Chinese hamster ovary (CHO) cells overexpressing CD36 show that AGE-BSA acts as a high-affinity ligand for CD36 and undergoes receptor-mediated endocytosis, indicating that CD36 is an AGE receptor (*14*). This conclusion is consistent with the recent finding that a significant increase in CD36 expression during cell differentiation of adipocytes from preadipocytes is accompanied by a parallel increase in the endocytic uptake of AGE proteins by these adipocytes; endocytic degradation of AGE-BSA by human subcutaneous adipocytes and 3T3-L1 adipocytes was inhibited by >80% by a neutralizing anti-CD36 antibody, indicating that CD36 plays a major role in the endocytosis of AGE-BSA by these cells (*23*).

In the present study, GA-modified BSA (GA-BSA) and MG-modified BSA (MG-BSA) were used as AGE proteins to determine the contribution of CD36 to endocytic uptake of these AGE proteins by mouse LECs in *in vivo* plasma clearance experiments, and in *in vitro* binding and endocytosis experiments using isolated LECs.

MATERIALS AND METHODS

Chemicals—BSA (fraction V) was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). RPMI 1640 medium, Dulbecco's modified Eagle's medium (DMEM), biotinamidohexanoic acid *N*-hydroxysuccinimido ester (BNHS), collagenase and mouse IgAκ (TEPC 15) were obtained from Sigma Chemical Co. (St. Louis, MO). $^{111}InCl_3$ (74 MBq/ml in 0.02 M HCl) was donated by Nihon Medi-Physics (Takarazuka, Japan). $Na^{125}I$ (3.7 GBq/ml in NaOH) and [9,10(*n*)-^{3}H]Oleic acid (370 GBq/mmol) were purchased from Amersham Pharmarcia Biotech (Little Chalfont, Bucks., U.K.). Mouse anti–murine CD36 monoclonal antibody (clone 63) was purchased from Cascade Bioscience (Winchester, MA, USA). All reagents used were of the highest grade available from commercial sources.

Ligand Preparation—To prepare GA-BSA, 5 mg/ml BSA was incubated with 50 mM GA at 37°C for 3 days in phosphate-buffered saline (PBS), followed by dialysis against PBS (*3*). MG-BSA was prepared under identical conditions, except that 50 mM carbonate buffer (pH 9.0) was used instead of PBS. LDL (d = 1.019–1.063 g/ml) was isolated by sequential ultracentrifugation of fresh plasma from normolipidaemic subjects after overnight fasting, and was dialysed against 0.15 M NaCl/1 mM EDTA (pH 7.4) (*24*). Ac-LDL was prepared by chemical modification of LDL with acetic anhydride as described previously (*24*). Ox-LDL was prepared by dialyzing LDL against PBS to remove EDTA, incubating the LDL (0.1 mg/ml) with 5 μM $CuSO_4$ at 37°C for 24 h, adding 1 mM EDTA, and cooling.

Protein Labeling with ^{111}In and ^{125}I—BSA, GA-BSA and MG-BSA were labeled with ^{111}In using diethylenetriaminepentaacetic acid (DTPA) as the bifunctional chelating agent as described previously (*25*). DTPA was attached to BSA by dissolving the protein (5 mg) in 1.0 ml of 0.1 M Hepes buffer (pH 7.0), and then adding 50 μg of DTPA anhydride in 10 μl of dimethyl sulfoxide. After stirring for 1 h at room temperature, unreacted DTPA was removed by passing the solution through a Sephadex G-25 column (1 × 40 cm) with 0.1 M sodium acetate buffer (pH 6.0). Fractions containing DTPA-BSA (based on absorption at 280 nm) were combined and concentrated using an ultrafiltration apparatus from Advantec (Dublin, CA). Then, 20 μl of $^{111}InCl_3$ solution, 60 μl of DTPA-BSA and 20 μl of 1 M sodium acetate buffer (pH 6.0) were added. After incubation for 30 min at room temperature, unreacted $^{111}InCl_3$ was removed by loading the solution on a PD-10 column and eluting with 0.1 M sodium acetate buffer (pH 6.0). The ^{111}In-enriched fractions were identified on the basis of their radioactivity, and were concentrated by ultrafiltration. Radioactivity was measured with a well counter. The specific activities of ^{111}In-BSA, ^{111}In-GA-BSA and ^{111}In-MG-BSA were 12, 11 and 11 × 10^6 cpm/mg protein, respectively.

GA-BSA and MG-BSA were labeled with ^{125}I using Iodo-Gen (Pierce). A solution containing 10 μl of $Na^{125}I$ solution and 0.5 mg of BSA in 0.2 ml of 0.1 M sodium phosphate buffer (pH 7.4) in an Iodo-Gen–coated test tube was incubated for 30 min at room temperature. Unreacted $Na^{125}I$ was removed by loading the solution on a PD-10 column and eluting with PBS. The ^{125}I-labeled fractions were combined after being identified on the basis of their radioactivity, which was measured with a well counter. The specific activities of GA-BSA and MG-BSA were 800 and 900 cpm/ng protein, respectively.

In Vivo Experiments—Prior to intravenous administration, trace amounts of ^{111}In-GA-BSA or ^{111}In-MG-BSA were diluted with saline, and the protein concentration was adjusted to 0.01 mg/ml. Each radiolabeled protein (0.1 mg/kg, 30 kcpm) was injected as a bolus into the tail vein of male ddY mice, with or without co-administration of unlabelled ligands (10 mg/kg) including GA-BSA, MG-BSA, Ox-LDL and Ac-LDL or mouse anti-murine CD36 monoclonal antibody (0.5 mg/kg) and mouse IgAκ (0.5 mg/kg), under anesthesia with diethylether (*26*). At 10 min after injection, the animals were killed and the liver was excised, followed by measurement of radioactivity.

To determine hepatocellular distribution of intravenously injected GA-BSA and MG-BSA, the animals were sacrificed at 5 min after administration of ^{111}In-GA-BSA or ^{111}In-MG-BSA. The preparation of pure cultures of functionally intact Kupffer cells, LECs and parenchymal

cells from a single rat liver is described in detail elsewhere (*27*). The portal vein was cannulated with a polyethylene catheter (diameter, 0.9 mm), 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, 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 GBSS containing 1% (w/v) BSA (medium A). The cell suspension was applied on top of a 70% Percoll gradient diluted with PBS, followed by centrifugation at 800 × *g* for 10 min. Only pure, intact parenchymal cells penetrated the gradient solution and were recovered from the pellet. Cells, including nonparenchymal cells and damaged parenchymal cells, that remained on top of the Percoll solution were collected and suspended in medium A, followed by centrifugation at 800 × *g* for 10 min. The cells in the pellet were resuspended in 10 ml of medium A 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. Pure nonparenchymal cells banded at the interface between the two density cushions. Pure cultures of Kupffer cells were obtained by seeding purified nonparenchymal cells onto the surface of tissue culture plastic and incubating them for 15 min at 37°C. Pure cultures of LECs were obtained by transferring the nonadherent cells from these dishes to dishes coated with fibronectin and incubating them for 1 h at 37°C. To determine the hepatocellular distribution of each ^{111}In-labeled protein, the radioactivity of parenchymal cells, Kupffer cells and LECs was measured.

Preparation of Biotinylated Antibody against GA-BSA—The monoclonal anti–GA-BSA antibody was prepared and purified by protein G-immobilized Sepharose gel chromatography to IgG1, as described previously (*6*). This monoclonal antibody was shown to be specific for the glycolaldehyde-derived pyridine (GA-pyridine) structure (*6*), and is referred to here as the anti–GA-pyridine antibody (*6*). The anti–GA-pyridine antibody (0.5 mg) was biotinylated by reacting it with 50 μg of BNHS for 1 h at room temperature in 0.1 M carbonate buffer (pH 8.4), followed by dialysis against PBS to remove unreacted BNHS. The immunoreactivity of the biotinylated antibody for GA-BSA was equal to that of the unlabeled anti–GA-pyridine antibody, as determined by ELISA.

Immunohistochemistry—GA-BSA (5 mg/kg) was injected as a bolus into the tail vein of male ddY mice at 7 weeks of age, after they were anaesthetized with diethylether. The animals were killed at 5 min after the injection. The liver was removed and fixed with a 2% periodate/lysine/paraformaldehyde fixative at 4°C for 5 h, and was then washed with PBS containing a graded series of sucrose (10, 15 and 20%). After immersion in PBS containing 20% sucrose, to inhibit ice crystal formation, the tissues were embedded in OCT compound (Sakura Fine Technical Co., Tokyo, Japan), frozen in liquid nitrogen, and stored at –80°C until used. Sections were cut at a thickness of 5 μm using a cryostat (HM-500M; Microm, Walldorf, Germany), and were mounted on poly-L-lysine–coated slides.

For immunohistochemical analysis, cryostat sections were prepared and examined using the indirect immunoperoxidase method. Briefly, after inhibition of endogenous peroxidase activity using the method of Isobe *et al.* (*28*), the sections were incubated with 10 μg/ml biotinylated anti–GA-pyridine antibody, washed with PBS, and reacted with streptavidin /HRP (DAKO). After visualization with 3,3′-diaminobenzidine (Dojin Chemical Co., Kumamoto, Japan), the sections were counterstained with hematoxylin (Mutoh Chemical Company, Tokyo, Japan). Two types of negative controls were produced: (i) by omitting antibody when performing the immunohistochemical procedures; (ii) by using nonimmune mouse IgG1 instead of anti–GA-pyridine antibody (staining of these controls produced no evidence of an immunoreaction). For the immunoabsorption test, 10 μg/ml of biotinylated anti–GA-pyridine antibody was preincubated with 10 μg/ml of GA-BSA at 37°C for 1 h, then reacted with Streptavidin/HRP.

Cellular Experiments with LECs—LECs isolated from mouse liver as described above were plated onto human fibronectin-coated 24-well plates (Becton Dickinson Lab., Bedford, U.K.) at 2 × 10^6 cells/well, and were grown in serum-free RPMI 1640 for 40 min at 37°C. Each well was then washed 3 times with 0.75 ml of PBS to remove nonadherent cells, and was incubated for 2 h with serum-free RPMI 1640 (*10*). Except for the binding experiments described below, all cellular experiments were performed at 37°C in a humidified atmosphere of 5% CO_2 in air.

In the binding experiments, the confluent LECs were washed twice with 1.0 ml of PBS and incubated for 90 min at 4°C with 0.5 ml of DMEM supplemented with 3% (w/v) BSA containing the indicated concentrations of ^{125}I-GA-BSA or ^{125}I-MG-BSA with or without 50-fold excess of unlabeled GA-BSA or MG-BSA. After washing twice with ice-cold PBS containing 1% (w/v) BSA and washing twice more with PBS, the cells were dissolved with 0.5 ml of 0.1 M NaOH, and the cell-bound radioactivity and cellular proteins were assayed.

In the uptake and degradation experiments, the confluent cells were washed twice with PBS and incubated at 37°C with 0.5 ml of DMEM supplemented with 3% (w/v) BSA containing the indicated concentrations of ^{125}I-GA-BSA or ^{125}I-MG-BSA with or without 50-fold excess of unlabeled GA-BSA or MG-BSA. After incubation for the indicated times, culture medium (0.375 ml) was taken from each well and mixed with 0.15 ml of 40% trichloroacetic acid on a vortex mixer. To this solution was added 0.1 ml of 0.7 M $AgNO_3$, followed by centrifugation. The resulting supernatant was used to determine trichloroacetic-acid–soluble radioactivity, which was used as an index of cellular degradation. The remaining cells in each well were washed three times with 0.75 ml of PBS containing 1% (w/v) BSA, and twice more with PBS. The cells were lysed at 37°C for 30 min with 0.5 ml of 0.1 M NaOH. One portion of the sample was used to measure the radioactivity of the cell-associated ligand, and the other portion was used to assay cellular proteins using a bicinchoninic acid protein assay reagent (Pierce) (*10*).

In the [^{3}H]oleate transport experiments, the confluent cells were washed twice with PBS and incubated at 37°C with 0.5 ml of DMEM containing 1 μM [^{3}H]oleate in the absence (control) or presence of 10 μg/ml mouse anti-murine CD36 monoclonal antibody and mouse IgAκ. After incubation for 2 h, cells in each well were washed three times with 0.75 ml of PBS. The cell pellet was

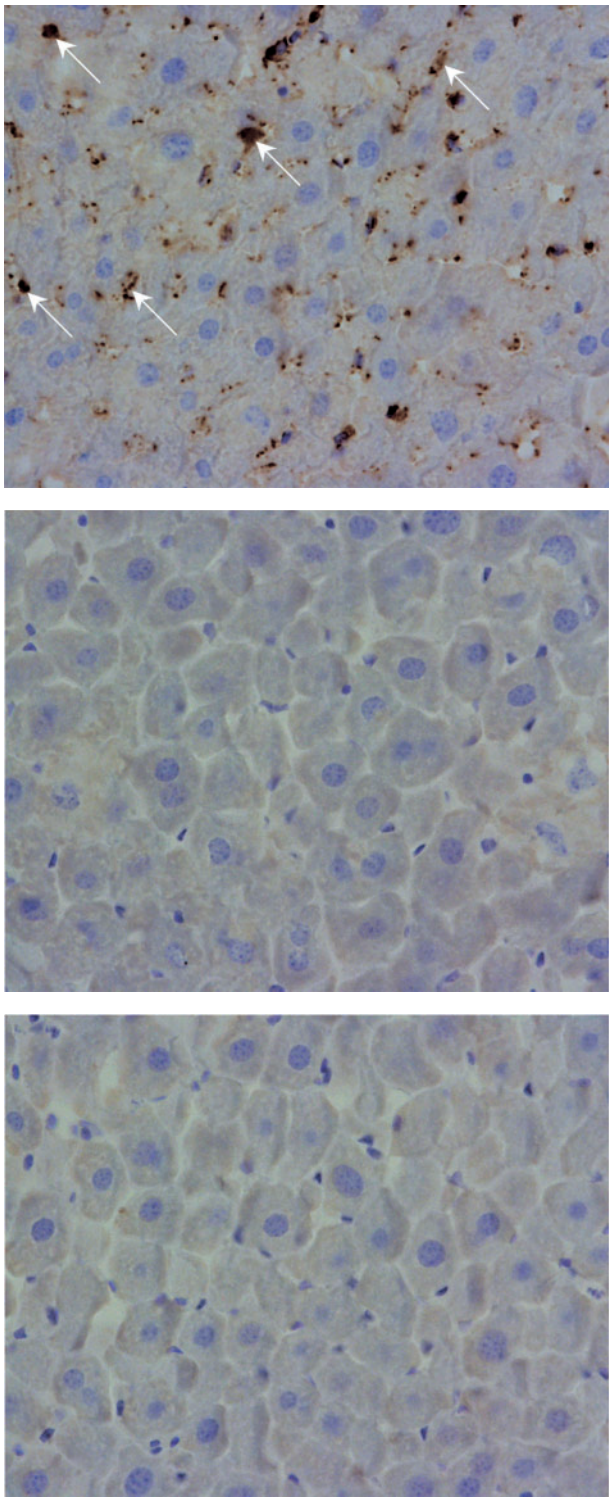

Fig. 1. **Immunohistochemical localization in liver of GA-BSA after its intravenous administration to mice.** The liver was removed 5 min after injection of a bolus of GA-BSA or BSA through the tail vein of mice. A: Anti–GA-pyridine antibody–positive products were observed in non-parenchymal cells, especially LECs obtained from mice that had been administered GA-BSA. B: Such positive reactions were abolished by preincubation of the antibody with 10 μg/ml GA-BSA. C: No immunoreactivity of anti–GA-pyridine antibody was detected in the LECs obtained from mice administered BSA.

Table 1. **Hepatocellular distribution of intravenously injected ^{111}In-GA-BSA and ^{111}In-MG-BSA to mice.**

	Uptake per cell population (% of total recovered)	
	GA-BSA	MG-BSA
Parenchymal cells	8.94	23.45
Nonparenchymal cells		
Kupffer cells	11.94	16.23
Liver endothelial cells	79.12	60.32

Uptake per cell population is shown as the relative radioactivity per total recovered radioactivity as parenchymal cells, kupffer cells and liver endothelial cells from the intact liver.

treated for 30 min with 0.2 ml of 1 M NaOH at room temperature. The solution was neutralized with 0.25 ml of 1 M HCl, and the radioactivity incorporated into the cells was counted using a liquid scintillation spectrophotometer.

In the inhibition assays, LECs were incubated at 37°C for 6 h with 2.5 μg/ml of ^{125}I-GA-BSA or ^{125}I-MG-BSA in the absence (control) or presence of a 40-fold concentration (100 μg/ml) of unlabeled ligands including GA-BSA, MG-BSA, Ox-LDL, Ac-LDL and LDL or 10 μg/ml mouse anti–murine CD36 monoclonal antibody and mouse IgAκ. The extent of cellular endocytic uptake and degradation of ^{125}I-GA-BSA and ^{125}I-MG-BSA was determined as described above.

Statistical Analysis—Data were expressed as mean ± SD. Differences between groups were evaluated using the paired Student's *t*-test. A *p* value of less than 5% was considered to indicate a statistically significant difference.

RESULTS

Hepatocellular Distribution of Intravenously Injected GA-BSA and MG-BSA—Table 1 shows the hepatocellular distribution of intravenously administered ^{111}In-GA-BSA and ^{111}In-MG-BSA. Mice were sacrificed at 5 min after injection of GA-BSA or MG-BSA. The liver was then perfused with the collagenase-containing buffer and separated into parenchymal cells and non-parenchymal cells (Kupffer cells and LECs). GA-BSA was mainly distributed among nonparenchymal cells, particularly LECs (79%). Similarly, MG-BSA accumulated in LECs (60%).

In order to verify the above results, the distribution of intravenously administered GA-BSA in the liver was examined immunohistochemically using the biotinylated anti–GA-pyridine antibody, as described in "MATERIALS AND METHODS." At 5 min after an intravenous injection of GA-BSA, positive immunoreactivity was detected in non-parenchymal cells, especially LECs (Fig. 1A). This positive immunoreactivity in the liver was abolished by pretreatment of the biotinylated antibody with 10 μg/ml GA-BSA (Fig. 1B). Furthermore, no positive immunoreactivity was detected in the liver of mice that received an intravenous injection of BSA (Fig. 1C).

These results indicate that LECs are responsible for hepatic uptake of intravenously injected GA-BSA and MG-BSA.

GA-BSA and MG-BSA Undergo Receptor-Mediated Endocytosis by LECs—To determine the mechanism of hepatic uptake of intravenously injected GA-BSA and

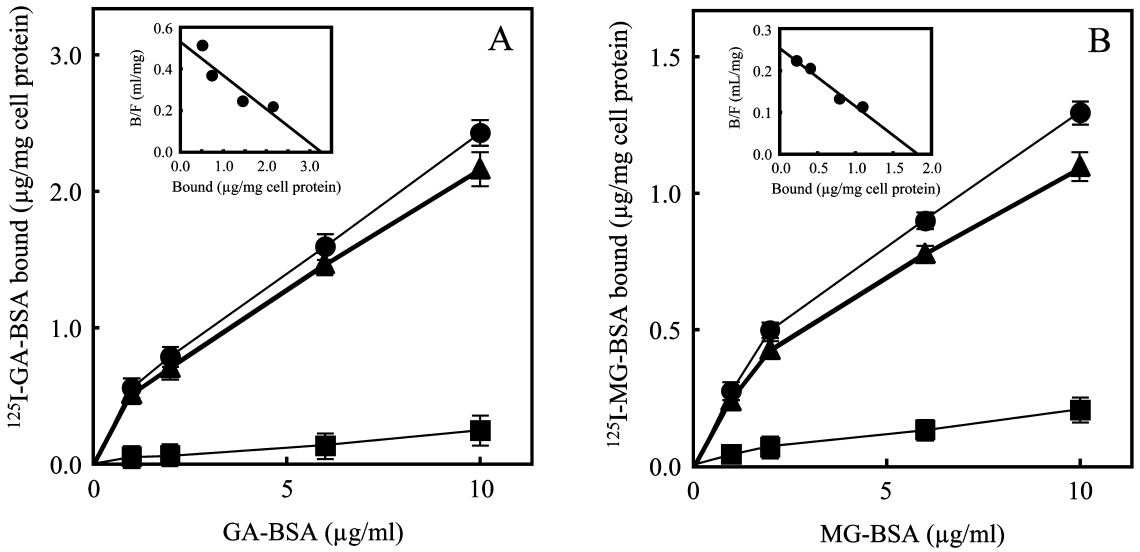

Fig. 2. **Binding of ^{125}I-GA-BSA (A) and ^{125}I-MG-BSA (B) to mouse LECs.** LECs were incubated at 4°C for 90 min with the indicated concentrations of ^{125}I-GA-BSA (A) or ^{125}I-MG-BSA (B) in the presence (closed squares) or absence (closed circles) of 50-fold unlabeled ligands. Closed triangles indicate the specific binding of each ligand. Inset, Scatchard analysis based on specific binding of GA-BSA (A) and MG-BSA (B) to LECs. Results are the means ± SD of three separate experiments.

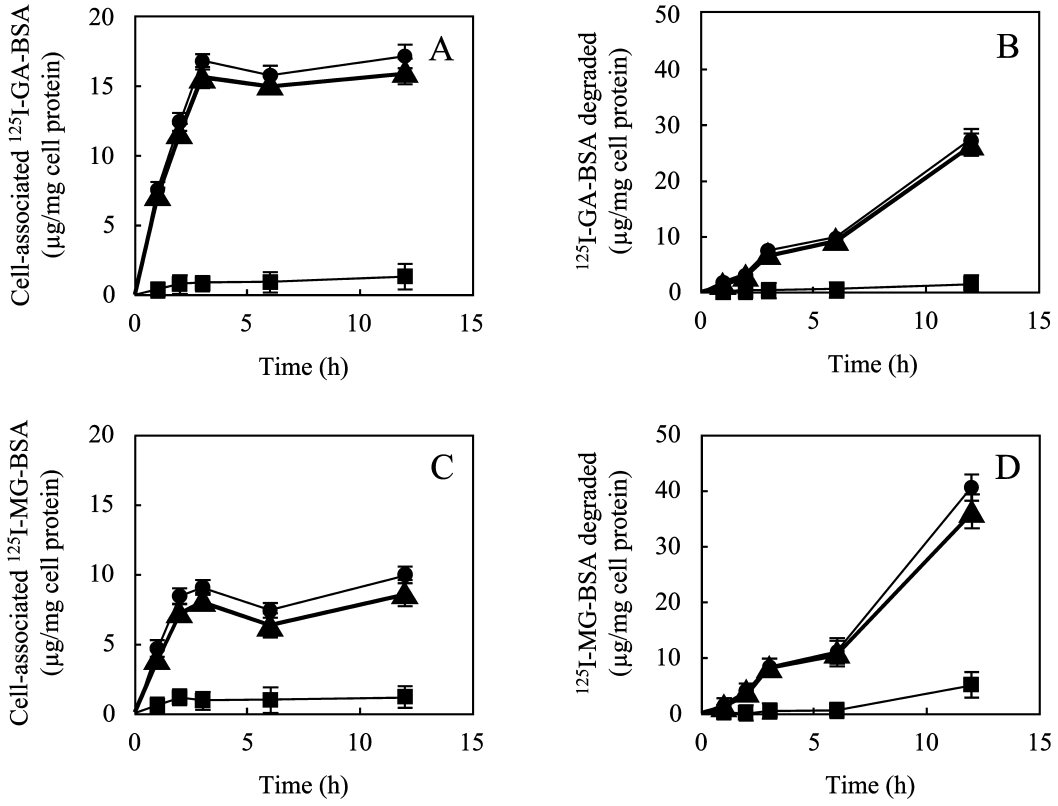

Fig. 3. **Time-dependent endocytic uptake (A and C) and degradation (B and D) of ^{125}I-GA-BSA (A and B) and ^{125}I-MG-BSA (C and D) by mouse LECs.** LECs were incubated at 37°C for the indicated time periods with 5 μg/ml of ^{125}I-GA-BSA or ^{125}I-MG-BSA in the presence (closed squares) or absence (closed circles) of 50-fold unlabeled ligands. Closed triangles indicate the specific association or degradation of each ligand. The amounts of cell-associated ^{125}I-GA-BSA and ^{125}I-MG-BSA and their degradation were measured as described in the "MATERIALS AND METHODS" section. Results are the means ± SD of three separate experiments.

MG-BSA, we examined the interaction of GA-BSA and MG-BSA with isolated LECs. The total binding of ^{125}I-GA-BSA to these cells at 4°C was inhibited by >90% by an excess amount of unlabeled GA-BSA (Fig. 2A). The specific binding, which was obtained by subtracting nonspecific binding from the total binding, showed a saturation curve whose Scatchard analysis revealed a binding site with an apparent K_d of 6.22 μg/ml and maximal bind-

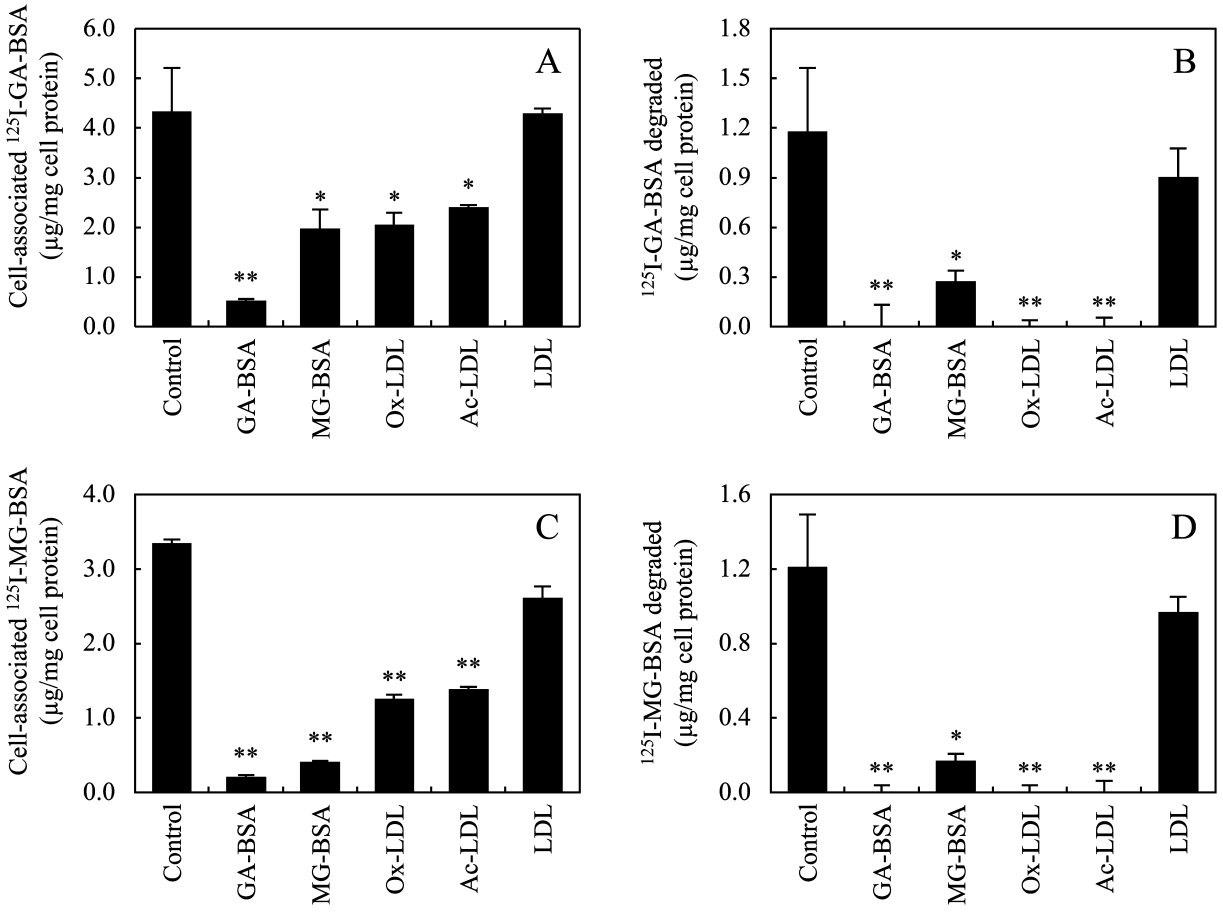

Fig. 4. **Effects of several ligands on endocytic uptake (A and C) and degradation (B and D) of ^{125}I-GA-BSA (A and B) and ^{125}I-MG-BSA (C and D) by mouse LECs.** LECs were incubated at 37°C for 6 h with 2.5 μg/ml ^{125}I-GA-BSA or ^{125}I-MG-BSA in the absence (control) or presence of 40-fold unlabeled ligands including GA-BSA, MG-BSA, Ox-LDL, Ac-LDL and LDL. The extents of cellular association of ^{125}I-GA-BSA and ^{125}I-MG-BSA and degradation were measured as described in the "MATERIALS AND METHODS" section. Results are means ± SD for three separate experiments. *P < 0.05, **P < 0.01, compared with the control.

ing of 3.31 μg/mg of cell protein (Fig. 2A, inset). The specific binding of MG-BSA to LECs was also saturable (Fig. 2B), and Scatchard analysis indicated a binding site with an apparent K_d of 7.14 μg/ml and maximal surface binding of 1.82 μg/mg of cell protein (Fig. 2B, inset). These results indicate that LECs possess a high-affinity binding site for GA-BSA and MG-BSA.

In order to characterize the post-binding kinetics of GA-BSA and MG-BSA, these LECs were incubated with ^{125}I-GA-BSA and ^{125}I-MG-BSA at 37°C. The amount of cell-associated ^{125}I-GA-BSA increased with time (Fig. 3A), followed by a parallel increase in the amount of ^{125}I-GA-BSA degraded by these cells (Fig. 3B). In a similar fashion, the amount of ^{125}I-MG-BSA associated with these cells (Fig. 3C) and the amount of ^{125}I-MG-BSA degraded by them (Fig. 3D) increased with time. These results suggest that LECs express receptors specific for GA-BSA and MG-BSA, and those ligands bound to these receptors undergo receptor-mediated endocytosis.

To characterize the receptor for GA-BSA and MG-BSA on LECs, we tested the effects of several ligands of the scavenger receptors, including Ac-LDL and Ox-LDL. The endocytic uptake and degradation of ^{125}I-GA-BSA were effectively inhibited not only by unlabeled GA-BSA but also by MG-BSA, Ac-LDL and Ox-LDL, whereas LDL had no significant effect (Fig. 4, A and B). Similarly, the endocytic uptake and degradation of ^{125}I-MG-BSA were effectively inhibited by unlabeled GA-BSA, MG-BSA, Ac-LDL and Ox-LDL, but not by LDL (Fig. 4, C and D). These results suggest that GA-BSA and MG-BSA are recognized by LECs *via* a scavenger receptor(s) and undergo receptor-mediated endocytosis.

We also examined the effects of the scavenger receptor ligands *in vivo*. Hepatic uptake of ^{111}In-GA-BSA was 44% of the injected dose at 10 min after intravenous administration, and was significantly inhibited by co-injection of unlabeled GA-BSA, MG-BSA, Ox-LDL or Ac-LDL (Fig. 5A). Similarly, hepatic uptake of intravenously injected ^{111}In-MG-BSA was 28% of the injected dose, and was inhibited by unlabeled GA-BSA, MG-BSA, Ox-LDL and Ac-LDL (Fig. 5B). The results of these *in vivo* experiments indicate that hepatic uptake of GA-BSA and MG-BSA from the circulation is mediated by a scavenger receptor.

Effects of Anti-Murine CD36 Monoclonal Antibody on the Hepatic Uptake of GA-BSA—To assess the relative contribution of CD36 to endocytic uptake and degradation of ^{125}I-GA-BSA by LECs, we examined the effects of

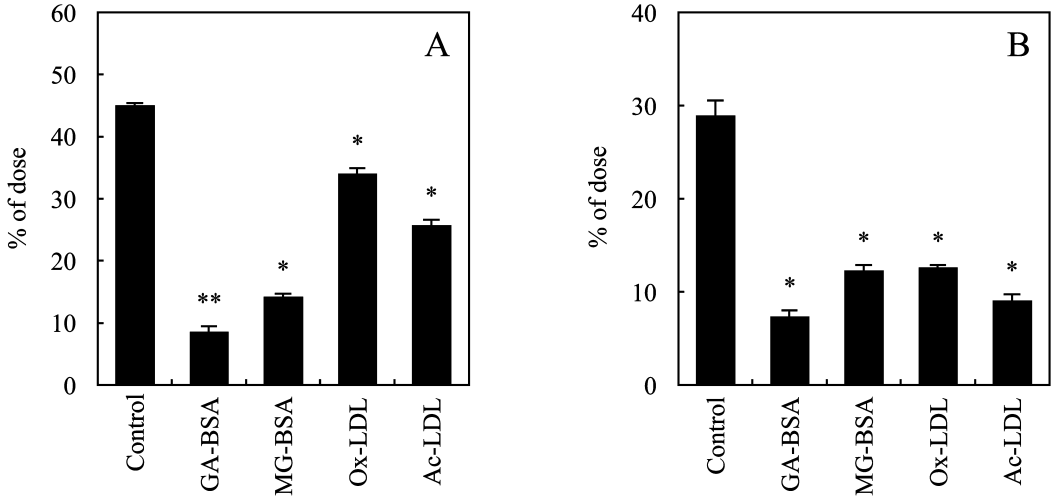

Fig. 5. **Effects of several ligands on hepatic uptake of intravenously injected ^{125}I-GA-BSA (A) and ^{125}I-MG-BSA (B).** ^{111}In-GA-BSA (A) and ^{111}In-MG-BSA (B) were injected as a bolus (0.1 mg/kg) into the tail vein of mice, with or without co-administration of GA-BSA, MG-BSA, Ox-LDL or Ac-LDL (10 mg/kg). Relative radioactivity in the liver at 10 min after intravenous administration was measured as described in the "MATERIALS AND METHODS" section. Results are means ± SD for three separate experiments. *P < 0.05, **P < 0.01, compared with the control.

anti-CD36 antibody on these processes. Abmurad et al. reported that CD36 binds and transports long-chain fatty acids, especially oleate (*29*). Therefore, in order to determine whether anti-CD36 antibody inhibits the activity of CD36 in LECs, we performed a preliminary experiment to assess inhibition of [^{3}H]oleate transport by anti-CD36 antibody in LECs. The anti-CD36 antibody inhibited the uptake of [^{3}H]oleate by LECs by 25%, whereas the non-immune IgAκ had no effect (Fig. 6). Although the results showed that CD36 was not the only mediator of uptake of oleate, the neutralizing antibody proved effective as an inhibitor of [^{3}H]oleate transport in LECs. The anti-CD36 antibody also effectively inhibited endocytosis of ^{125}I-GA-BSA by 3T3-L1 adipocytes, as reported previously (data not shown) (*23*). Unexpectedly, the quantity of cell-associated ^{125}I-GA-BSA was not significantly affected by anti-CD36 antibody or non-immune IgAκ (Fig. 7A). The extent of endocytic degradation of ^{125}I-GA-BSA with co-administration of anti-CD36 antibody was almost the same as the extent observed without co-administration of the antibody (Fig. 7B). These results suggest that GA-BSA is endocytosed by LECs via a route that does not involve CD36.

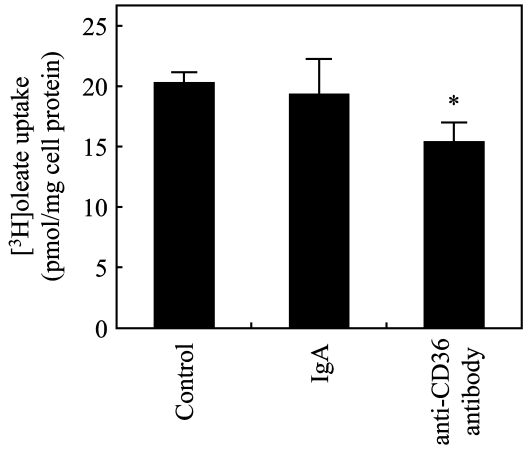

Fig. 6. **Effects of anti-CD36 antibody on transport of [^{3}H]oleate by mouse LECs.** LECs were incubated at 37°C for 2 h with 1 μM [^{3}H]oleate in the absence (control) or presence of 10 μg/ml mouse anti-murine CD36 monoclonal antibody or mouse IgAκ. Transport of [^{3}H]oleate was measured as described in the "MATERIALS AND METHODS" section. Results are means ± SD for three separate experiments. *P < 0.05, compared with the control.

To examine the contribution of CD36 to hepatic uptake of AGE proteins *in vivo*, we measured the hepatic uptake of ^{111}In-GA-BSA with simultaneous administration of anti-CD36 antibody. The results shown in Fig. 8 indicate that anti-CD36 antibody and control IgAκ did not affect hepatic uptake of GA-BSA. These results suggest that hepatic clearance of AGE proteins from the circulation was not mediated to a significant extent by the CD36 pathway.

DISCUSSION

There are five reasons for our specific examination of the role of CD36 in the present study. First, CD36 is expressed by LECs (*21*). Second, the capacity of monocyte-derived macrophages obtained from CD36-deficient patients to endocytose Ox-LDL is <50% of that of monocyte-derived macrophages obtained from normal subjects (*30*). Third, endocytic degradation of Ox-LDL and AGE-BSA by adipocytes is inhibited by >80% by a neutralizing anti-CD36 antibody (*20*, *23*). Fourth, a recent experiment using peritoneal macrophages obtained from SR-A/CD36 double knockout mice clearly showed that the vast majority of Ac-LDL and Ox-LDL is taken up and degraded by pathways involving SR-A or CD36 (*18*). Finally, SR-A does not significantly contribute to the endocytic uptake of AGE-BSA by LECs (*10*). Also, in a recent experiment, we found no difference in hepatic uptake of intravenously injected GA-BSA between SR-A knockout mice and litter-

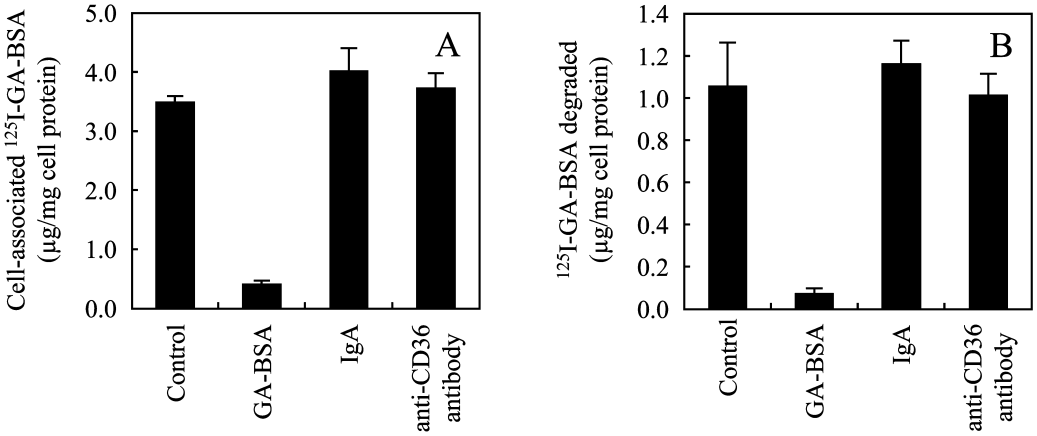

Fig. 7. **Effects of anti-CD36 antibody on endocytic uptake (A) and degradation (B) of ^{125}I-GA-BSA by mouse LECs.** LECs were incubated at 37°C for 6 h with 2.5 μg/ml ^{125}I-GA-BSA or ^{125}I-MG-BSA in the absence (control) or presence of 10 μg/ml mouse anti–murine CD36 monoclonal antibody or mouse IgAκ. The extent of cellular association of ^{125}I-GA-BSA and ^{125}I-MG-BSA and degradation were measured as described in the "MATERIALS AND METHODS" section. Results are means ± SD for three separate experiments.

mate normal mice (data not shown). Therefore, to specify the receptor responsible for hepatic uptake of AGE proteins, we examined the effects of a neutralizing anti-CD36 antibody on endocytic uptake of AGE proteins by LECs. However, the anti-CD36 antibody did not affect the endocytic uptake of GA-BSA by LECs *in vitro* (Fig. 7) or hepatic clearance of GA-BSA *in vivo* (Fig. 8), suggesting that endocytic uptake of AGE proteins by LECs occurs via a pathway that does not involve CD36.

Ohgami *et al.* showed that SR-BI, a member of the class B scavenger receptor family, mediates endocytic uptake and subsequent intracellular degradation of AGE proteins (*15*). Because SR-BI is expressed by LECs, it has been assumed that it contributes to endocytosis of AGE proteins by LECs. The expression level of SR-BI in LECs is half the level of parenchymal cells at both the mRNA and protein level (*21*, *31*). Thus, the previous reports and present findings that LECs are mainly responsible for hepatic uptake of GA-BSA and MG-BSA from circulation, whereas parenchymal cells have a small contribution (Table 1 and Fig. 1), suggest that SR-BI plays a minor role in endocytosis of GA-BSA and MG-BSA by LECs.

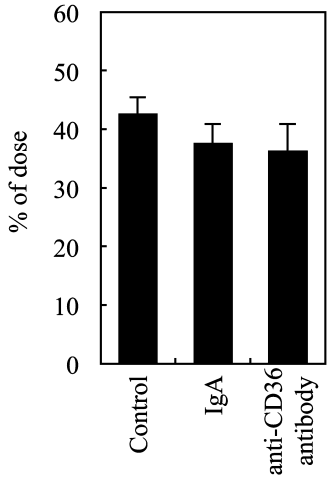

Fig. 8. **Effects of anti-CD36 antibody on hepatic uptake of intravenously injected ^{125}I-GA-BSA.** ^{111}In-GA-BSA was injected as a bolus (0.1 mg/kg) into the tail vein of mice, with or without co-administration of mouse anti–murine CD36 monoclonal antibody (0.5 mg/kg) or mouse IgAκ (0.5 mg/kg). Relative radioactivity in the liver at 10 min after intravenous administration was measured as described in the "MATERIALS AND METHODS" section. Results are means ± SD for three separate experiments.

Previously, LOX-1 has been shown to recognize AGE proteins and Ox-LDL as ligands, but not Ac-LDL (*32*). In this context, the finding that endocytic uptake of ^{125}I-GA-BSA and ^{125}I-MG-BSA by LECs is significantly inhibited by Ac-LDL (Fig. 4) appears to exclude the possibility that LOX-1 participates in endocytosis of AGE proteins by LECs. However, at the 2004 annual meeting of the Japanese Biochemical Society, several groups reported that analysis using BIA core indicates that LOX-1 recognizes Ac-LDL as well as Ox-LDL. These studies showed that Ox-LDL and Ac-LDL had very similar association/dissociation constants. Moreover, Jono *et al.* reported that specific binding of AGE-BSA to cultured bovine aortic endothelial cells was suppressed by 67% by anti-LOX-1 antibody, indicating that LOX-1 mediates a substantial fraction of the endocytosis of AGE-BSA by endothelial cells (*16*). Such findings suggest that LOX-1 has some roles in the uptake of AGE proteins by LECs.

RAGE and 80K-H/OST-48/galectin-3 complexes have been characterized as AGE receptors that are independent of members of the scavenger receptor family. In immunoblot analyses, galectin-3 has been detected in LECs at a level equal to that of peritoneal macrophages, whereas RAGE was not detected in LECs (*10*). Thus, it appears unlikely that RAGE is involved in endocytic uptake of AGE proteins by LECs, although further study is needed to determine the contribution of galectin-3 to this process.

McCourt *et al.* have purified hyaluronan scavenger receptor (HA/S-R) from liver, and have shown that polyclonal antibody against HA/S-R inhibits binding and degradation of both formaldehyde-modified BSA and hyaluronan by cultured sinusoidal liver endothelial cells (*33*). Hansen *et al.* reported that polyclonal antibody

against HA/S-R inhibited uptake of ^{125}I-AGE-BSA by rat LECs by 44% (*34*). These findings strongly suggest that the HA/S-R is one of the mediators of endocytosis of GA-BSA and MG-BSA by LECs.

In conclusion, the present results indicate that GA-BSA and MG-BSA are eliminated by LECs, via scavenger receptor-mediated endocytosis involving a pathway that does not include CD36. Both SR-A and CD36, which are important mediators of uptake of AGE proteins by macrophages, appear to play no role in endocytosis of AGE proteins by LECs, indicating a marked difference between endothelial cells and macrophages in the receptors they use for endocytosis of AGE proteins. Thus, LOX-1, one of the major AGE receptors in endothelial cells, (*16*), appears to be a promising candidate for AGE receptor in LECs. Identification of AGE receptor(s), other than SR-A and CD36, involved in endocytic uptake of AGE proteins by LECs may clarify the mechanisms involved in clearance of chemically modified proteins produced *in vivo*.

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