

Expression Cloning of a Novel Scavenger Receptor from Human Endothelial Cells*

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Scavenger receptors mediate the endocytosis of chemically modified lipoproteins, such as acetylated low density lipoprotein (Ac-LDL) and oxidized LDL (Ox-LDL), and have been implicated in the pathogenesis of atherosclerosis. The evidence that endothelial cells possess scavenger receptor activity is substantial, and this property is widely used in the isolation of endothelial cells from vascular tissues. In the current study, we have isolated, by expression cloning, the cDNA encoding a novel type of scavenger receptor expressed by endothelial cells (SREC), which mediates the binding and degradation of Ac-LDL. The primary structure of the molecule has no significant homology to other types of scavenger receptors, including the recently cloned endothelial cell Ox-LDL receptor, a member of the C-type lectin family. The cDNA encodes a protein of 830 amino acids with a calculated molecular mass of 85,735 Da (mature peptide). Chinese hamster ovary cells stably expressing SREC bound ¹²⁵I-labeled Ac-LDL with high affinity ($K_d = 3.0 \mu\text{g/ml}$, approximately 1.7 nM) and degraded them via an endocytic pathway. Association of DiI-Ac-LDL were effectively inhibited by Ox-LDL, malondialdehyde-modified LDL, dextran sulfate, and polyinosinic acid, but not by natural LDL and heparin. The cloned receptor has several characteristic domain structures, including an N-terminal extracellular domain with five epidermal growth factor-like cysteine pattern signatures and an unusually long C-terminal cytoplasmic domain (391 amino acids) composed of a Ser/Pro-rich region followed by a Gly-rich region.

Chemically modified low density lipoprotein (LDL)¹, such as

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¹ The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; Ac-LDL, acetylated LDL; Ox-LDL, oxidized LDL; DiI, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate; SREC, scavenger receptor expressed by endothelial cells; HUVECs, human umbilical vein endothelial cells; CHO, Chinese hamster ovary; CAEs, coronary arterial endothelial cells; CASMs, coronary

arterial smooth muscle cells; poly I, polyinosinic acid; kb, kilobase pair(s); PBS, phosphate-buffered saline; MDA-LDL, malondialdehyde-modified LDL; EGF, epidermal growth factor.

acetylated LDL (Ac-LDL) and oxidized LDL (Ox-LDL), can be rapidly taken up by cultured macrophages and some other cells via receptor-mediated endocytosis. The receptor involved in this pathway is called a scavenger receptor. The first scavenger receptors to be purified and cloned were the macrophage type I and type II receptors (1, 2). Receptors that recognize chemically modified LDL, but which are distinct from the type I and II macrophage scavenger receptors, have been identified in macrophages and some other cell types (3–6). It is well recognized that endothelial cells possess scavenger receptor activity, and the uptake by endothelial cells of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI)-labeled Ac-LDL (DiI Ac-LDL) is widely used to isolate endothelial cells from vascular tissues. However, several lines of evidence suggest that the receptor present on their surface is distinct from the scavenger receptors described to date (3–5, 7).

Recently, an endothelial receptor for Ox-LDL was cloned, using an expression cloning strategy, from cultured bovine aortic endothelial cells (8). The cDNA for the receptor encodes a protein of 270 amino acid residues, with a calculated molecular mass of 30,872 Da. It has a type II membrane protein structure with a short N-terminal cytosolic domain and a long C-terminal extracellular domain and belongs to the C-type lectin family. Although the ligand specificity or the competitor sensitivity of the receptor is not yet known, the cloned receptor recognizes Ox-LDL with a high affinity, but not Ac-LDL. Similarly, it was demonstrated that rabbit venous endothelial cells in primary culture and a bovine aortic endothelial cell line had no detectable mRNA of type I and type II scavenger receptors (9). Based on these observations, we speculated that a scavenger receptor(s) other than those that have been cloned to date is expressed by endothelial cells.

In the present study, we have isolated, by expression cloning, the cDNA encoding a new type of scavenger receptor expressed by endothelial cells (SREC) which mediates the binding and degradation of Ac-LDL.

EXPERIMENTAL PROCEDURES

Expression Cloning—A cDNA library was constructed using the pcDNA3 vector (Invitrogen) with poly(A)⁺ mRNA isolated from human umbilical vein endothelial cells (HUVECs). To obtain long cDNA, the entire pooled cDNA library was digested with *NotI* and size-fractionated using 0.7% agarose gel electrophoresis. Large DNAs (>8 kb, corresponding to >3 kb of cDNA) were recircularized with a DNA ligation kit (Takara, Tokyo, Japan). The resultant transformants were divided into small pools (approximately 1,000 clones/pool), and we obtained the plasmid DNA using a plasmid mini-kit (QIAGEN). Plasmid DNA was transfected into Chinese hamster ovary (CHO) cells, and the transiently transfected cells were screened visually for endocytosis of fluorescent DiI Ac-LDL. On day 0, CHO cells were plated into six-well plates (1×10^5 cells/well) in minimum essential medium α (medium A) (Life Technologies, Inc.) with 10% fetal calf serum. On day 1, the cells in each plate were transfected with 1 μg of expression library plasmid DNA from a single pool employing LipofectAMINE reagent (Life Technologies, Inc.). On day 3, the monolayers were refed with medium A containing 2 $\mu\text{g/ml}$ protein of DiI Ac-LDL (Biomedical Technologies Inc.) and 5% lipoprotein deficient serum. After a 3-h incubation at 37 °C, the plates were washed twice with medium A containing 10% fetal calf serum and then twice with PBS, and the cells were fixed with 3.0% formaldehyde in PBS for 15 min at room temperature. The pres-

arterial smooth muscle cells; poly I, polyinosinic acid; kb, kilobase pair(s); PBS, phosphate-buffered saline; MDA-LDL, malondialdehyde-modified LDL; EGF, epidermal growth factor.

located in the N-terminal half of the cytoplasmic domain. It is difficult to speculate on the biological significance of this region at present; however, this domain contains several serine and threonine residues that could potentially be phosphorylated by various protein kinases, suggesting that some biological signals may be delivered via the domain. Downstream of the Ser/Pro-rich region is a glycine-rich region (Gly-rich region). There are 26 glycine residues distributed throughout a domain of 177 amino acids. In the C-terminal end of the domain, there is a

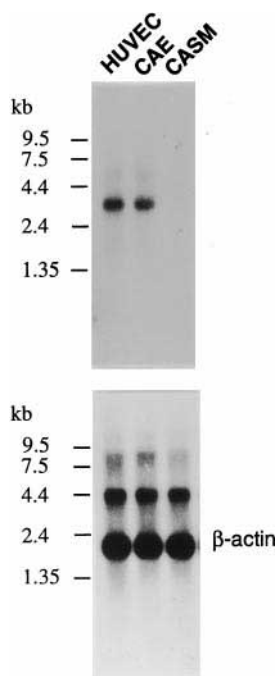


FIG. 2. Northern blot analysis of SREC mRNA expressed in human endothelial cells. HUVECs, CAEs, and CASMs were obtained from Clonetics and cultured according to the supplier's instructions. The amount of poly(A)⁺ RNA from these cells was 0.5 μ g. The filter-bound RNA was hybridized with ³²P-labeled human SREC cDNA. Hybridization to a human β -actin probe (CLONTECH) is shown as a control. Molecular sizes are indicated on the left (in kilobases).

tyrosine residue (Tyr⁸²²) fitting the consensus sequence (RXX-EXXY) for a site phosphorylated by tyrosine kinases (19), suggesting that it may play a role in some signal transduction processes.

We compared the expression level of the message in HUVECs, CAEs, and CASMs. The 3.5-kb transcript was clearly detected in HUVECs and CAEs, whereas we did not detect any bands in CASMs (Fig. 2).

The expression plasmid containing SREC DNA was expressed in CHO cells (CHO-SREC). The binding of ¹²⁵I-Ac-LDL was assessed following incubation of the transformed cells with ¹²⁵I-Ac-LDL. Saturation binding of ¹²⁵I-Ac-LDL was observed at 4 °C (Fig. 3A). Scatchard analysis (Fig. 3A, inset) showed the presence of a single class of receptors and the calculated K_d was 3.0 μ g/ml ¹²⁵I-Ac-LDL (approximately 1.7 nM ¹²⁵I-Ac-LDL), which is comparable with the value of 2.7 nM for scavenger receptors on HUVECs (20). We have also performed binding experiments employing DiI Ac-LDL and obtained the same result (K_d is 3.2 nM). Incubation of the transfectants at 37 °C with DiI Ac-LDL led to the uptake of fluorescent lipid (DiI) into the cytoplasm, producing a punctate pattern (Fig. 4, A and B). Diffuse labeling, most likely of the cell surface, was also observed. When CHO-SREC cells were incubated with ¹²⁵I-Ac-LDL at 37 °C for various lengths of time, chloroquine-sensitive cellular association of radioactivity and acid-soluble radioactivity in the medium continued to appear at a linear rate, reflecting the continuous uptake and degradation of ¹²⁵I-Ac-LDL (Fig. 3B).

To further characterize the binding properties of the SREC, the receptors activity was measured in the presence of various materials known as type I and type II macrophage scavenger receptor inhibitors (Fig. 5). As is the case with type I macrophage scavenger receptors, unlabeled Ac-LDL as well as poly I and dextran sulfate, but not native lipoproteins such as LDL and HDL, reduced the binding of DiI Ac-LDL on the cells to the

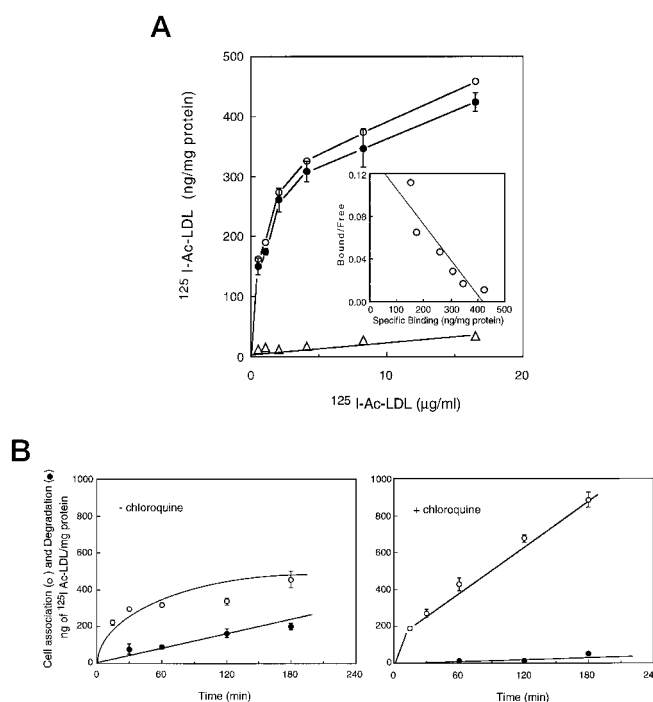


FIG. 3. Binding and degradation of ¹²⁵I-Ac-LDL by control and SREC-expressing CHO cells. A, specific binding of ¹²⁵I-Ac-LDL to SREC-expressing CHO cells. CHO-SREC cells grown to confluence in 12-well plates were incubated with 0.52–16.5 μ g/ml ¹²⁵I-Ac-LDL at 4 °C for 2 h. Total binding (open circles) represents the binding at increasing concentrations of ¹²⁵I-Ac-LDL and the nonspecific binding (triangle) represents the binding in the presence of a 20-fold excess of unlabeled Ac-LDL. Specific binding (closed circle) is obtained by the subtraction of the nonspecific curve from the total curve. Specific binding of ¹²⁵I-Ac-LDL to control CHO cells was less than 5% that obtained with transfectant cells. Inset shows the Scatchard analysis of the data ($r = -0.911$). The maximal number of binding sites equaled 411 ng/mg protein for ¹²⁵I-Ac-LDL. B, time course of ¹²⁵I-Ac-LDL association with and degradation by CHO-SREC cells. CHO-SREC cells and control cells grown to confluence in 24-well plates were incubated with 2 μ g/ml ¹²⁵I-Ac-LDL. After incubation at 37 °C for the indicated time, degradation (closed circles) and association (open circles) were determined for triplicate wells. Degradation (closed circles) and association (open circles) in the presence of 100 μ M chloroquine were also shown. In each case binding and degradation by control cells were negligible.

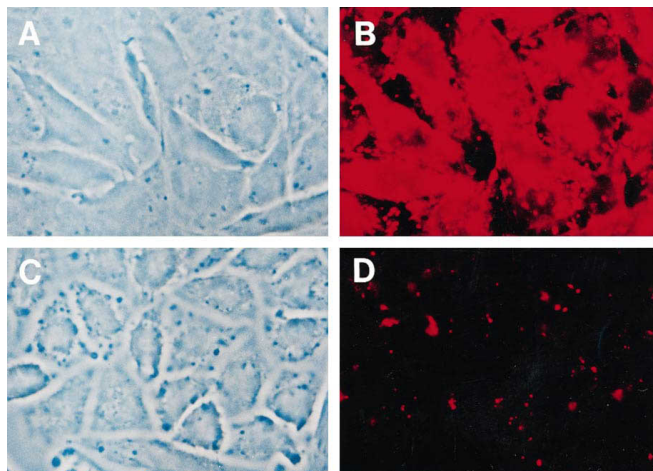


FIG. 4. Uptake of DiI Ac-LDL by CHO-SREC cells. CHO-SREC (A, B) or control CHO cells (C, D) were seeded on day 0 at 1×10^5 cells/well in medium A. On day 2, medium containing 2 μ g/ml protein of DiI Ac-LDL was added to the monolayers. After incubation at 37 °C for 3 h, the cells were washed, fixed in 3% formaldehyde in PBS, and the uptake of DiI Ac-LDL was observed using fluorescence microscopy. Phase-contrast light micrographs of CHO monolayers (A, C) and the fluorescence of cell-associated DiI Ac-LDL (B, D) are shown.

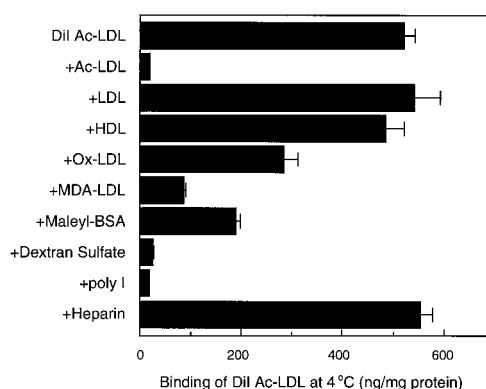


FIG. 5. **Inhibition of DiI Ac-LDL binding to CHO-SREC cells by various compounds.** The CHO-SREC cells were incubated with 2 μ g/ml DiI Ac-LDL at 4 °C for 2 h in the presence of 200 μ g/ml various inhibitors. Inhibitors used are Ac-LDL, LDL, HDL, Ox-LDL, MDA-LDL, maleyl-bovine serum albumin (Maleyl-BSA), dextran sulfate, poly I, and heparin.

basal level. Ox-LDL, maleyl-bovine serum albumin, and MDA-LDL partially reduced the binding of DiI Ac-LDL on the cells. These results show that the SREC has a binding specificity similar to those of the type I and type II macrophage scavenger receptors.

In this study, we cloned the cDNA encoding a new type of scavenger receptor that is expressed by endothelial cells. Interestingly, the SREC has an unusually long C-terminal cytoplasmic domain that contains a Ser/Pro-rich domain and a Gly-rich domain. Together with the fact that the molecule also contains a tyrosine phosphorylation site in the C-terminal end, these characteristics tempt us to speculate that the cytoplasmic domain may mediate some biological signals. It has been demonstrated by several investigators that stimulation of scavenger receptors of endothelial cells induces the expression of proteins related to vascular functions. For example, Ac-LDL and Ox-LDL stimulate the release of endothelin, a potent vasoconstrictor peptide (21). Indeed, the circulating levels of endothelin are increased in patients with hyperlipidemia or atherosclerosis (22, 23). Alternatively, poly I, a scavenger receptor agonist significantly induces the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and E-selectin by a human endothelial cell line, EA hy.926 (24). Adherence of circulating monocytes to the arterial endothelium

is first seen in high cholesterol diet-induced experimental atherosclerosis in rabbits (25). The molecular mechanism underlying such physiological changes of vascular endothelial cells in the pathogenesis of atherosclerosis is not well understood. The present report of the isolation of cDNA for endothelial cell scavenger receptor should help our understanding of the molecular mechanisms involved in a development of atherosclerosis.

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