Analysis of Macrophage Scavenger Receptor (SR-A) Expression in Human Aortic Atherosclerotic Lesions

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Abstract—The class A scavenger receptors (SR-As) are trimeric, integral membrane glycoproteins that exhibit unusually broad ligand-binding properties. A number of studies have suggested that these receptors may play an important role in host defense and in many macrophage-associated pathological processes, including atherosclerosis and Alzheimer's disease. The study of the expression and function of these receptors in human disease has been hampered by the lack of suitable antibodies recognizing human SR-A. This has generated questions regarding the nature of receptors responsible for scavenger receptor activity detected in a variety of cell types, including monocytes, macrophages, smooth muscle cells, and endothelial cells. To address these questions, we have produced high-titer antisera recognizing human SR-A by using mice deficient for SR-A (SR-A −/−). We show that SR-A −/− mice produce a significantly higher-titer immune response than do wild-type (SR-A +/+) littermates, with antisera of the former having a broad species reactivity and recognizing SR-A from humans, mice, and rabbits. The antisera recognize both type I and II SR-A in a wide range of immunological techniques. Using these antisera we show that the expression of SR-A protein is induced during monocyte to macrophage differentiation and that SR-A mediates 80% of the uptake of acetylated low density lipoprotein by human monocyte–derived macrophages. We also establish that human SR-A is expressed by tissue macrophages in liver and lung and by macrophage-derived foam cells within aortic atherosclerotic lesions, with little detectable expression by smooth muscle cells or aortic endothelium. **(*Arterioscler Thromb Vasc Biol.* 1999;19:461-471.)**

Key Words: macrophages ■ scavenger receptors ■ atherosclerosis ■ antibodies ■ knockout mice

The class A scavenger receptors (SR-As)[1] are trimeric, integral membrane glycoproteins that have been implicated in various macrophage functions, including endocytosis,[1,2] adhesion,[3] phagocytosis,[4] and intracellular signaling.[5] There are 3 forms of the receptor derived by alternative splicing of a single gene.[6–8] The 3 isoforms each contain 6 predicted structural domains[9–11]: cytoplasmic, transmembrane, spacer, α-helical coiled coil, collagenous, and a type-specific carboxyl terminus. Type I SR-A has the 110–amino acid scavenger receptor cysteine-rich domain, a highly conserved protein motif found in many other immunological proteins.[12] Type II SR-A has a short carboxyl-terminal domain that is relatively nonconserved between species. Type III SR-A has a truncated form of the scavenger receptor cysteine-rich domain and has been shown to have dominant-negative properties.[8] Both type I and type II SR-A bind a diverse array of macromolecules, including modified lipoproteins (acetylated [Ac] or oxidized LDL), bacterial surface lipids (endotoxin and lipoteichoic acid), proteins modified by advanced glycation (advanced glycation end products), and β-amyloid fibrils.[2,13–17] The SR-A–mediated uptake of modified LDL is not regulated by cellular cholesterol levels, unlike the native LDL receptor, and therefore leads to intracellular cholesterol accumulation and the formation of foam cells. The generation of macrophage-derived foam cells is hypothesized to be a key step in the pathogenesis of atherosclerosis.[1,18] The in vivo role of SR-A in atherosclerosis has recently been highlighted by Kodama and colleagues in SR-A–deficient mice.[15]

Immunological tolerance prevents an immune response against self-antigens and epitopes conserved between immunogen and recipient species. The gene-targeting technique[19] allows the inactivation of specific genes in the mouse germ line. In such animals, owing to the absence of the murine protein, immunological tolerance to any determinant of that gene product cannot be established. The product of the gene inactivated in the germ line would therefore be recognized as "foreign" by the immune system and elicit an efficient antibody response on immunization. The approach of using gene-inactivated mice has been utilized to generate monoclonal antibodies against other highly conserved proteins.[20–23]

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The study of the role of SR-A in human macrophage physiology and pathology has been hampered by the lack of suitable antibodies recognizing human SR-A.[24] Much of our knowledge of scavenger receptor expression by human cells has been gained by using functional assays measuring the uptake of modified LDL. With such techniques it has been shown that in vitro cultured endothelial and smooth muscle cells, in addition to macrophages, have scavenger receptor activity.[25–28] However, the recent cloning of several receptors with the ability to mediate uptake of modified LDL[29–33] has meant that the nature of the receptors responsible for these activities is unclear. In this current study, we describe the generation and characterization of high-titer polyclonal antisera against the human SR-A protein by using SR-A–deficient mice. We show that the antisera can be used to study modified LDL uptake by human monocyte–derived macrophages and to detect SR-A expression in human tissues. Using these antisera we show SR-A expression by macrophages in human and rabbit aortic atherosclerotic lesions, with little detectable SR-A protein in smooth muscle or endothelial cells.

Methods

Cell Culture and Transfection

A20 cells were maintained in RPMI 1640 medium supplemented with 50 IU/mL penicillin G, 50 μg/mL streptomycin, 2 mmol/L glutamine (PSG; all from Life Sciences), and 10% FCS (Sigma). Chinese hamster ovary (CHO)-K1 cells were routinely cultured in Ham's F-12 medium supplemented as above. Expression constructs for full-length human SR-A with and without the FLAG epitope tag in the pcDNA3 expression vector (Invitrogen) were generated as described elsewhere.[8] Transfection of the A20 cell line was achieved by electroporation. In brief, A20 cells were harvested, washed twice in ice-cold PBS (137 mmol/L NaCl, 2.7 mmol/L KCl, 8.1 mmol/L Na_2HPO_4, and 1.5 mmol/L KH_2PO_4, pH 7.3), and resuspended at a final density of 10^7 cells/mL in PBS. Aliquots of 7×10^6 cells were mixed with 50 μg of plasmid DNA, added to a 0.4-cm electrode-gap electroporation cuvette (Bio-Rad), and shocked in a Bio-Rad Gene-Pulser (300 V, 960 μF) at room temperature. Cells were recovered in 10 mL of cell growth medium that had been prewarmed to 37°C and distributed into T25 flasks. Transfection of CHO cells was performed with lipofectamine (Life Sciences) using an adapted version of the manufacturer's protocol. In brief, 3×10^6 CHO-K1 cells were plated in 9-cm tissue-culture plastic Petri dishes (Nunc) 2 days before transfection. A transfection mix was prepared by addition of 25 μL of lipofectamine and 5 μg of plasmid DNA to 5 mL of serum-free Optimem (Life Sciences), mixed by inversion, and allowed to stand for 15 minutes. The cells were washed twice in PBS to remove serum, and the transfection mix was added. The cells were incubated at 37°C for 4 hours, after which the transfection mix was aspirated and replaced with growth medium. For transient analysis, cells were harvested 24 hours after transfection with the use of PBS, 5 mmol/L EDTA, and 0.1% trypsin; washed once in PBS; and plated in either 6-well clusters or 24-well plates (Falcon, Becton Dickinson Labware) at a density of 5×10^5 or 2×10^5 cells per well, respectively.

For both A20 and CHO cells, stable expression of SR-A was selected 48 hours after transfection by continuous culture in normal growth medium supplemented with 1 mg/mL G418 (geneticin; Life Sciences). High levels of SR-A expression were achieved by further selection in "MAC medium": basal growth medium containing PSG, 3% lipoprotein-deficient FCS, 250 μmol/L mevalonate, 40 μmol/L mevastatin (all from Sigma), and 3 μg/mL AcLDL (Biogenesis) 2 weeks after selecting cells in geneticin. This medium provides nutritional selection for cells expressing functional scavenger receptors.[34] Cells expressing high levels of type I and II SR-A were obtained by culture of cells in MAC medium for 9 months.

Quantification of AcLDL Uptake

To assay AcLDL uptake, cells in 24-well or 6-well plates were washed twice in PBS and then incubated in basal culture medium supplemented with PSG and 2% lipoprotein-deficient FCS in the presence or absence of 2 μg/mL DiI (1,1′-dioctadecyl-1-3,3,3′,3′-tetramethylindocarbocyanine perchlorate)-labeled AcLDL (DiI-AcLDL; Biogenesis) for between 1.5 and 4 hours at 37°C. In experiments to measure inhibition of DiI-AcLDL uptake, cells were preincubated for 30 minutes with inhibitors at the indicated concentrations before labeling for 90 minutes in the presence of inhibitors. Cells were washed 5 times in PBS; harvested with either PBS, 5 mmol/L EDTA, and 0.1% trypsin (for CHO cells) or PBS, 10 mmol/L EDTA, and 10 mg/mL lidocaine-HCl (Sigma; for human macrophages); fixed in a 4% (wt/vol) solution of paraformaldehyde in PBS; and analyzed on a FACScan (Becton Dickinson) using the FL2 photomultiplier.

Animals and Immunization

Mice deficient in type I and type II SR-A (SR-A −/−) were produced as described previously.[15] SR-A −/− and wild-type SR-A +/+ mice of the same strain were bred and maintained at the Sir William Dunn School of Pathology animal facility and used between 6 and 10 weeks of age. Animals were immunized by 3 intraperitoneal injections of 5×10^7 live cells at monthly intervals. Animals were completely exsanguinated 9 days after the final injection, and serum was collected from the retracted clot. All mice were handled in accordance with guidelines issued by the home office (UK), and were killed by CO_2 asphyxiation. Sera from SR-A −/− mice are referred to as SRKO followed by a number indicating the specific animal involved. Although individual titers varied, results with different antisera were very reproducible. Sera were adsorbed by incubating 100 μL of serum with 2.5×10^8 A20 cells or A20 cells expressing high levels of SR-A for 1 hour at room temperature on a rotating wheel. Cells used for adsorption had been previously fixed in 4% paraformaldehyde, quenched, and permeabilized in PBS plus 10% normal goat serum (NGS) and 0.2% Triton X-100.

New Zealand White rabbits (n=4) were fed a hypercholesterolemic diet containing 0.25% cholesterol (Fluka) starting 2 weeks before the operation until they were humanely killed. Balloon injury was performed with a 4.0F arterial embolectomy catheter (Sorin Biomedical). The catheter was introduced via the right iliac artery, maneuvered to the aortic arch, and inflated twice to denude the aorta. Animals were killed 14 days after balloon injury under anaesthesia induced by intravenous injection of phentanyl-fluanisone (0.3 mg/kg, Hypnorm, Jansen Pharmaceuticals) and midazolam (1 mg/kg, Dormicum, Hoffman-La Roche), and the aortas were removed and snap-frozen in LN_2. All rabbit studies were approved by Experimental Animal Committee of the University of Kuopio.

Immunoblotting

Cells were washed 3 times in PBS before lysis on ice in 150 mmol/L NaCl, 10 mmol/L EDTA, 10 mmol/L NaN_3, 10 mmol/L Tris (pH 8.0), 1 mmol/L PMSF, 5 mmol/L iodoacetamide, and 1% NP-40. Lysates were centrifuged at 15 000*g* for 10 minutes to remove debris and stored in aliquots at −20°C. Lysates were boiled for 5 minutes in nonreducing sample buffer (10 mmol/L Tris [pH 6.8], 2% SDS, 20% glycerol, and 0.001% [wt/vol] bromophenol blue) and resolved by 6% SDS–polyacrylamide gel electrophoresis (PAGE) with protein lysate from an equal number of cells loaded per lane. Separated proteins were transferred to nitrocellulose membranes (Hybond-C; Amersham International) and blocked for 1 hour at room temperature in PBS plus 3% (wt/vol) powdered milk and 0.1% Tween 20. Primary antibody was added at the indicated dilution in blocking buffer and incubated on a rocking platform for 1 hour at room temperature. Binding was detected by incubation with peroxidase-conjugated anti-mouse IgG (Sigma), diluted 1:1000 in blocking buffer, and visualized by chemiluminescence (ECL; Amersham International).

ELISA

Generation and purification of a soluble, secreted form of the extracellular portion of the type I human SR-A including the

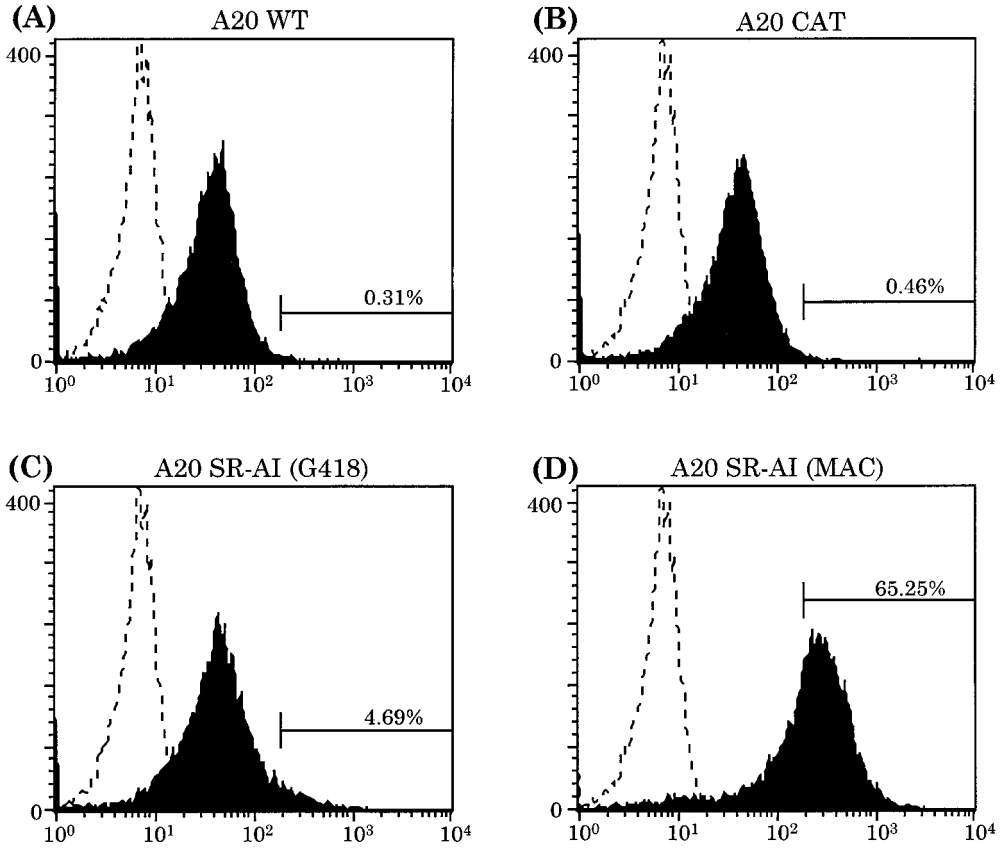


Figure 1. FACScan analysis of DiI-AcLDL uptake by transfected A20 cells. A20 cells were transfected with expression constructs for the full-length coding sequence of type I SR-A and for an identical expression vector containing the CAT reporter gene. Stable expression was selected by incubation in G418 or MAC medium as described in Methods. The histograms depict fluorescence per cell on the *x* axis in arbitrary fluorescence units and numbers of cells in a given fluorescence channel on the *y* axis. Dotted lines indicate autofluorescence of cells not incubated with DiI-AcLDL. Identical gates in each panel were chosen to represent the percentage of cells deemed to be positive for DiI-AcLDL uptake. A, A20 wild-type. B, A20 CAT, G418-selected for 1 month. C, A20 SR-A type I, G418-selected for 1 month. D, A20 SR-A type I, MAC-selected for 2 months.

8–amino acid FLAG epitope will be described elsewhere (P.J.G. et al, unpublished observations, 1997). Ninety-six–well microtiter plates (Sterilin) were coated with 100 ng of purified protein (diluted in PBS) per well for 3 hours at room temperature in a humidified atmosphere. Plates were washed twice in PBS before being blocked overnight with PBS plus 10% NGS (Sigma). Mouse sera were diluted in PBS plus 10% NGS and incubated for 2 hours at room temperature. Plates were washed 4 times with PBS, and binding antibodies were detected with peroxidase-conjugated anti-mouse IgG diluted 1:500 in PBS plus 10% NGS, followed by addition of *o*-phenylenediamine 2HCl. Optical density was determined by an automatic plate reader (Anthos HTII, Denley Instruments) measuring the absorbance at 450 nm. The anti-FLAG monoclonal antibody M2 (IBI Ltd, Cambridge, UK) was used as a positive control in all experiments.

FACS Analysis of Transfected CHO-K1 Cells

Forty-eight hours after transfection cells were harvested with PBS, 5 mmol/L EDTA, and 0.1% trypsin; washed twice with PBS; and fixed in a 4% (wt/vol) solution of paraformaldehyde in PBS. Cells were stained with the anti-FLAG monoclonal antibody M2 diluted to 10 μg/mL or with mouse sera at the indicated dilution in PBS containing 10% NGS and 0.1% (vol/vol) Triton X-100 by incubation at room temperature for 60 minutes. Cells were washed 3 times in PBS containing 0.1% Triton X-100 before incubation with FITC-conjugated goat anti-mouse IgG $F(ab')_2$ [Chemicon, Harrow, UK] diluted 1:100 in PBS containing 0.1% Triton X-100. Fluorescence-activated cell sorting (FACS) analysis was performed with a FACScan utilizing the FL1 photomultiplier.

Human Monocyte Isolation and Culture

Buffy coats were obtained through the National Blood Transfusion Center at the John Radcliffe Hospital, Oxford, UK. Mononuclear cells were obtained by Ficoll-paque centrifugation (Pharmacia LKB) and were washed 5 times with PBS to remove platelets. Cells were resuspended in RPMI 1640 medium supplemented with 5% heat-inactivated autologous human serum and PSG, and monocytes were enriched by adherence for 90 minutes at 37°C in 75-cm^2 polystyrene cell-culture flasks (Falcon). Nonadherent cells were removed by washing the flask 6 times with RPMI 1640 prewarmed to 37°C. The cells were subsequently cultivated for a further 24 hours at 37°C in RPMI 1640 medium supplemented with 5% heat-inactivated, autologous human serum and PSG before detachment by incubation at 4°C for 1 hour. Cells were replated in 6-well plates at a density of 3×10^6 cells per well in X-Vivo 10 (Bio-Whittaker) supplemented with 1% autologous human serum and PSG, and incubated for the indicated times.

Immunocytochemistry

Human tissues were obtained from the Cellular Pathology Department of the John Radcliffe Hospital and Autopsy Services, University of Kuopio, Kuopio, Finland; snap-frozen in LN_2; and stored at −70°C. Watanabe heritable hyperlipidemic rabbit atherosclerotic lesions and liver tissue sections were obtained from rabbits that had been perfused with PBS, followed by a 10-minute perfusion-fixation with 4% paraformaldehyde. Cryostat sections of 5- to 8-μm thickness were cut, collected on glass multiwell slides, and dried overnight at room temperature. The slides were then wrapped in aluminum foil and stored at −20°C until required. Before being stained, the sections were thawed at room temperature for 10 minutes

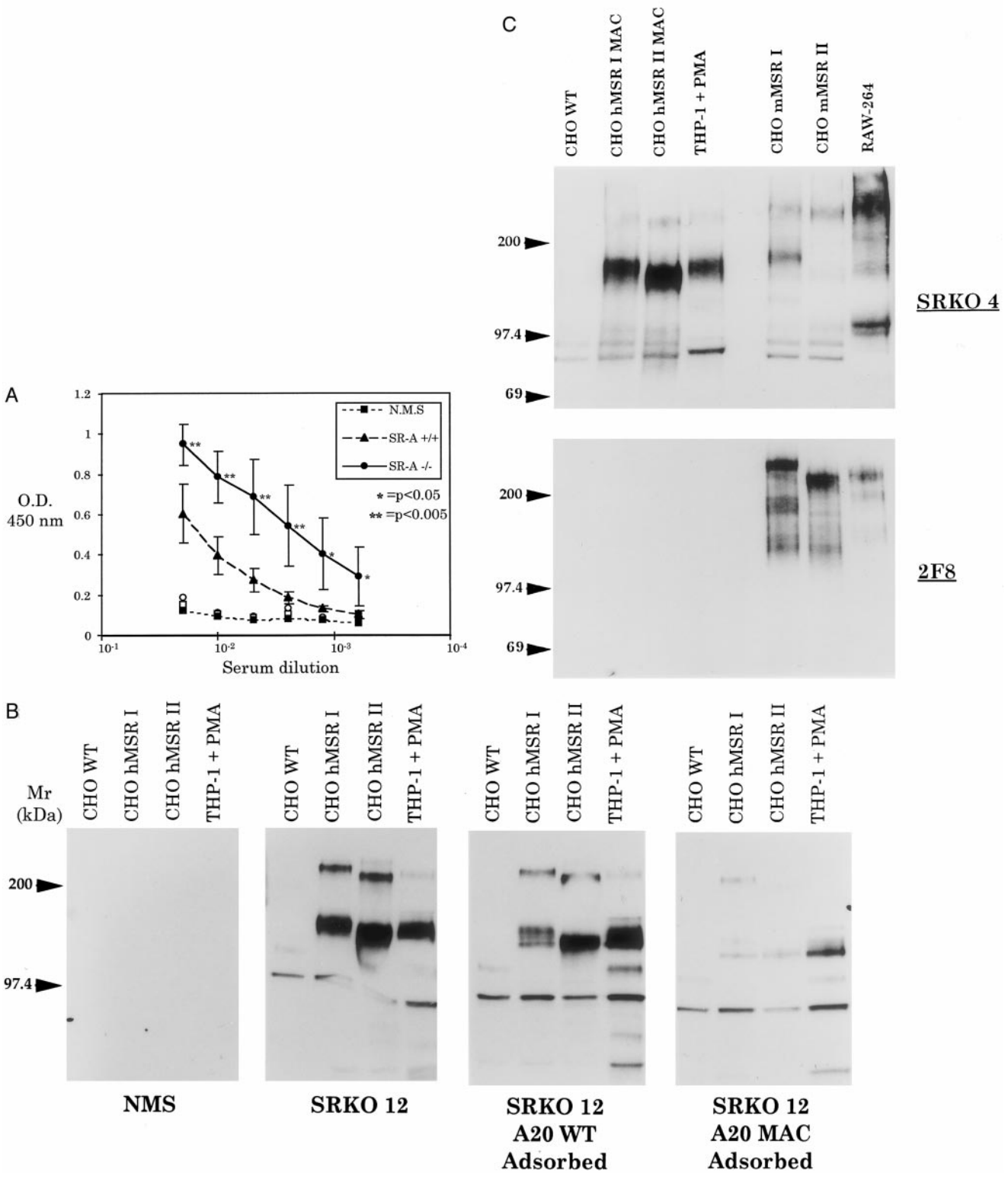


Figure 2. A, Titration of anti-human (h) SR-A antibody production by SR-A +/+ and SR-A −/− mice. Sera from mice immunized as described in Methods were bound to plates coated with a purified form of the extracellular portion of type I human SR-A. Each point represents the mean absorbance values of sera from 5 mice, with error bars representing the SD at each point. Reactivity of NMS and SR-A −/− immune sera against plates coated with PBS alone are shown by hollow squares and circles, respectively. *$P<0.05$ and **$P<0.005$, by nonpaired Student's t test. B, Immunoblot analysis of anti–SR-A antibody activity. Total protein from CHO-K1 cells (CHO WT), CHO cells transfected with type I or II human SR-A (CHO hMSR I and CHO hMSR II, respectively), and THP-1 cells treated with PMA for 4 days (THP-1+PMA) were separated by 6% nonreducing SDS-PAGE. Blots were probed with NMS (1:1000), SRKO-12 (1:1000), SRKO-12 adsorbed against A20 wild-type cells (1:1000), and SRKO-12 adsorbed against A20 cells stably transfected with type I human SR-A (1:1000) as described in Methods. C, Cross-species reactivity of anti-human SR-A antisera. Total protein lysates as for Figure 2B, from CHO cells stably transfected with type I or type II murine SR-A (CHO mMSR I and CHO mMSR II, respectively), and the murine macrophage cell line RAW-264 were separated by 6% nonreducing SDS-PAGE. Blots were probed with NMS (1:500; data not shown), SRKO-4 (1:500), and anti-murine SR-A monoclonal antibody 2F8 (10 μg/mL).

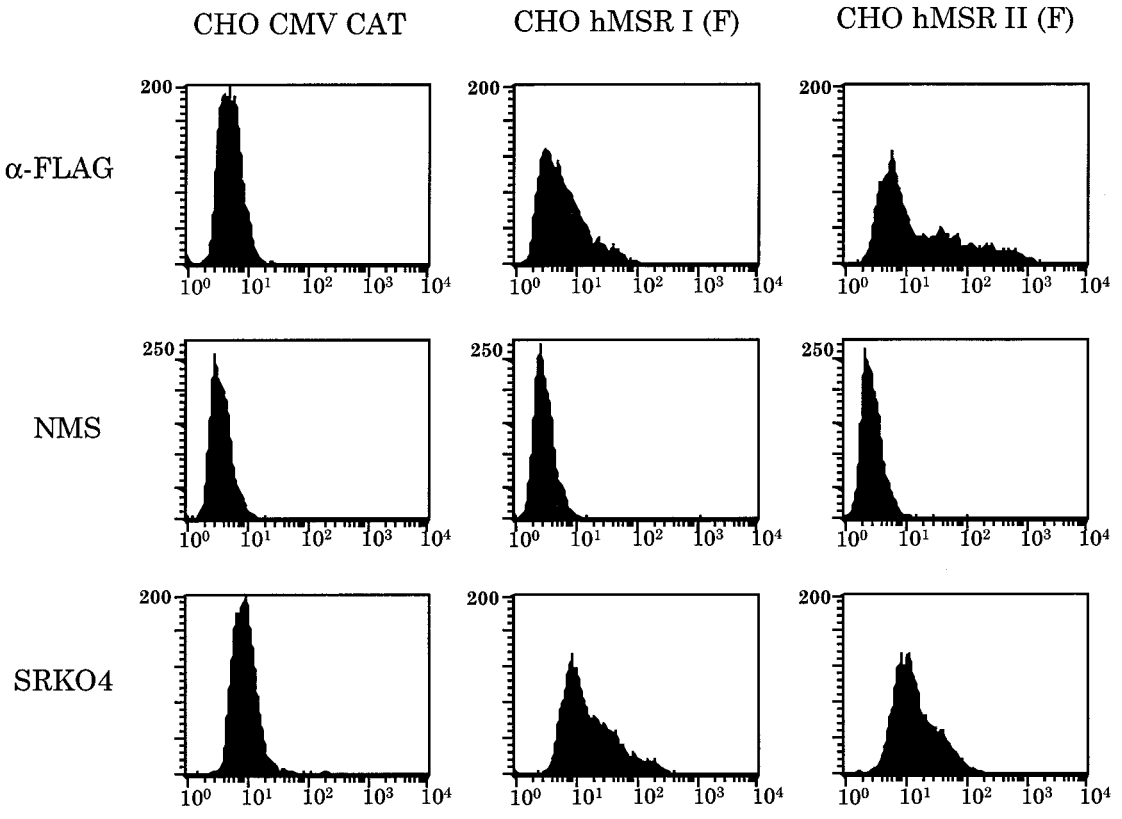


Figure 3. FACScan analysis of SR-A expression in transiently transfected CHO-K1 cells. Cells were transfected with expression constructs containing the full-length coding sequence of type I and II SR-A including the FLAG epitope and an identical expression vector containing the CAT reporter gene. Cells were stained with either anti-FLAG monoclonal antibody M2, NMS (1:250), or anti–SR-A polyclonal serum (1:250) as described in Methods. Results are from a single experiment performed in duplicate and are representative of 3 independent experiments.

and fixed for 10 minutes in acetone at room temperature. Sections were incubated with antibody diluted as indicated in PBS plus 10% NGS (blocking buffer) for 1 hour at room temperature in a humidified atmosphere. Binding was detected either by incubation of sections with peroxidase-conjugated anti-mouse IgG diluted 1:100 in blocking buffer or biotinylated goat anti-mouse IgG (Jackson ImmunoResearch Labs, West Grove, Pa) diluted 1:100 in blocking buffer, followed by avidin-biotin-peroxidase complex (ABC elite; Vector) and 0.5 mg/mL diaminobenzidine (Polysciences Inc) with 0.024% H_2O_2 in 10 mmol/L imidazole in PBS. Sections were counterstained with crystal violet.

Results

Generation of A20 Cells Expressing High Levels of Human SR-A

To generate a high-titer specific immune response against human SR-A, we generated murine A20 cells expressing high levels of type I human SR-A to use as an immunogen. A20 cells were transfected with type I SR-A or the CAT reporter gene in the same mammalian expression vector. Stable expression was selected by 2-week culture in G418, and levels of SR-A were analyzed by assaying the uptake of the fluorescently labeled SR-A ligand DiI-AcLDL (Figure 1A through 1C). Levels of expression were low and cells were transferred to MAC medium, a medium that provides nutritional selection for cells expressing functional SR-A activity.[34] After 2 months of culture in this medium, A20 cells expressing high levels of SR-A activity were obtained (Figure 1D), and these cells served as the immunogen for all subsequent experiments.

Characterization of Anti–SR-A Antisera

Initial experiments with BALB/c mice showed a very-low-titer anti–human SR-A response (data not shown), so we used SR-A −/− mice in an attempt to overcome tolerance to the human SR-A protein. SR-A −/− and SR-A +/+ mice of the same strain were immunized with A20 cells expressing high levels of SR-A. SR-A–specific antibodies were analyzed by ELISA against plates coated with a soluble, secreted form of type I SR-A. Sera from SR-A −/− mice showed a significantly higher specific antibody titer at all dilutions tested (Figure 2A) compared with sera from SR-A +/+ mice. Anti–human SR-A antibody titers produced by SR-A −/− mice were highly reproducible, and antisera from several mice were used for the subsequent experiments.

To demonstrate anti–human SR-A specificity, sera from SR-A +/+ and −/− mice were used for immunoblot analysis on cell lysates from CHO cells transfected with type I or type II human SR-A and the human cell line THP-1 treated with phorbol 12-myristate 13-acetate (PMA) to induce expression of SR-A.[35] Sera from SR-A +/+ mice did not yield SR-A–specific bands at any dilution used (data not shown), unlike sera from SR-A −/− mice that were able to detect SR-A–specific bands from both transfected CHO cells and treated THP-1 cells (Figure 2B). Under the nonreducing SDS-PAGE conditions used, SR-A should migrate predominately as a mixture of monomers (M_r ≈90 to 70 kDa) and disulfide-linked dimers (M_r ≈170 to 150 kDa) with a small amount of

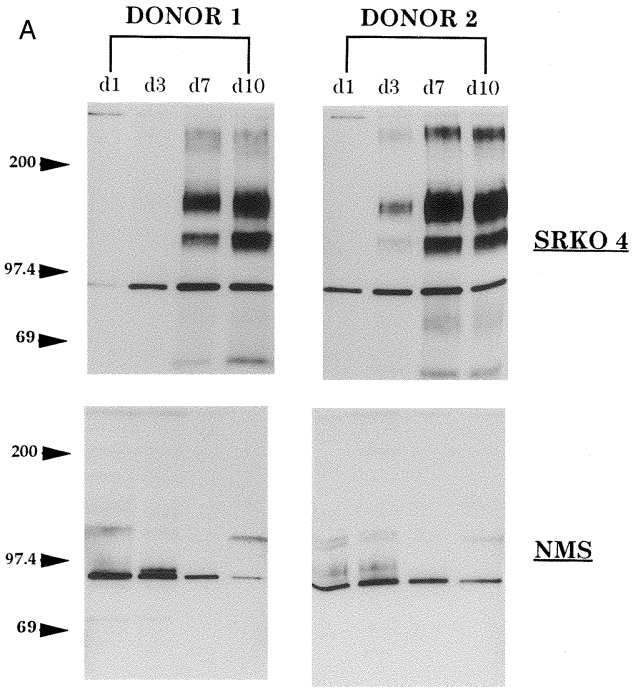
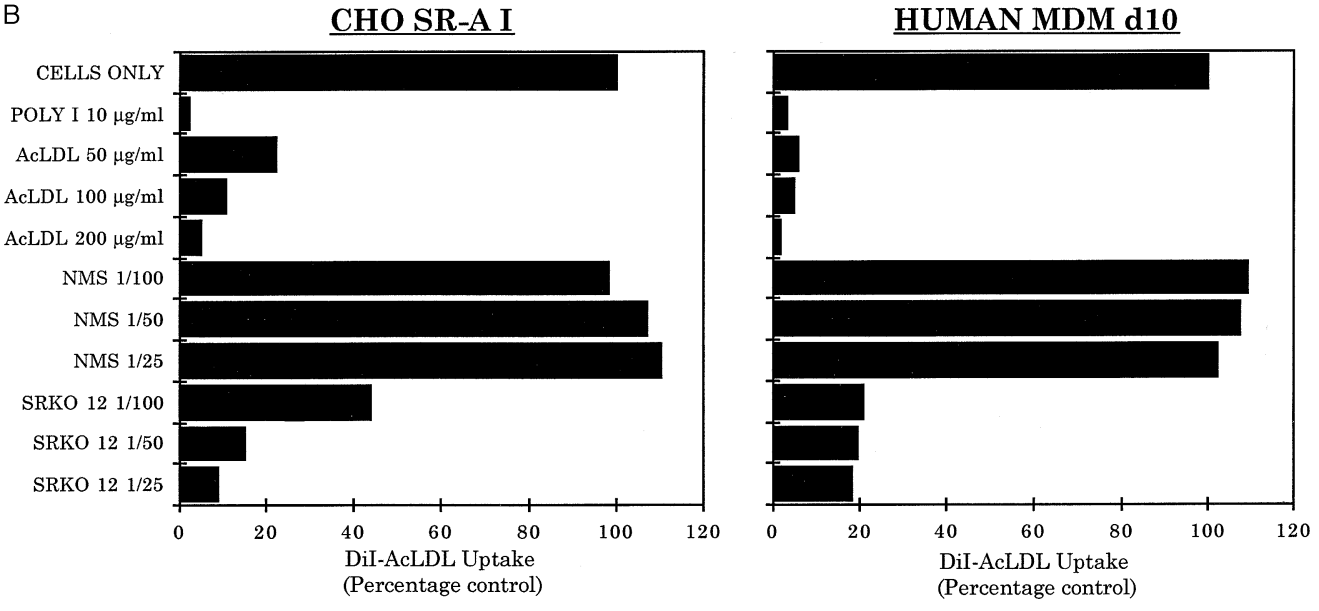


Figure 4. A, Immunoblot analysis of SR-A expression by human monocyte–derived macrophages. Total cellular protein (30 μg) from human monocyte–derived macrophages from 2 different donors cultured for the indicated times was separated by 6% nonreducing SDS-PAGE. Blots were probed with NMS (1:500) and SRKO-4 (1:500). B, SRKO-12 inhibition of DiI-AcLDL uptake. Transfected CHO cells expressing high levels of type I SR-A and human monocyte–derived macrophages cultured in vitro for 10 days were preincubated with inhibitors at the indicated concentrations for 30 minutes before labeling in the continued presence of inhibitors for 90 minutes as described in Methods. Specific fluorescence intensity was calculated by subtracting autofluorescence from fluorescence intensity of DiI-AcLDL–labeled cells by using CellQuest software (Becton Dickinson) and expressed as a percentage of uptake by control cells labeled in the absence of inhibitors in the same experiment. Results are from a single experiment and are representative of 3 independent experiments.



trimers (M_r ≈240 to 220 kDa). The antibody recognized both the trimeric and dimeric forms of both type I and type II SR-A but did not recognize the monomeric form of either receptor. No SR-A–specific bands were visualized after immunoblotting under reducing conditions (data not shown). To confirm the specificity of the anti–SR-A antisera, sera were adsorbed against A20 cells or A20 cells expressing high levels of type I SR-A to deplete nonspecific or specific antibodies, respectively. Immunoblotting with antisera adsorbed against wild-type A20 cells gave a banding pattern similar to that of the unadsorbed antisera, indicating that the antisera were largely monospecific. The SR-A–specific bands were almost abolished after immunoblotting with serum adsorbed against cells expressing type I SR-A, indicating that the banding pattern was due to specific anti–SR-A antibodies.

The cross-species reactivity of antisera from SR-A −/− mice was tested by immunoblotting against lysates from CHO cells transfected with either human or murine SR-A or from human or murine macrophage cell lines. The anti–SR-A antisera were able to recognize both human and murine SR-A, unlike the anti-murine SR-A monoclonal antibody 2F8 that only recognized murine SR-A (Figure 2C). The anti–SR-A immunoblotting highlights the species differences in intratrimer disulfide linkages. The majority of immunoreactive material for the human receptor was in the dimeric form, unlike that in the mouse, which was predominately trimeric. This difference is due to the presence of an additional cysteine residue (Cys87) in the murine spacer domain, leading to the formation of covalently linked trimers, in comparison with the covalently linked dimer and noncovalently associated third chain of the human form of the receptor.[11]

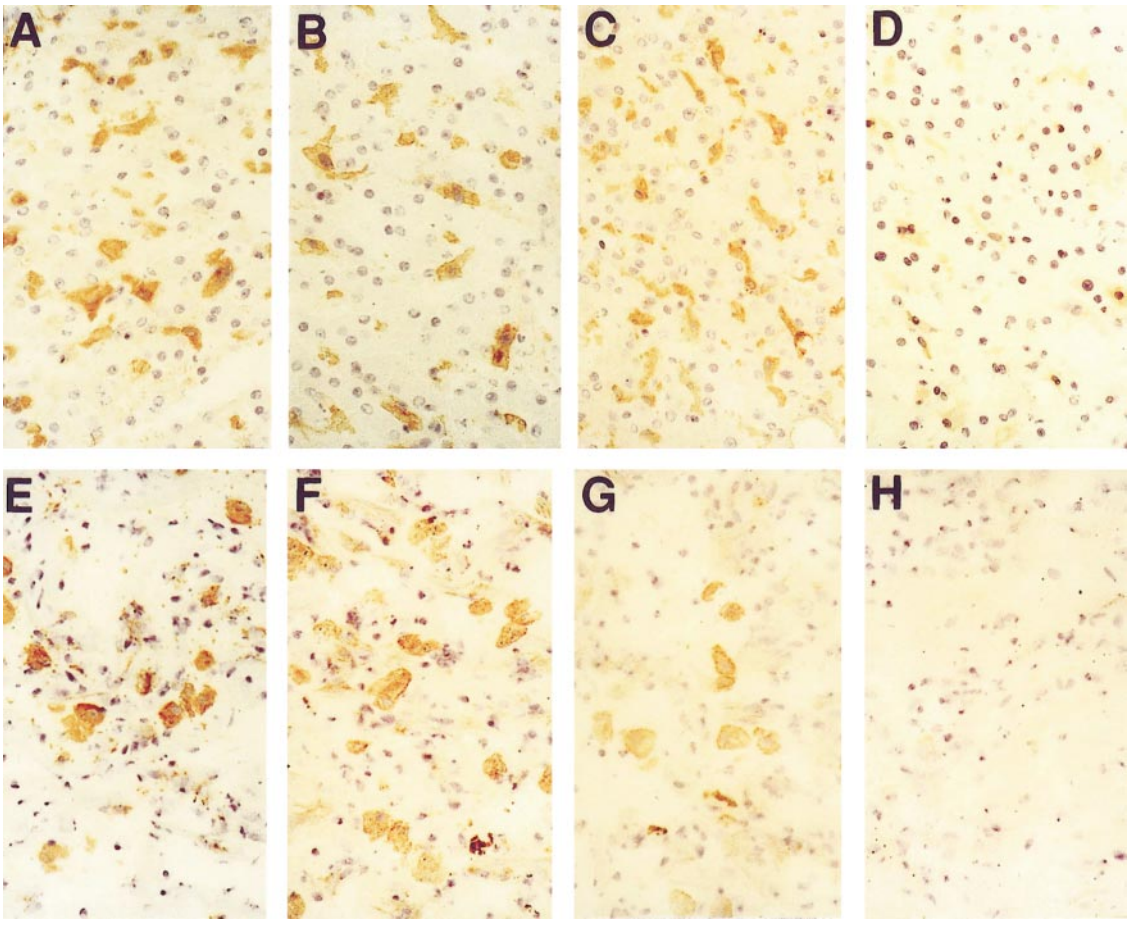


Figure 5. Immunohistochemistry using anti–SR-A antisera. Normal human liver and lung were stained with anti-CD68 (clone EBM-11; A and E), unadsorbed KO12 antiserum (1:350; B and F), KO12 antiserum adsorbed against wild-type A20 cells (1:350; C and G), and KO12 adsorbed against A20 cells expressing high levels of type I SR-A (1:350; D and H). Magnification ×80.

The anti–SR-A antisera were further characterized by FACS analysis of CHO cells transiently transfected with type I or type II human SR-A containing a FLAG epitope tag between the initiator methionine and the second residue glutamate of SR-A. FACS analysis using the anti-FLAG antibody M2 showed that cells transfected with either of the FLAG-tagged SR-A constructs displayed a similar heterogeneous pattern, with the antibody recognizing the transfected subpopulation (Figure 3). Staining with anti–SR-A antiserum gave an identical pattern of fluorescence in contrast to serum from nonimmunized mice.

In Vitro Analysis of Human SR-A Expression

To examine SR-A expression by primary human cells, we cultured monocyte-derived macrophages, prepared total protein lysates at various time points, and used immunoblotting to detect SR-A protein. Figure 4A shows that cells from 2 donors revealed no SR-A expression at day 1, but receptor expression increased with time in culture. The pattern of SR-A–specific bands detected by the antiserum from primary macrophage cell lysates is different from that for transfected CHO cells. A band appeared, corresponding to the precursor of the dimeric form of the receptor ($M_r \approx 130$ kDa), and some monomeric protein was also detected.

We examined the ability of anti–SR-A antisera to block SR-A–mediated endocytosis by both CHO cells expressing high levels of type I SR-A and human monocyte–derived macrophages cultured for 10 days (Figure 4B). Uptake of DiI-AcLDL by transfected CHO cells was completely inhibited by polyinosinic acid (Poly I); uptake was also inhibited in a concentration-dependent manner by excess unlabeled AcLDL. Nonimmune mouse serum did not significantly alter DiI-AcLDL uptake in comparison with anti–SR-A antiserum that largely abrogated endocytosis of this SR-A ligand. In several experiments with CHO transfectants, complete inhibition of ligand uptake was not obtained, presumably due to an inability to obtain saturating levels of antibody, as highlighted by the decreasing levels of inhibition seen with decreasing concentration of antibody. Endocytosis of DiI-AcLDL by human monocyte–derived macrophages was also completely inhibited by Poly I and excess unlabelled AcLDL but was decreased to only 80% by the anti–SR-A antiserum. This finding indicates that SR-A is responsible for the large majority of AcLDL uptake by macrophages, the remaining 20% presumably mediated by a non–SR-A but Poly I–inhibitable scavenger receptor.

In Vivo Analysis of Human SR-A Expression in Normal Human Tissue and Atherosclerotic Lesions

The usefulness of anti-human SR-A antisera for studying SR-A expression in human tissues was examined by immunocytochemistry. Figure 5A shows specific staining of

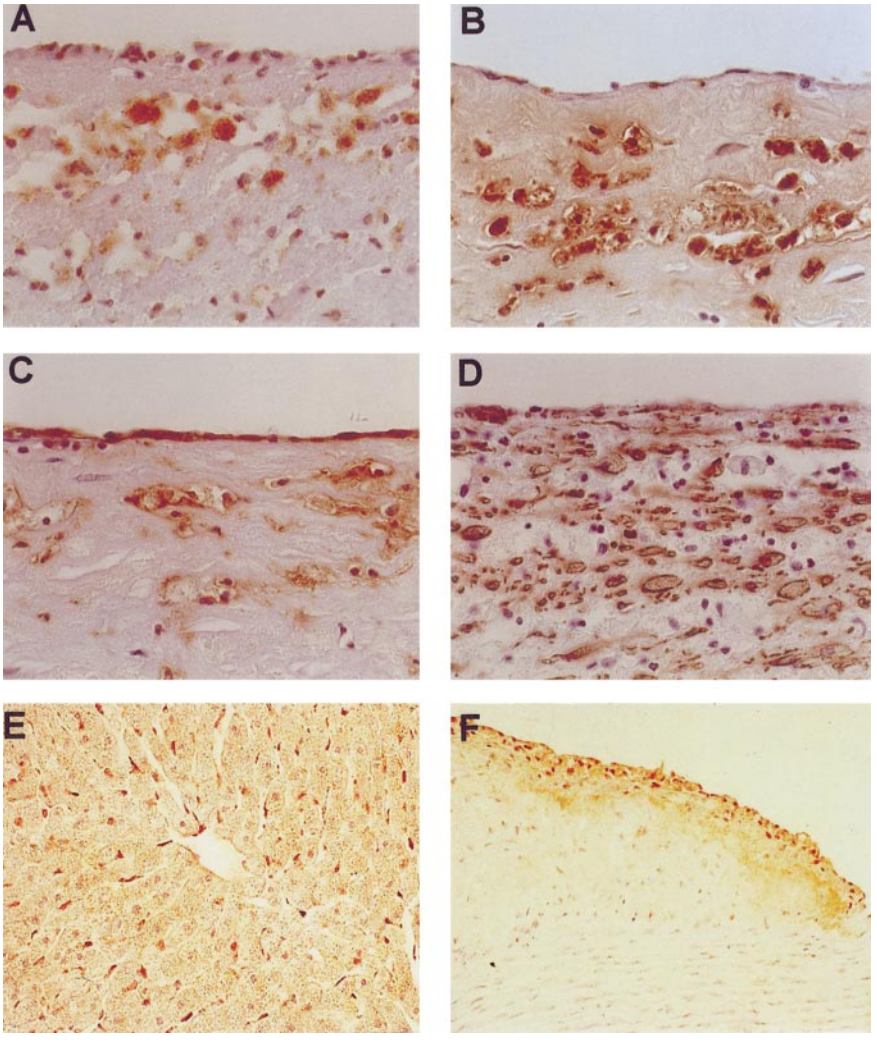


Figure 6. Staining of human and rabbit aortic atherosclerotic lesions with anti–SR-A antisera. Serial sections of a lesion from the thoracic aorta of a 36-year-old female 6 to 8 hours postmortem were stained with unadsorbed KO8 antiserum (1:200; A), anti-CD68 (1:50, clone EBM-11; B), anti-CD31 (1:50, clone JC/70A; C), and anti–α-actin (1:50, clone HHF-35; D). Watanabe heritable hyperlipidemic rabbit liver (E) and atherosclerotic lesions (F) were stained with KO4 antibody (1:500). Magnification: A to D, $\times$80; E, $\times$60; and F, $\times$40.

Kupffer cells in human liver with the anti-CD68 monoclonal antibody EBM-11. Human liver stained with anti–SR-A antiserum yielded a very similar staining pattern, with little or no reactivity with sinusoidal endothelial cells and no staining of hepatocytes (Figure 5B). Serum that had been adsorbed with A20 cells displayed an identical pattern, whereas staining was abolished when SR-A–specific antibodies were specifically removed by adsorption of anti–SR-A serum with A20 cells expressing high levels of SR-A (Figure 5C and 5D). Alveolar macrophages stained strongly positive for SR-A, with the adsorption of specific antibodies abolishing staining; their distribution was very similar to that of CD68-positive macrophages (Figure 5E through 5H).

Having established the specificity of the anti–SR-A-antiserum in the staining of normal human tissues, we examined SR-A expression within human aortic atherosclerotic lesions. Figure 6A shows that anti–SR-A immunostaining was localized to the same areas that contained CD68-positive macrophages (Figure 6B). Aortic endothelium, as identified by anti-CD31 staining (Figure 6C), did not show any immunoreactivity with anti–SR-A antiserum. Macrophages in lesions were also positive for CD31 staining. The distribution of α-actin–positive smooth muscle cells (Figure 6D) was also different from anti–SR-A staining.

To further examine the species specificity of the anti–SR-A antiserum, we performed immunocytochemistry on rabbit liver sections. The expression of SR-A in rabbit liver was very similar to that in human liver, with Kupffer cells staining strongly (Figure 6E). SR-A was expressed in Watanabe heritable hyperlipidemic rabbit atherosclerotic lesions (Figure 6F) in areas that contained macrophages, as judged by macrophage-specific immunostaining of serial sections (RAM-11 antibody; data not shown). Again, no SR-A–specific immunostaining was detected in endothelium or medial smooth muscle cells (data not shown).

The expression of SR-A by smooth muscle cells was further examined by immunohistochemistry of aortic lesions from hypercholesterolemic rabbits 14 days after balloon injury. This approach was chosen to produce fibrous atheromatous lesions, rich in smooth muscle cells, in a relatively short time. Immunohistochemistry showed that the lesions contained numerous intimal smooth muscle cells and very few macrophages, as judged by HHF-35 and RAM-11 staining, respectively (Figure 7B and 7C), and did not have an intact endothelium (Figure 7D). SR-A expression was observed in a few cells in the very superficial part of the thickened intima (Figure 7A). Analysis of serial sections revealed that the few SR-A–positive cells colocalized with smooth muscle cells.

Discussion

In this study, we have described the production, characterization, and application of the first high-quality anti-

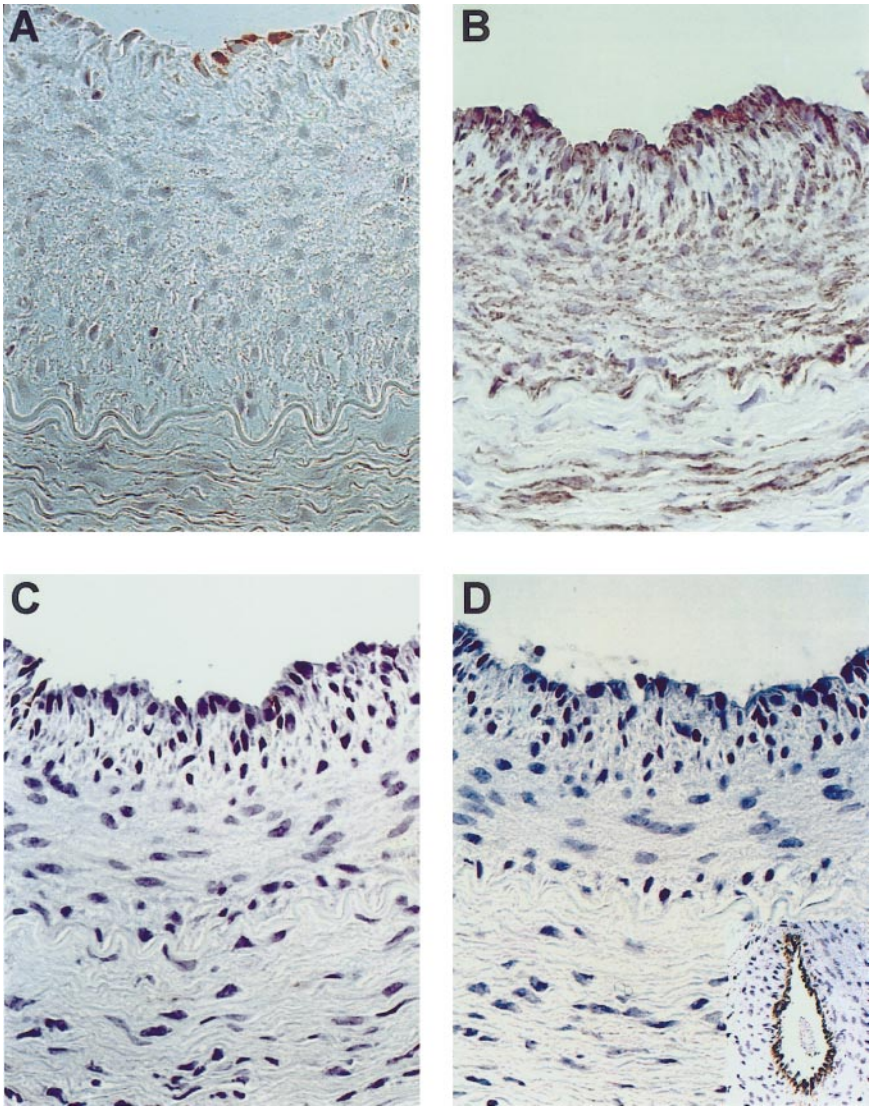


Figure 7. Staining of balloon-injured aorta from a hypercholesterolemic rabbit with anti–SR-A antisera. Serial sections of an aortic lesion from hypercholesterolemic New Zealand White rabbits 14 days after balloon injury were stained with unadsorbed KO8 antiserum (1:200; A), anti–α-actin (1:50, clone HHF-35; B), RAM-11 (1:100), which recognizes an unidentified antigen on rabbit macrophages (C), and anti-CD31 (1:50, clone JC/70A; D). Insert in panel D shows positive staining of a vessel from the adventitia of the same section. Magnification $\times$60.

body to recognize both type I and II human SR-A. Our earlier rat anti-murine SR-A monoclonal antibody (2F8) does not react with the human receptor molecule. We have overcome the problems of tolerance caused by the high degree of similarity between human and murine SR-A proteins (70% amino acid identity) by utilizing mice deficient in SR-A. The polyclonal antisera produced by SR-A −/− mice in response to immunization with a murine cell line expressing high levels of human SR-A had a significantly higher antibody titer than did those from SR-A +/+ mice and were qualitatively different, in that they recognized murine, rabbit, and human SR-A. This reagent has enabled us to use immunoblotting and immunocytochemistry to study SR-A expression by human monocyte–derived macrophages in vitro and by individual macrophage populations within normal and diseased human and rabbit tissues. The ability of anti–SR-A antisera to block SR-A–mediated endocytosis has also allowed us to examine the contribution of SR-A to the uptake of modified LDL in cultured, human monocyte–derived macrophages.

Analysis of the expression of SR-A protein by in vitro cultured human monocyte–derived macrophages revealed that monocytes express no detectable SR-A protein, with expression of SR-A being significantly induced by macrophage differentiation. Previous studies have shown that monocytes do have some detectable scavenger receptor activity as measured by AcLDL uptake, although the nature of the receptor involved was not characterized.[36] Our present results are consistent with other studies that have utilized reverse transcription–polymerase chain reaction to examine SR-A expression.[37] Because the anti–SR-A antisera do not efficiently recognize the monomeric form of the receptor, the immunoblot approach used here is not suitable for studying receptor isoform expression by cultured monocyte-derived macrophages. By using the anti–SR-A antisera to immunoprecipitate SR-A from metabolically labeled monocyte-derived macrophages, we were able to resolve type-specific bands by reducing the immunoprecipitated protein and treating it with *N*-glycanase to remove all *N*-linked glycosylation. This approach has shown that both type I and type II receptors are expressed by cultured macrophages at comparable levels (data not shown). Future studies using this approach will allow us to elucidate the factors that affect receptor isoform expression and their significance for SR-A function.

Using the anti–SR-A antiserum to block SR-A–mediated endocytosis, we have shown that SR-A mediates ≈80% of AcLDL endocytosis by human macrophages. This figure is comparable to that seen for macrophages from SR-A −/− mice.[15] Several receptors have been shown to recognize modified LDL, including CD36, SR-B1, LOX-1, SREC, and CD68.[29–33,38] Our antiserum will allow the contribution of SR-A in the uptake of other ligands, eg, oxidized LDL and advanced glycosylation end product modified–BSA to be examined.

Immunostaining of normal human tissue shows that human alveolar macrophages and Kupffer cells of the liver express large amounts of SR-A protein, whereas there was little or no receptor expressed by sinusoidal endothelial cells. Previous studies of bovine and murine SR-A protein expression have shown similar results.[39,40] SR-A was detected on murine sinusoidal endothelium by using the rat anti-mouse monoclonal antibody 2F8,[39] and with the use of antipeptide antiserum it has recently been reported that SR-A is present on rabbit aortic endothelium.[41] These observations may reflect species differences, variability in tissue extraction expression or preparation, or different antibody specificities. We have attempted to address these questions by utilizing our polyclonal antisera that do blot murine SR-A to examine SR-A expression in mouse tissue but have been hampered by background problems created by using a mouse reagent on murine tissue (data not shown). Several investigators have shown that modified LDL can be metabolized by endothelial cells in vitro[28,42] and that sinusoidal endothelial cells take up AcLDL injected intravenously.[43] However, there is increasing evidence that in vivo clearance of AcLDL is mediated by another scavenger receptor.[15,44–46] The recent cloning of 2 novel, endothelium-specific scavenger receptors may offer an explanation for the apparent discrepancy between scavenger receptor activity and the absence of SR-A protein.

Our findings show that SR-A is expressed by macrophages and foam cells in human and rabbit atherosclerotic lesions, with little detectable expression by smooth muscle cells or aortic endothelium. There have been many previous studies investigating the expression of SR-A by smooth muscle cells in vitro. Pitas and colleagues have shown that in vitro cultured human and rabbit smooth muscle cells express low levels of SR-A but that expression can be markedly induced by treatment with phorbol esters or platelet secretion products, resulting in lipid accumulation and foam cell formation.[25,26,47,48] Tumor necrosis factor-α, interferon-γ, and human cytomegalovirus infection have also been shown to induce expression of SR-A by smooth muscle cells in vitro.[49,50] In contrast, several studies of SR-A expression in human and rabbit atherosclerotic lesions using immunohistochemical and in situ hybridization techniques have failed to show significant receptor expression.[24,51–53] To date, there is only 1 report of significant SR-A expression by smooth muscle cells in rabbit atherosclerotic lesions.[49] These findings were based on staining with an anti-peptide antibody of lesions from normal and hypercholesterolemic rabbits, with and without balloon injury, similar to those used in Figure 7. In contrast to our observations of a relatively small subpopulation of smooth muscle cells expressing SR-A in the balloon injury lesions from hypercholesterolemic rabbits, Li et al showed that a significant proportion of smooth muscle cells express SR-A. The lesions generated by balloon injury shown in Figure 7 are relatively deficient in macrophages compared with those generated in the study by Li et al, despite their use of similar protocols. It is possible that these differences in basic lesion morphology can explain the discrepancies in the results with respect to SR-A expression; however, differences in the specificity of the antisera cannot be ruled out. Although our results show no evidence for the SR-A expression by more than a small minority of smooth muscle cells, we cannot rule out the possibility that this expression can be further induced under certain lesion conditions by factors analogous to those regulating SR-A in in vitro cultured smooth muscle cells.

The generation and characterization of specific antisera recognizing human SR-A have allowed us to establish the cell types that express SR-A within human atherosclerotic lesions. This reagent will be useful for examining the regulation of SR-A expression and its relevance to the pathogenesis of atherosclerosis.

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