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Coexpression of Type I and Type II Human Macrophage Scavenger Receptors in Macrophages of Various Organs and Foam Cells in Atherosclerotic Lesions

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Macrophage scavenger receptors are trimeric membrane glycoproteins implicated in the pathologic deposition of cholesterol in arterial walls during atherogenesis. Two types of cDNAs for functional human receptors have been cloned, but their physiologic roles remain obscure. To study the expression of these receptors, the authors generated antibodies against scavenger receptor type-specific synthetic peptide. Immunohistochemical examination using these antibodies and other anti-human receptor antibodies shows that type I and type II receptor proteins can be detected in foam cells in various stages of atherosclerosis, most evidently in fatty streaks. Coexpression of the two types of receptor protein was also detected in macrophages of various organs. Both types of the protein were detected on the surface and the membrane of endosomes in macrophages. These results indicate that both type I and type II scavenger receptors are expressed and functionally active in physiologic and pathologic conditions. (Am J Pathol 1992, 141:591–599)

Macrophage scavenger receptors mediate the recognition and uptake of a wide range of negatively charged macromolecules and are implicated in the pathologic deposition of cholesterol in arterial walls during atherogenesis through receptor-mediated endocytosis of modified low-density lipoproteins (LDL).[1–5] Molecular cloning of cDNAs for bovine,[6,7] murine,[8] and human[9] scavenger receptors has disclosed several unexpected features. Two types of C-terminally different receptor subunits exist, and both of these receptor proteins contain two extracellular domains that are predicted to form a long triple-stranded alpha-helical coiled coil and a collagenlike triple helix. In the C-terminal type specific domain of the type I receptor, all six cystein residues and the length of the gaps between them are completely conserved in all animal species studied. Conversely, human type II receptor has a 17-residue C-terminals,[9] which is bigger than the six-residue C-terminals of the bovine and murine receptors. When expression vectors containing either type I or type II cDNA were transfected into cultured cells, both receptors mediated the endocytosis of modified LDL.[6,7,9] The scavenger receptor protein[9] and mRNA[10] are detectable in the macrophage-rich areas of atherosclerotic lesions, and the expression of LDL receptor mRNA was deeply suppressed in these lesions.[10] These results suggest the importance of a scavenger receptor pathway for cholesterol deposition in atherosclerosis, but little is known about the type-specific expression, or its activity during atherogenesis. We have generated four antibodies against synthetic receptor peptides, two of which recognize either type I or type II receptor protein specifically. Here we report that type I and type II receptor proteins are coexpressed in macrophages of various human organs and in foam cells in various stages of atherosclerosis, and that they are localized on membranes of the cell surface and endocytotic vesicles, suggesting that both types of receptors actually function *in vivo.*

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Materials and Methods

Generation of Anti-human Scavenger Receptor Antibodies

Synthetic peptides hSRI-1, 2, 3 and hSRII-1 and their bovine serum albumin conjugates were purchased from Peptide Institute (Osaka, Japan). The amino acid sequences of hSRI-1, 2, 3, and hSRII-1 correspond to the sequences in the α-helical coiled coil domain (residue 199–209), the collagenous domain, (325–342) and the C-terminal domain (401–419) of the type I scavenger receptor, and the C-terminal domain (342–358) of the type II receptor, respectively. Antibodies were generated by subcutaneous injections of 500 μg bovine serum albumin (BSA)-coupled peptide emulsified in Freund's complete adjuvant RM606-1 (IATRON, Tokyo, Japan) into three 2-month-old male rabbits (Csk:JW/Ina) for a first immunization followed by repeated injection of 200 μg peptide emulsified in Freund's incomplete adjuvant (Difco, Detroit, MI) four times every 3 weeks. Blood was sampled 1 week after each immunization, and IgG was purified by Protein A chromatography (Protein A. IG Pure Kit, Religen Corp., Cambridge, MA). The titer of the antibody was determined by enzyme-linked immunosorbent assay (Nunc-immuno plate Maxisorp F96, Nunclon, Roskilde, Denmark). An antigen-linked plate was prepared by pouring 50 μl 0.1 mol/l (molar) $NaHCO_3$ containing synthetic peptides at a concentration of 10 μg/ml into each well of the plates and incubating at 4°C overnight. After washing with phosphate-buffered saline (PBS), blocking was performed by 300 μl of 3% gelatin (Gelatin, Fine powder, Nakarai Tesque, Kyoto, Japan) dissolved in PBS. After removal of the blocking agent, 100 μl of each gradient-diluted sample (rabbit anti-peptide serum or purified IgG) was added as primary antibody and incubated for 1 hour at room temperature. After three washes with PBS, 100 μl peroxidase-conjugated goat anti-rabbit IgG (Organon Teknika Corp., West Chester, PA) diluted 1:2,000 or 1:3,000 was added as a secondary antibody and incubated for 1 hour. After four washes with PBS, 100 μl 0.3% O-phenylendiamine dihydrochloride dissolved in substrate ($NaHPO_4 \cdot H_2O$ 12 g, salicylate acid 12 g, citric acid 3.8 g, 0.05% H_2O_2 1l, pH 6.0) was added and incubated for 10 to 20 minutes at room temperature under light-shielded conditions. The reaction was stopped by adding 25 μl 8 N H_2SO_4. Light absorbance was measured by using a Microplate Reader (MPR A4; Tosoh, Tokyo, Japan) at OD492/610. Antisera or purified IgG from the antisera showing a value more than 0.05 different from the negative control (normal rabbit serum or IgG) were judged to be positive.

Tissues and Cells

Lung, liver, spleen, lymph node, and aortic tissues were obtained from four surgical and three autopsy cases of traumatic or nonsystemic diseases. Aortic tissues with various degree of atherosclerotic lesions were obtained from 20 autopsy cases ranging in age from 25 to 82 years. Alveolar macrophages were obtained from three healthy volunteers by bronchoalveolar lavage. Monocytes were collected by gradient centrifugation using Lymphoprep (Ohtsuka, Tokyo, Japan); 1×10^5 cells were cultured in plastic dishes (35 mm; Nunclon, Roskilde, Denmark) containing RPMI without fetal calf serum for 1 hour.

Histochemistry

Human aortic tissue specimens fixed and frozen for immunohistochemistry as described below were cut into sections 6 to 8 μ thick and stained with Oil red O and Sudan IV.

Immunohistochemistry

Human tissue specimens were fixed for 4 hours with 2% periodate-lysine-paraformaldehyde solution. After washing in PBS containing a graded series of sucrose, the tissues were rinsed in PBS containing 20% sucrose and 10% glycerol for 1 hour. The tissues then were embedded in OCT compound (Miles, Elkhart, IN) and frozen in dry ice-acetone. Cryostat sections were cut 6 to 8 μm thick and dried in air. After inhibition of endogenous peroxidase activity by the method of Isobe et al,[11] the specimens were incubated for 1 hour with rabbit anti-human macrophage scavenger receptor antisera, hSRI-1, 2, 3, and II-1, at a dilution of 1:800–1,200. After washing in PBS, they were covered with species-specific goat anti-rabbit Ig [F(ab')^2] conjugated with peroxidase (Amersham, Amersham, UK) for 1 hour. Peroxidase activity was visualized using 3-3'-diaminobenzidine (DAB) as substrate. For control, tissue slides were incubated with non-immunized rabbit serum or PBS instead of primary antibody and subsequently processed by the same procedure as described above. To visualize the type I and type II scavenger receptors in alveolar macrophages simultaneously, immunohistochemical double staining was performed using hSRI-3 and hSRII-1. Firstly, cytospine specimens were fixed with pure acetone for 10 minutes and incubated with hSRI-3, followed by peroxidase-conjugated goat anti-rabbit Ig [F(ab')^2] and DAB to stain positive cells brown. Before the second staining, specimens were washed in glycine-HCl buffer for 1 hour. Sec-

ondly, specimens were incubated with hSRII-1, biotinylated alkaline phosphatase, and Fast blue BB substrate to stain them blue. For comparison, either the first or second step was performed. As the negative control, primary antibodies were replaced by nonspecific rabbit serum. To identify macrophages, an anti-bovine and human macrophage monoclonal antibody, EBM11 (Dakopatts, Glostrup, Denmark), was also used as primary antibody, followed by incubation with anti-mouse Ig[F(ab′)^2] conjugated with peroxidase (Amersham, Amersham, UK). To identify smooth muscle cells, anti-desmin (Dakopatts, Glostrup, Denmark), anti-myosin (IATRON, Tokyo, Japan), and anti-actin (IATRON, Tokyo, Japan) monoclonal antibodies were used as primary antibodies. As the secondary antibodies, anti-mouse and anti-rabbit Ig[F(ab′)^2] conjugated with peroxidase (Amersham, Amersham, UK) were used, respectively.

Immunoelectron Microscopy

Human alveolar macrophages and monocytes were fixed for 30 minutes in 2% periodate-lysine-paraformaldehyde solution. Immunostaining was performed as described above with a minor modification. After demonstration of peroxidase activity with DAB, the cells were postfixed with 1% osmium tetroxide for 60 minutes. The cells then were dehydrated through a graded series of ethanols and embedded in Epon 812 (E. Fullam, Latham, NY) and sectioned. The ultrathin sections were cut by an Ultrotome NOVA (LKB, Uppsala, Sweden) and observed through a JEM 2000EX electron microscope (JEOL, Tokyo, Japan) without staining.

Results

Antibodies

The serum reacted with the peptides in an enzyme immunoassay at a dilution of 1:1,000 (hSRI-1, 3, hSRII-1) and at dilutions greater than 1:10,000 (hSRI-2), which is at least 100-fold higher than that of nonimmune sera or sera from animals immunized against other peptides. IgG was purified from the serum by using staphylococcal protein A.

Expression of Scavenger Receptors in Tissue Macrophages

Antibodies against synthetic peptides, hSRI-1, 2, 3 and hSRII-1, were reacted with macrophages in various tis-

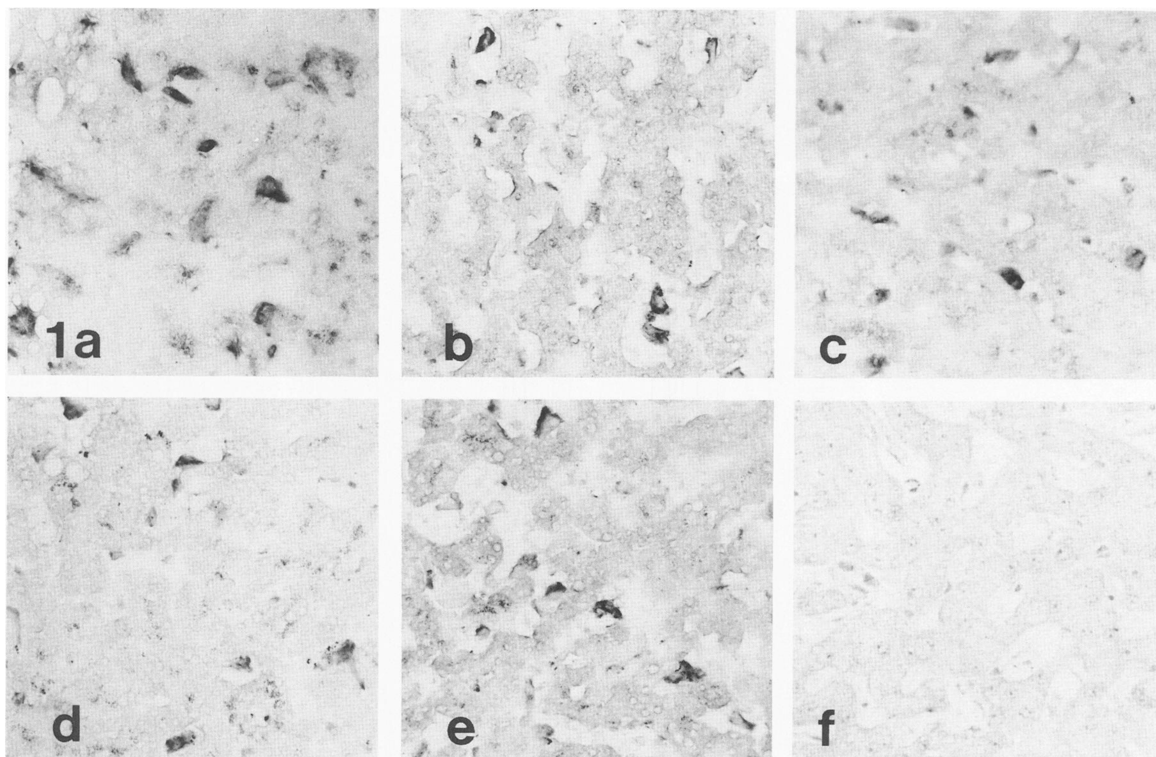
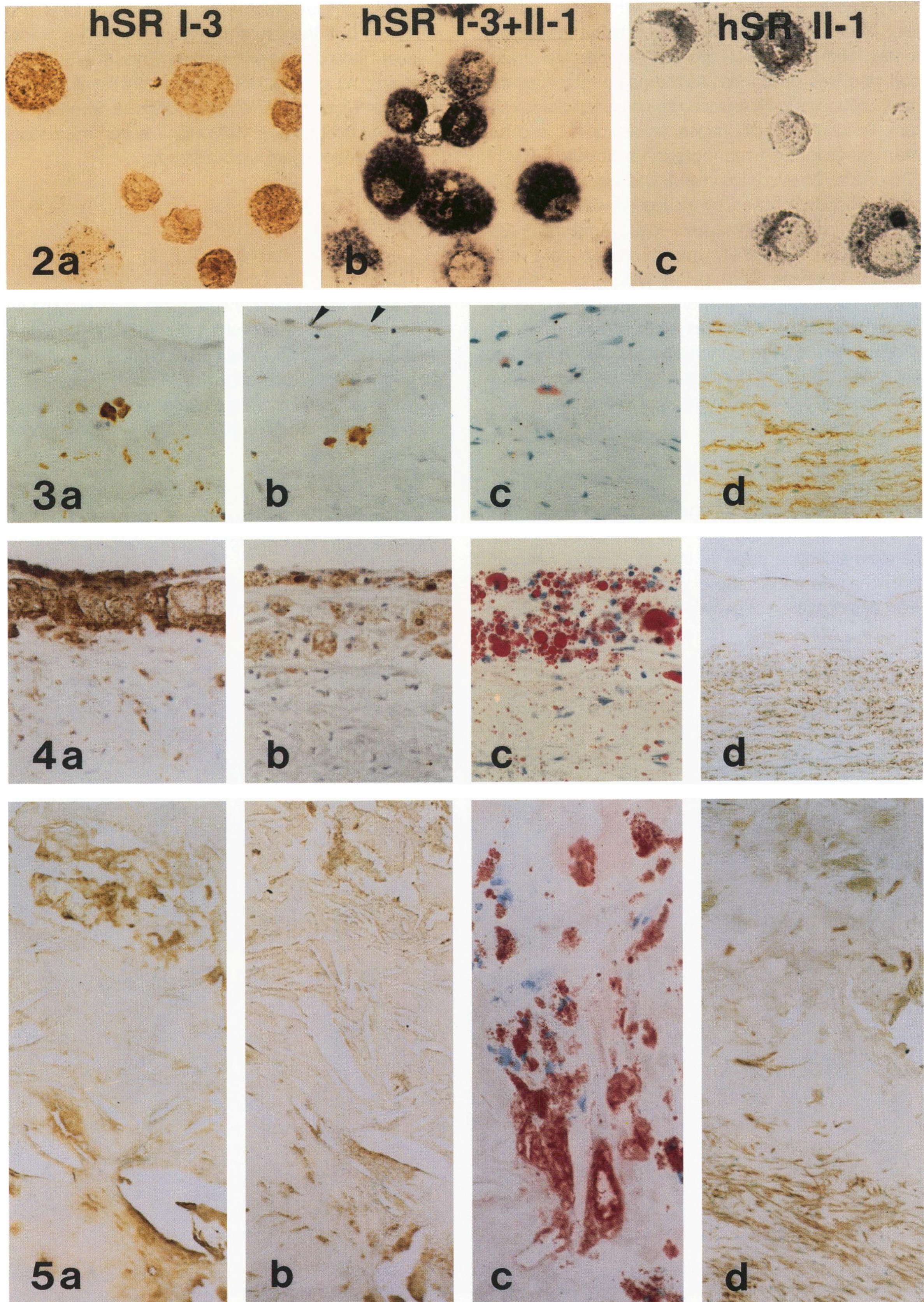


Figure 1. *Immunohistochemical staining of Kupffer cells using EBM11* (a), *hSRI-1* (b), *I-2* (c), *I-3* (d), *and hSRII-1* (e). *Kuppfer cells were reactive to these antibodies. Control* (f); *Kupffer cells were not immunostained with nonimmune serum, ×200.*


hSR I-3
hSR I-3+II-1
hSR II-1
2a
b
c
3a
b
c
d
4a
b
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d
5a
b
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d

sues, such as Kupffer cells in the liver (Figure 1), alveolar macrophages, macrophages in the lymphatic sinuses, macrophages in the red pulp of the spleen, dermal macrophages, and macrophages in the interstitium of the adrenals, kidneys, endometrium, and testis. Kupffer cells and alveolar macrophages reacted more intensely than macrophages in other tissues. Their distribution was very similar to that of EBM11-positive macrophages in these tissues, as observed previously in bovine tissues.[12] Other cell types, such as endothelial cells or fibroblasts, did not react to these antibodies. Among four antisera, hSRI-2 showed the most intense immunohistochemical reaction at a dilution of 1:1,000. Alveolar macrophages were positive for hSRI-3 (Figure 2a) and hSRII-1 (Figure 2c) respectively. Control specimens using nonspecific serum were not stained. By the immunohistochemical double staining, alveolar macrophages were shown to be positive for both hSRI-3 and hSRII-1, indicating that type I and type II scavenger receptors were coexpressed in macrophages (Figure 2b).

Expression of Scavenger Receptors in Atherosclerosis

The atherosclerotic lesions were classified into three major types based on the previous histologic studies.[13,14]

Diffuse Intimal Thickening

It is difficult to detect this slight thickening elevation on gross inspection. Histologically, these lesions were composed predominantly of spindle-shaped cells interspersed with various amounts of collagenous fibers.

Fatty Streaks

These are grossly identifiable as raised yellow streaks. The intimal lesions were characterized by layers of lipid-laden foam cells. The thickness of the lesions was generally less than half that of the media.

Atherosclerotic (Fibrous) Plaques

These advanced plaques were composed of foam cells and spindle-shaped cells and were characterized by the presence of a varying amount of atheromatous substances and fibrous caps. The thickness of these lesions exceeded the thickness of the former two types of lesions and they were often thicker than the media.

In the normal area of the aorta, there were no positive cells for anti-macrophage, anti-scavenger receptor or anti-smooth muscle cell antibodies in the intima. Smooth muscles in the media reacted to three anti-smooth muscle cell antibodies.

In the diffuse intimal thickening (Figure 3), macrophages were rarely detected immunohistochemically. A few small and round macrophages positive for EBM11 were found, however, just beneath the endothelial cells (Figure 3a), together with some lymphoid cells. These macrophages were negative or weakly positive for hSRI-2 (Figure 3b). Spindle cells in the intima were stained with anti-actin antibody, but not with anti-desmin and anti-myosin antibodies.

In the fatty streaks (Figure 4), a varying number of foam cells accumulated in the intima. Foam cells heavily laden with cholesterol ester and other lipids were stained red with Oil red O or Sudan IV (Figure 4c). Foam cells expressed a much more intense immunoreactivity against hSRI-2 (Figure 4b) than did those of diffuse intimal thickening and advanced atherosclerotic plaques. In general, small or medium-sized foam cells reacted more strongly than large ones. Almost all the foam cells in the fatty streaks were positive for EBM11 (Figure 4a) and negative for the anti-smooth muscle antigens, suggesting that these cells were derived from macrophages. A few spindle-shaped cells found in some fatty streaks stained only slightly with any anti-smooth muscle cell antibodies.

With accumulation of foam cells from the intima to the media, the internal elastic lamina became attenuated, fragmented, and disappeared. In the center of the atherosclerotic plaques, various amounts of atheromatous materials became amorphous and contained a few foam cells, cell debris, and cholesterol clefts (Figure 5). In the atherosclerotic plaques, foam cells showed less promi-

Figure 2. *Alveolar macrophages showed a positive reaction for hSRI-3* (a) *and II-1* (c). *Immunohistochemical double staining of alveolar macrophages using both antibodies demonstrated that two types of scavenger receptors are coexpressed on all the cells* (b), *×400.*
Figure 3. *In diffuse fibrous thickening, a few macrophages were positive for EBM11* (a). *hSRI-2-positive cells were few and endothelial cells (arrowheads) were not reactive to hSRI-2* (b). *Cells laden with cholesterol and stained red by Oil red O were scarcely found* (c). *The spindle-shaped cells in aortic intima were stained with anti-actin antibody* (d), *×200.*
Figure 4. *Foam cells in fatty streaks showed a positive reaction for EBM11* (a) *and hSRI-2* (b). *A few small round macrophages positive for EBM11 are negative or weakly positive for hSRI-2. Foam cells were laden with cholesterol and stained red by Oil red O* (c). *The aortic media was stained with anti-actin antibody* (d), *×200.fig4*
Figure 5. *Foam cells in atheromatous plaques.* a: *The foam cells were positive for EMB11.* b: *A few foam cells positive for hSRI-2 were present in the atheromatous substance.* c: *The foam cells were filled with cholesterol as shown by Oil red O staining.* d: *Around the atheromatous material, actin-positive spindle-shaped cells (smooth muscle cells) were present, ×200. Each image in Figures 3–6 was taken from serial sections of the same specimen.*

nent immunoreactivities against EBM11 (Figure 5a) and hSRI-2 (Figure 5b) compared with those of fatty streaks (Figure 4). hSRI-2-positive cells were fewer than EBM11-positive cells. Foam cells in the center of atherosclerotic plaques did not react to anti-smooth muscle cell antibodies. Around the central atheromatous substances, however, especially on the side of the media, numerous spindle cells positive for anti-actin antibody were observed. In the fibrous cap, there were several spindle-shaped cells positive for anti-actin antibodies, among which a few foamy cells positive for the antibody were detected together with a few macrophages. In ulcerated atherosclerotic plaques devoid of fibrous cap, similar findings were confirmed.

In atherosclerotic lesions of various stages, foam cells were positive for each antipeptide antibody, indicating that both type I and II receptors were expressed on cholesterol-laden foamy macrophages (Figure 6). Observation on adjacent serial sections reconfirmed the expression of both types of scavenger receptors in the same foam cells. In all stages of the atherosclerotic lesions, endothelial cells, smooth muscle cells, and connective tissues of the adventitia were not reactive to any antisera.

Ultrastructural Localization of Scavenger Receptors in Macrophages

Immunoelectron microscopy showed the localization of reaction products for each antibody on the cell surface membrane of all the alveolar macrophages (Figure 7a, b) and occasionally on the membrane of vesicles. Monocytes showed no positive reaction (Figure 7c), whereas positive reactions for the antibodies appeared on the cell surface and the membrane of endocytic vesicles of monocyte-derived macrophages after 4 days of culture (Figure 7d).

Discussion

Both type I and type II human macrophage scavenger receptors consist of six domains (Figure 8). They share five domains, and the only structural difference in the two receptors is that the type I receptor contains an extra cystein-rich domain of 110 amino acids at the C-terminus, but type II has a short C-terminus without cystein. In this study, we generated four antibodies against synthetic peptides corresponding to different extracellular domains as indicated in Figure 9. Human type II receptor has a 17-residue C-terminus domain, which is longer than the six-residue domain of bovine[7] or murine[8] type II receptor. This situation is suitable for generating type-specific antibodies. Among the antibodies obtained, two antibodies (hSRI-1 and hSRI-2) can recognize both receptors, whereas two others (hSRI-3 and hSRII-1) are specific for either type I or type II receptor protein, respectively. These antibodies both react with their antigen peptides specifically, and recognize human tissue macrophages specifically.

Using these antibodies, both type I and type II receptor proteins are detected in the membrane of cell surface and endocytic vesicles of tissue macrophages and foam cells. Reactivity on the cell membrane of macrophages against C-terminal type specific domains clearly show that this domain is preserved when it is transferred to the cell surface. This fact is in contrast to the processing of procollagen to collagen involving removal of the N- and C-terminal precursor-specific regions.[15] These results also indicate that both proteins are transported on the endocytic pathway, suggesting that both types are functionally active in physiologic and pathologic conditions.

Coexpression of two types of receptor subunits suggests that adding to the homotrimers, which are known to be functionally active,[6,7,9] mixed oligomers may exist *in vivo*. In various types of collagen molecules,[15] heterotri-

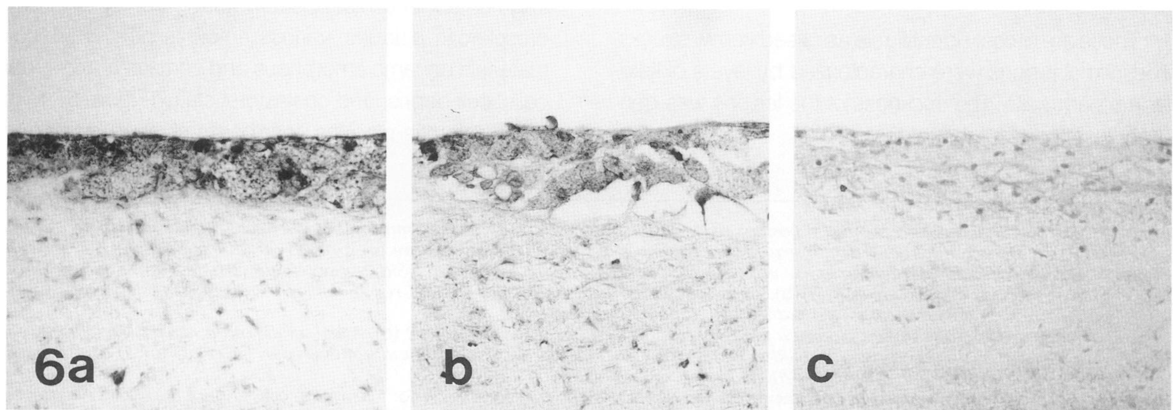

Figure 6. *Immunohistochemical staining of aortic intima using hSRI-3 and hSRII-1. Foam cells in fatty streaks showed a positive reaction for hSRI-3* (a) *and II-1* (b), *indicating that two types of scavenger receptors are coexpressed in macrophage-derived foam cells. A control section* (c) *incubated with nonspecific serum showed no reaction,* ×*200.*

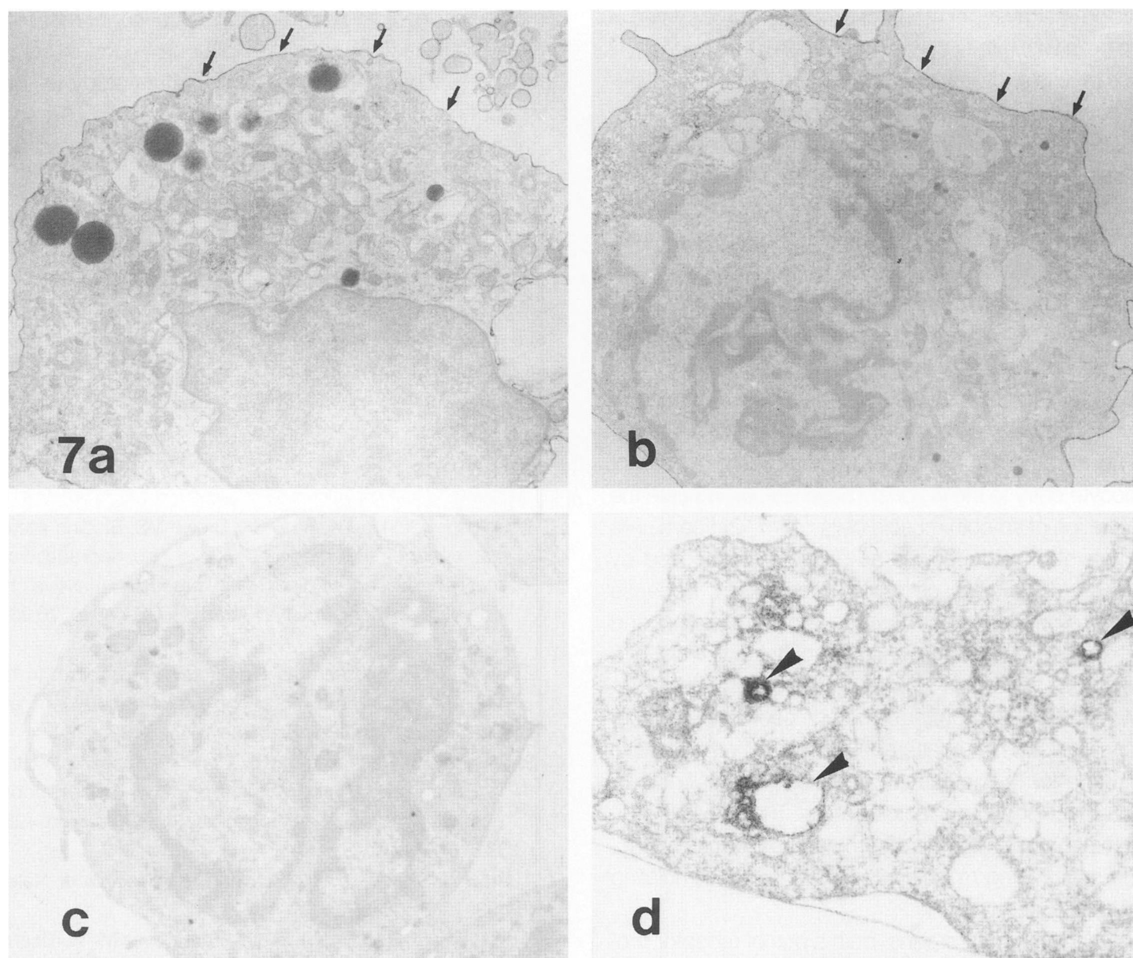

Figure 7. *Alveolar macrophages showed a positive reaction for hSRI-3* (**a**) *and II-1* (**b**) *on the cell membrane and on the membrane of a few endosomes. Monocytes were not stained with hSRI-2* (**c**) *but reaction products were found on the membrane of endosomes (arrowheads) and cell membrane after 4 days of culture* (**d**). *a–c: ×6,500,* **d**, *×12,000.*

meric structures are more common than homotrimer structures. The type-specific antibodies obtained in this study will be useful tools to analyze the higher structures of scavenger receptors.

Atherosclerosis is characterized by the accumulation of foam cells in lesions, especially in fatty streaks. Monocyte and smooth muscle cell origins have been proposed for the origin of foam cells.[16–23] In the current study, foam cells reacted to anti-scavenger antibodies were also positive for the anti-macrophage–specific antibody EBM11. Although increases in the number of smooth muscle cells and smooth muscle cell-derived foam cells were observed in the late stages of atherosclerotic lesions as reported previously,[21–23] macrophage-derived foam cells were a major cell component in the early stages of atherosclerosis.

We studied the expression of scavenger receptors in three major stages of atherosclerosis. In diffuse intimal thickening, macrophages positive for EBM11 are smaller than those in fatty streaks, and these small macrophages showed a relatively low level of the expression of scavenger receptors. This observation is compatible with our *in vitro* observation that monocytes do not express scavenger receptor proteins, and only after several days of cultivation were they detectable. The most prominent ex-

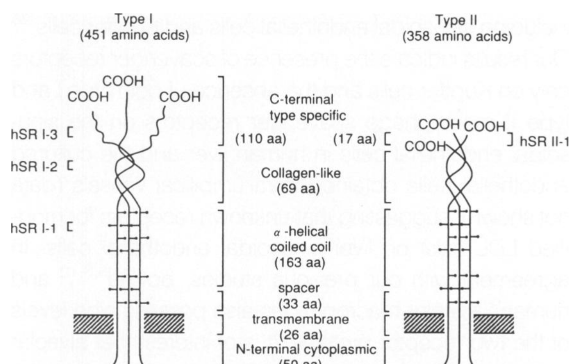

Figure 8. *Primary structure of human scavenger receptors and four antibodies against synthetic peptides corresponding to each domain.*

pression of scavenger receptor proteins was observed in foam cells within fatty streaks as previously reported.[9,10] These results and previous studies suggest that scavenger receptors are not involved in the recruitment of macrophages into arterial walls. Once both types of receptor proteins are expressed, they rapidly uptake cholesterol and change into foam cells. In advanced atherosclerotic lesions, we observed that much more amorphous atheromatous materials had accumulated, whereas the number of foam cells decreased and the levels of expression of scavenger receptor proteins were lower than those in fatty streaks.

Several factors may be related to this decrease in scavenger receptor expression in the advanced lesions. 1) The fibrous caps of atheromatous lesions prevent monocyte entry to the lesions. These lesions indicate the proliferation of smooth muscle cells.[21,24,25] 2) Foam cells may leave from the lesions as previously proposed by Gerrity.[18,19] 3) Foam cells may die *in loco,* because monocyte-derived macrophages are nondividing and short-lived and their turnover time is on the order of 1 or 2 weeks.[26] In advanced lesions, however, scavenger receptor-positive macrophages can be seen in the deeper lesions in groups, or scattered. Ross et al[27] reported that macrophages in all stages of atherosclerotic lesions synthesize platelet-derived factor B chain. These results suggest that macrophages in atheromatous plaques are active in scavenging modified lipoproteins and stimulating neighboring cells.

In various human organs, both types of receptor proteins are detectable only in macrophages. They are not detectable in monocytes. After 4 days of cultivation and differentiation into macrophages, both types of proteins are detected, consistent with the ligand blot and Northern analysis results of our previous studies.[5,6] Both types of receptor protein are richly present in Kupffer cells and alveolar macrophages. These cells are particularly active in the clearance of foreign bodies and modified materials. When modified LDL are injected into experimental animals, they are rapidly taken up by sinusoidal liver cells, including sinusoidal endothelial cells and Kupffer cells.[28] Our results indicate the presence of scavenger receptors only on Kupffer cells and the absence of both type I and type II macrophage scavenger receptors on the sinusoidal endothelial cells in human liver and the cultured endothelial cells obtained from umbilical vessels (data not shown), suggesting that unknown receptors for modified LDL exist on liver sinusoidal endothelial cells. In agreement with our previous studies, bovine[5,6,12] and human[9] alveolar macrophages also possess high levels of the two receptor proteins. It is of interest that alveolar surfactant apoprotein and scavenger receptors share the collagenlike structure. Collagen is a major component of extracellular matrix in the human body, and binds many molecules, including modified LDL. Scavenger receptors in alveolar macrophages may play an important role in the removal of alveolar surfactant. Further study will provide further important information on the role of macrophage scavenger receptors in both physiologic and pathologic conditions.

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