CD36, a Novel Receptor for Oxidized Low-Density Lipoproteins, Is Highly Expressed on Lipid-Laden Macrophages in Human Atherosclerotic Aorta

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Abstract—CD36 has been reported to be a receptor for oxidized LDL (Ox-LDL). In our previous study, the uptake of Ox-LDL in CD36-deficient macrophages was reduced by approximately 50% compared with that in control macrophages, suggesting an important role of CD36 as a receptor for Ox-LDL in humans. In the current study, we examined the immunohistochemical localization of CD36 in human aorta in comparison with that of scavenger receptor class A type I and type II (SRA). Cryostat sections were made from aortic tissues. For immunohistochemical staining, the following antibodies were used: (1) FA6-152, anti-CD36 antibody, and (2) SRI-2, which recognizes both type I and type II SRAs. Immunohistochemical staining for CD36 and SRA was performed using labeled streptavidin method. In macrophages scattered in aortic walls without atherosclerotic lesions, the expression of CD36 was hardly observed, whereas that of SRA was detected weakly but consistently. In contrast, in atherosclerotic lesions, macrophages around the core region showed a weak immunoreactivity to CD36 and a strong immunoreactivity to SRA. Furthermore, lipid-laden macrophages, which mainly existed in the core region, had a strongly positive immunoreactivity to CD36, but a low or moderate level of immunoreactivity to SRA. The distributions of CD36 and SRA were different from each other, and especially foamed, large-sized macrophages in atherosclerotic plaques tended to more abundantly express CD36 protein. These data demonstrate, for the first time, that the expression of both CD36 and SRA might be differentially regulated in aortic walls, and might play different roles in the formation of foam cells in atherosclerotic lesions. (Arterioscler Thromb Vasc Biol. 1999;19:1333-1339.)

Key Words: CD36 ■ atherosclerosis ■ scavenger receptor ■ oxidized LDL ■ macrophage

A number of epidemiological studies have demonstrated that increased levels of LDLs are associated with an increased risk of atherosclerosis. The exact mechanisms by which increased plasma LDL concentrations lead to the development of atherosclerosis have not been clarified. It has recently been shown that modified LDLs, especially oxidized LDL (Ox-LDL), play important roles during the formation of atherosclerotic lesions. LDL must be modified before LDL becomes pathogenic.1,2 Several studies have indicated that Ox-LDL is generated in vitro through oxidation by Cu2+ ion or by incubation of LDL with cells such as endothelial cells, macrophages, and smooth muscle cells.3–7 The latter mechanism was attributed to the generation of oxidants by the cells.8,9 Several studies have shown that Ox-LDL exists in vivo in atherosclerotic arterial walls.10–12 The importance of Ox-LDL in atherogenesis has also been suggested in animal studies by administration of antioxidants such as probucol.13–15

Once LDL particles are oxidized, they are no longer recognized by the LDL receptor. Instead, Ox-LDL is recognized by receptors on macrophages. Scavenger receptor class A type I and type II (SRA), which was originally cloned by Kodama et al,16 has been considered a major receptor for Ox-LDL in vivo. Both acetyl-LDL (Ac-LDL) and Ox-LDL can bind to SRA. The uptake of Ac-LDL was completely blocked by an excessive amount of Ac-LDL in Ac-LDL receptor (SRA) cDNA transfected cells, although the uptake of Ox-LDL was not completely blocked by Ac-LDL in macrophages and isolated Kupffer cells. Therefore, it was suggested that Ox-LDL might also be taken up by other receptors.17,18

CD36 is a glycoprotein with a molecular weight of 88 kDa and is expressed on platelets, monocyte-macrophages, and capillary endothelial cells.19 The physiological function of CD36 was proposed to be a receptor for both thrombospordin and collagen,20–22 to mediate cytoadherence of Plasmodium...
CD36 in Human Aorta

*Plasmodium falciparum*-parasitized erythrocytes, and to be a transporter of long-chain fatty acids. Endemann et al have recently reported that CD36 binds Ox-LDL. However, the pathophysiological role of CD36 in terms of atherogenesis has not been clarified. We have identified CD36-deficient subjects and clarified their gene abnormalities. We have recently demonstrated that in CD36-deficient macrophages, the uptake of Ox-LDL was reduced by approximately 50% compared with that in control macrophages and that cholesteryl ester accumulation was also reduced in CD36-deficient macrophages. These findings suggested that CD36 is involved in the formation of foam cells in atherosclerotic lesions. CD36 belongs to the class B scavenger receptor gene family, in which LIMPII (lysosomal integral membrane glycoprotein II) and CLA-I (CD36 and LIMPII analogous-I) are included. In immunohistochemical analyses, SRA was detected in macrophages of aortic intima, especially in fatty streaks and atherosclerotic plaques, suggesting a significant role of SRA in the process of atherogenesis. However, it has not been clarified whether the tissue distributions of these 2 major receptors, CD36 and SRA, are the same or not. In the present study, we investigated the expression of CD36 and SRA in human aorta. We demonstrated that CD36 is expressed on macrophages in atherosclerotic lesions of aorta and that the distributions of CD36-positive and SRA-positive macrophages are different in human atherosclerotic lesions, suggesting the differential contributions of these receptors to foam cell formation.

**Methods**

**Materials**

Human aortic tissues (mainly descending thoracic aorta) were obtained from 22 autopsied cases. Autopsies were performed within 5 hours after death. Informed consents were obtained from the bereaved families. Table 1 depicts the age, sex, and cause of death of the subjects. Aortic tissues were embedded in Tissue-Tek OCT compound (Miles Inc), frozen in liquid nitrogen, and stored at −80°C until use. Cryostat sections were cut 7 or 8 μm thick and air-dried for immunohistochemical staining and for oil red O staining.

**Antibodies**

For immunohistochemical staining, the following antibodies were used: (1) AF6-152, anti-human CD36 monoclonal antibody (mouse IgG fraction) from Cosmo Bio Co, (2) SRI-2, anti-human SRA antibody (mouse serum fraction), which was a gift from Dr Tatsuhiko Kodama, Tokyo University, and (3) HAM56, anti-macrophage antibody (mouse IgM fraction) from DAKO.

**Immunohistochemical Detection of CD36 and SRA**

Frozen sections were washed in 0.05 mol/L Tris-HCl buffer (pH 7.6), fixed for 10 minutes with acetone, and washed in 0.05 mol/L Tris-HCl buffer for 10 minutes. For detection of CD36, after incubation of the section with 10% normal goat serum for 5 minutes at room temperature, an alkaline phosphatase method was used, with mouse anti-CD36 monoclonal antibodies AF6-152 diluted 1/200 in Tris-HCl buffer containing 1% BSA (30 minutes at room temperature), and streptavidin alkaline phosphatase (10 minutes at room temperature). A positive reaction was visualized by incubation for 5 to 20 minutes at room temperature in peroxidase substrate solution containing DAB (3,3′-diaminobenzidine) (Zymed Laboratories, Inc). As a negative control, normal mouse IgG, (DAKO) and (mouse preimmune serum) were used for CD36 and SRA, respectively.

**Identification of Macrophages**

For identification of macrophages in aortic walls, frozen tissue sections were incubated for 20 minutes at room temperature with 1.5% normal goat serum diluted in PBS containing 1% BSA, followed by an incubation with mouse monoclonal antibodies against human macrophages (HAM56, Biomedia) diluted 1/50 in PBS containing 1% BSA. After washing the section in PBS, the ABC (avidin-biotin complex) method was applied to detect macrophages using biotinylated goat anti-mouse IgM, diluted 1/2000, and VECSTATIN ABC Reagent (Vector Laboratories, Inc). A positive reaction was visualized by incubation for 5 to 20 minutes at room temperature in peroxidase substrate solution containing DAB. As a negative control, normal mouse IgM (DAKO) was used. For detecting lipids accumulated in foam cells, oil red O staining was performed.

**Double Immunohistochemical Staining for CD36 and SRA**

Sections were stained immunohistochemically for SRA using the peroxidase method as described above and washed for 2 hours in 0.1 mol/L glycine hydrochloride buffer (pH 2.2). After further washing in PBS, the sections were incubated for 20 minutes at room temperature with 10% normal mouse serum diluted in PBS containing 1% BSA, followed by an incubation with FITC-labeled AF6-152 diluted 1/100 in PBS containing 1% BSA. The sections were washed in PBS for 30 minutes, mounted with Perma Fluor aqueous mounting medium (Immunon), and observed using a fluorescence microscope with epi-illumination (Olympus). As a negative control, normal mouse anti-SRA immunoglobulins diluted 1/500 in Tris-HCl buffer containing 1% BSA (30 minutes at room temperature), biotinylated anti-mouse immunoglobulins in PBS (10 minutes at room temperature), and streptavidin peroxidase (10 minutes at room temperature). A positive reaction was visualized by incubation for 5 to 20 minutes at room temperature in peroxidase substrate solution containing DAB. As a negative control, normal mouse IgM (DAKO) was used. For detecting lipids accumulated in foam cells, oil red O staining was performed.

<table>
<thead>
<tr>
<th>Clinical Status or Cause of Death</th>
<th>Case No.</th>
<th>Sex</th>
<th>Age (y)</th>
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<tr>
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<td>Male 61 Tongue cancer, diabetes mellitus</td>
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<tr>
<td>Male 71 Liver abscess</td>
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<td>Male 66 Acute myeloblastic leukemia</td>
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<td>Male 47 Glioblastoma</td>
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<tr>
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<tr>
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<td>7</td>
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<td>Male 63 Prostate cancer</td>
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<tr>
<td>Female 76 Acute renal failure, brain infarction</td>
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<tr>
<td>Female 52 Colon cancer</td>
<td>22</td>
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</table>
Some macrophages were observed in these regions, the that were identified as macroscopically normal. Although CD36 in the aortas of adults with atherosclerotic lesions. In the current study we first evaluated the localization of Localization of CD36 and SRA in Human Aorta for the analysis.

The intima of atherosclerotic plaque was arbitrarily divided into 3 lesions from the lumen to the media: cap lesion, transitional lesion, and core lesion in order. We could easily distinguish the fibrous cap from the lipid core. The fibrous cap was named as a cap lesion. The region between cap and core lesions where moderately foamed macrophages were scattered was named a transitional lesion. For semiquantification of the staining intensity, sections that were doubly immunohistochemically stained for CD36 and SRA were used. Each staining intensity of 3 lesions was graded from 0 to V (0 indicates none; I, very weak; II, weak; III, moderate; IV, strong; and V, very strong).

To determine the proportion of SRA- or CD36-positive cells in atherosclerotic plaques, we doubly immunohistochemically stained with HAM56 and CD36, and with HAM56 and SRA, in 2 consecutive sections. Nine nonoverlapping low-power fields (final magnification, ×600) were investigated in each plaque: 3 areas of the cap lesion, 3 areas of the transitional lesion, and 3 areas of the core lesion were examined. The results were expressed as the percentage of the positive immunoreactivity to CD36 or SRA relative to the total macrophages of each field.

Results

Localization of CD36 and SRA in Human Aorta

In the current study we first evaluated the localization of CD36 in the aortas of adults with atherosclerotic lesions. First, we analyzed the regions of aorta without atherosclerosis that were identified as macroscopically normal. Although some macrophages were observed in these regions, the immunoreactivity of CD36 in macrophages was very low or negative in almost all sections from 22 cases examined. Representative microscopic pictures are shown in Figure 1a and 1b.

The expression of SRA in the aorta was next examined using SRI-2 antibodies. SRA was expressed in macrophages from normal aorta without atherosclerosis (Figure 1c). Macrophages detected in the intima of macroscopically normal aorta were oil red O-negative (data not shown). In contrast to these observations, many macrophages in mildly atherosclerotic plaques and in the deep region of atherosclerotic plaques had a strongly positive immunoreactivity to CD36 protein, and these macrophages tended to be large and foamed (Figures 2 and 3). Macrophages were also observed scattering in the surface layer of atherosclerotic plaques or around these plaques (Figure 3b and 3d). These macrophages were, however, small and less foamed, and had little immunoreactivity to CD36 protein (Figure 3c). Concerning the expression of SRA in these atherosclerotic lesions, many macrophages in the areas of atherosclerotic plaques were positive for SRI-2 immunoreactivity.

Differential Localization of CD36 and SRA in Atherosclerotic Plaques

To examine the differential localization of CD36 and SRA more clearly, we performed a double immunohistochemical staining analysis of aortic plaques (Figure 4). A number of CD36-positive cells were restricted mainly to the core of plaques, whereas many SRA-positive cells were localized around the core of plaques. Macrophages with a CD36 immunoreactivity tended to have little immunoreactivity to SRA. Therefore, the cell populations consisting of CD36-positive cells and those consisting of strongly SRA-positive cells were relatively well separated from each other.

We further evaluated the differential distribution of SRA and CD36. Semiquantitative assessment of staining intensity for CD36 and SRA in the intima of atherosclerotic plaques of

Figure 1. Immunohistochemical localization of CD36 (a), macrophages (b), and SRA (c) in sections from an aorta without atherosclerosis (a 48-year-old man, case 1) (a, b, and c, ×70, bar=150 μm). In this section intimal cells had no immunoreactivity to CD36 (a), although a small number of macrophages could be detected in this area of the consecutive section (b). In contrast, intimal cells, presumably macrophages, with positive immunohistochemical staining for SRA protein could be observed (c). I indicates intima; M, media.

Figure 2. Immunohistochemical localization of CD36 (a), macrophages (b), and SRA (c) in sections from a mildly atherosclerotic plaque (a 66-year-old man, case 4) (a, b, and c, ×170, bar=60 μm). Many macrophages could be detected in the intima. CD36 protein was mainly localized in macrophages, which tended to be foamed. The macrophages in this area weakly expressed SRA protein. Abbreviations as in Figure 1.
human aorta is shown in Figure 5A. CD36 was strongest in the staining intensity in the core lesion, and gradually became less intense closer to the lumen. In contrast, SRA became more strongly intense closer to the lumen and was strongest in the cap lesion. The proportion of SRA- or CD36-positive cells in atherosclerotic plaques is shown in Figure 5B. As with staining intensity, CD36-positive macrophages were most frequently observed in the core lesion, and less frequently observed closer to the lumen. On the other hand, SRA-positive macrophages were most frequently recognized in the cap lesion and less frequently in the core lesion.

**Distribution of CD36-Positive Cells and Oil Red O-Positive Cells**

Next, we analyzed and compared the distribution patterns of lipid-laden foam cells stained with oil red O and those of SRA-positive and CD36-positive cells in consecutive sections of atherosclerotic lesions to examine whether CD36 expression is changed in the process of the formation of foam cells. As shown in Figure 6, the distribution of oil red O-positive area was similar to that of CD36 but had less relation to that of SRA. These immunohistochemical analyses demonstrate that the expression of CD36 may be induced in response to the accumulation of cholesteryl ester in macrophages through taking up Ox-LDL.

**Discussion**

In the development of atherosclerosis, macrophages play an important role in foam cell formation by taking up modified LDLs. Ox-LDL is one of the most plausible candidates among many suggested modified LDLs. In the response-to-injury theory by Ross, after mechanical or functional damage of vascular endothelium, expression of adhesion molecules is upregulated in the endothelium, resulting in adherence of monocytes and T cells to the endothelium and infiltration of these cells into the intima. Subsequently, monocytes differentiate to macrophages, and these macrophages take up Ox-LDL to form foam cells. Although the role of SRA in foam cell formation is well established, we have recently clarified that CD36 also plays an important role in the pathogenesis of atherosclerosis by taking up Ox-LDL. There have been several histochemical analyses on the distribution of SRA and CD36 in atherosclerotic lesions in humans, but the differential distribution of these receptors has not been clarified. In the present study, we investigated the localization, in the aorta, of both CD36 and SRA, which are 2 major scavenger receptors in humans. We found, for the first time, that these 2 receptors are differentially expressed in the atherosclerotic lesions of aorta.

First, macrophages, which were loaded with no or a small amount of lipid droplets in the aortic intima without atherosclerosis or around the core region of plaques, exhibited very weak immunoreactivity to CD36. In contrast, foam cells, which existed in the core region of plaques or mildly atherosclerotic lesions (fatty streaks), had strong immunoreactivity to CD36. In the presence of altered homeostasis, such as atherosclerosis, CD36 expression in macrophages may further be accelerated in response to a variety of inductive signals. One such inductive signal might be Ox-LDL itself. In our data, the expression of CD36 in human monocyte-derived macrophages was upregulated by Ox-LDL in vitro. Therefore, the current immunohistological data are consistent with the results of in vitro experiments. Although CD36 can be detected in peripheral monocytes, its expression in macrophages may further be accelerated in response to a variety of inductive signals. One such inductive signal might be Ox-LDL itself. In our data, the expression of CD36 in human monocyte-derived macrophages was upregulated by Ox-LDL in vitro.
directly correlate with our current study. In this in vitro model, monocytes-macrophages have not been exposed to any suppressive factors such as cytokines. According to our observation, interferon-\(\gamma\) suppressed CD36 expression in human monocyte-derived macrophages.\(^4,5\) We speculated that after the extravasation of macrophages into the aortic wall, the expression of CD36 in tissue macrophages was concomitantly suppressed.

Concerning SRA, its expression was weakly positive in the aortic intima without atherosclerotic lesions and strongly positive around the core of atherosclerotic lesions. Matsumoto et al.\(^3,3\) reported that SRA was distributed in the lipid-rich atherosclerotic lesions but not in the intima without atherosclerosis. Our observations on the expression of SRA may be partly similar to those reported by Matsumoto et al.\(^3,3\) However, these authors did not clearly mention the immunoreactivity of SRA in the core lesion nor the relationship between the extent of cholesterol accumulation and the expression of SRA. In the current study, we further demonstrated that the degree of immunoreactivity to SRA in foamed macrophages in the core lesion was relatively low compared with that for CD36. Although the sensitivities of both antibodies to detect CD36 and SRA by immunohistochemistry might be different, and undetectable levels of both proteins would be produced in macrophages with no immunoreactivity, it is apparent that these cells produce distinctly different amounts of these scavenger receptor proteins in the different portions of the aortic intima. The present results thus might suggest that the function of CD36 differs from that of SRA in the process of atherogenesis.

The expression of CD36 was also observed in foamed macrophages in fatty streaks in addition to the core region of atherosclerotic plaques. From these observations we suggest that the expression of CD36 in foam cells is not indicative of terminal atherosclerosis, but may be representative of active lipid accumulation. Wal et al.\(^4,1\) reported that OKM5-positive macrophages were localized only in the deep layer of atheromatous plaques, and suggested that this might result from the activation of macrophages by some inflammatory processes in atherosclerotic plaques. Our observations might correspond to their findings.

As mentioned above, our most striking finding in the present study is that the major distributions of SRA and CD36 can be relatively well discriminated in atherosclerotic lesions.
The distribution of lipid-laden macrophages seemed to be consistent with that of CD36-positive macrophages, rather than that of SRA-positive macrophages. These data suggest that CD36 may play a role in taking up Ox-LDL and in transforming macrophages to foam cells, especially in the late phase of plaque formation. One of the physiological roles of scavenger receptor has been thought to be removal of foreign materials or modified proteins. Because SRA was observed in normal aortic walls or around the atherosclerotic plaques, SRA might play such roles from an early phase of atherosclerosis. The expression of CD36 might be upregulated when the lipid content of atherosclerotic lesions increases. Our findings may also suggest that in CD36 deficiency, foam cells may be formed less severely than in control subjects, resulting in a reduced severity of atherosclerosis in CD36-deficient subjects. However, it may be necessary to follow up these CD36-deficient patients to properly evaluate the presence or absence of atherosclerosis. Taken together, the current study has demonstrated the differential contributions of CD36 and SRA in the development of atherosclerosis. The establishment of CD36 transgenic animal models would give us more insights into the physiological roles of CD36 in vivo.

Acknowledgments

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References


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