Scavenger Receptors for Oxidized Lipoprotein in Age-Related Macular Degeneration

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PURPOSE. The accumulation of macrophages is known to be involved in the pathogenesis of age-related macular degeneration (AMD), but the reasons why macrophages accumulate in AMD lesions have not been determined. Because the histopathology of AMD has some factors common with those of atherosclerosis, the authors hypothesized that macrophages accumulate to take up oxidized lipoproteins in the eyes of patients with AMD, as has been demonstrated in atherosclerosis.

METHODS. Immunohistochemistry was performed on 10 surgically excised choroidal neovascular (CNV) membranes from eyes with AMD. An antibody against oxidized lipoprotein and antibodies against the scavenger receptors SR-PSOX and LOX-1 were used. Antibodies against cytokerin, CD68, and von Willebrand factor were used to identify retinal pigment epithelium (RPE), macrophages, and vascular endothelial cells, respectively. RT-PCR was performed to detect the mRNAs of the scavenger receptors in the CNV membranes.

RESULTS. Oxidized lipoproteins were immunohistochemically detected in the CNV membranes. Intense immunostaining was observed at the surface of the CNV membranes with the SR-PSOX antibody, whereas LOX-1 immunostaining was weak. Cells expressing scavenger receptors were found to be predominantly macrophages with a minority of RPE. Both SR-PSOX and LOX-1 mRNAs were detected in CNV membranes.

CONCLUSIONS. Oxidized lipoproteins are present in AMD lesions. Macrophages and RPE in the CNV membranes express cell surface scavenger receptors for oxidized lipoproteins. These findings suggest that macrophages may accumulate to take up oxidized lipoproteins in AMD and that the control of oxidative stress and macrophage responses may therefore be potential treatments for AMD. (Invest Ophthalmol Vis Sci. 2007;48: 1801–1807) DOI:10.1167/iovs.06-0699

Age-related macular degeneration (AMD) is a leading cause of legal blindness in the elderly in the United States1 and Europe and is rapidly increasing in Asia.2 Several types of treatments, including photodynamic therapy,3 anti-VEGF therapy,4 and macular translocation surgery,5,6 have been recently developed to treat AMD. However, the pathogenesis of AMD has not been fully understood,7,8 which results in limited options for current therapies.

AMD is classified into two types: the dry type and the wet type. The wet type of AMD, which is characterized by formation of choroidal neovascular (CNV) membranes, affects 90% of patients with severe visual loss due to AMD. Earlier studies have demonstrated that macrophages accumulate in the tissues of eyes with the wet-type AMD, especially in the CNV membranes.9–12 They may play an important role by secreting cytokines and enzymes that induce and enhance neovascularization.13 In keeping with this idea, the depletion of macrophages reduces CNV formation in an animal model.14,15 Despite the suggestion that macrophages may play a crucial role in neovascular membrane formation in eyes with AMD, the reason that they accumulate in the AMD lesion remains unknown.

In a histopathological study, Killingsworth et al.16 observed that macrophages and phospholipid-containing debris were colocalized in Bruch’s membrane in eyes with AMD. Curcio et al.17 also demonstrated an age-related accumulation of cholesterol esters in Bruch’s membrane similar to that observed in the arterial intima. These and other studies18,19 suggest that the histopathology of AMD has some factors common with those of atherosclerosis.

The cellular uptake of oxidized low-density lipoprotein by macrophages and vascular endothelial cells plays a crucial role in the pathogenesis of atherosclerosis.20 Because the pathologic changes in AMD are similar to those in atherosclerosis16,19 and atherosclerosis may contribute to the pathogenesis of AMD,21,22 we hypothesized that macrophages accumulate to take up oxidized lipoproteins in the macular area of eyes with AMD as in the arterial intima in cases with atherosclerosis. To test this hypothesis, we investigated whether oxidized lipoproteins were present in the AMD lesions and whether oxidized lipoprotein-specific cell-surface receptors were expressed in AMD lesions. We also determined what types of cells express these scavenger receptors.

MATERIALS AND METHODS

Collection and Preparation of CNV Membranes

As a treatment for AMD, choroidal neovascular membranes were surgically excised from 13 eyes of 13 patients with AMD, ages 57 to 80 years (average, 73 years), as described previously.23 After an explanation

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of the purpose of the study, an informed consent was obtained from each patient to collect and study the excised tissues. The procedures used to collect and prepare the tissues conformed to the tenets of the Declaration of Helsinki.

Ten of the specimens were used for immunohistochemistry and three for mRNA extraction. For immunohistochemistry, the surgically excised CNV membranes were placed in balanced saline solution in the operating room, kept at 4°C, and embedded in optimum cutting temperature (OCT) compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) within 2 hours after excision. Cryosections, 8 μm thick, were made for immunohistochemistry. For RT-PCR analysis, the CNV membranes were placed in liquid nitrogen in the operating room immediately after surgical excision and kept at −80°C until RNA extraction.

**Immunohistochemistry for Oxidized Lipoproteins**

Indirect immunohistochemistry was performed on cryosections of surgically excised membranes with a monoclonal antibody (IgM) against oxidized lipoproteins, FOH1a/DLH3. The antibody was generated by immunizing a mouse against homogenates of human atheroma. It has been found to recognize oxidized phosphatidylcholine as an epitope and has been used for detecting oxidized lipoproteins in atherosclerotic lesions.24 The avidin-biotin complex immunoperoxidase technique (Vector Laboratories Inc., Burlingame, CA) was used. In brief, after the sections were fixed in cold 4% formaldehyde, the endogenous peroxidase activity was blocked by NaIO₄. The specimens were incubated with 0.3% BSA-PBS to block nonspecific immunoreaction and then with DLH3 at a dilution of 1:100, followed by incubation with a biotinylated horse anti-mouse IgM antibody. The sections were then incubated with streptavidin-biotin complex labeled with peroxidase. The immunoreactivity was made visible with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc.).

For further characterization of the positive cells, double staining was performed with DLH3 and anti-CD68 (Zymed, South San Francisco, CA) to detect the distribution of macrophages. After DLH3 staining, the specimens were incubated with anti-CD68 monoclonal antibody at a dilution of 1:100, followed by incubation with a biotinylated horse anti-mouse IgM antibody. The sections were then incubated with streptavidin-biotin complex labeled with peroxidase. The immunoreactivity was made visible with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc.). Sections incubated with nonimmune mouse IgM as a primary antibody served as negative controls.

For immunohistochemistry, sections were incubated with anti-CD68 monoclonal antibody at a dilution of 1:100, followed by incubation with a biotinylated horse anti-mouse IgM antibody. The sections were then incubated with streptavidin-biotin complex labeled with peroxidase. The immunoreactivity was made visible with 3-amino-9-ethylcarbazole (AEC; Vector Laboratories, Inc.).

**Immunohistochemistry for Scavenger Receptors**

To identify scavenger receptors and cells expressing these receptors in CNV membranes, double staining was performed with an anti-scavenger receptor antibody and a cell marker antibody. After fixation in cold 4% formaldehyde, the sections were incubated with the primary antibody mixtures including one of the anti-scavenger receptor antibodies and one of the cell markers. Antibodies against scavenger receptors included anti-LOX-1 (lectin-like Ox-LDL receptor-1) monoclonal antibody25 (1:50 dilution) and anti-SR-PSOX (scavenger receptor that binds phosphatidylserine and oxidized lipoprotein) polyclonal antibody26 (1:200 dilution). Cell marker monoclonal antibodies included CD68 (1:400 dilution; Zymed), pan cytokeratin (1:500 dilution; Sigma-Aldrich, St. Louis, MO), and von Willebrand factor (vWF; 1:1000 dilution; Dako Co., Glostrup, Denmark). In cases in which the primary antibody mixture contained both monoclonal antibodies (e.g., a mixture of anti-LOX-1 antibody and a cell marker), the primary antibodies were mixed with a labeling kit (Zenon; Invitrogen-Molecular Probes, Inc, Eugene, OR), according to the manufacturer’s instruction. The specimens were then incubated with the mixture for 1 hour at room temperature and then incubated with secondary antibodies, Alexa Fluor 488–conjugated anti-mouse IgG antibody and Alexa Fluor 546–conjugated anti-rabbit IgG antibody (Invitrogen-Molecular Probes), for 1 hour at room temperature. The specimens were examined with a confocal microscope. For control sections, a mixture of nonimmunized mouse IgG and rabbit IgG was applied as the primary antibody.

The intensity of the immunologic reaction was graded semiquantitatively according to a previous report.26 The grades for the degree of staining were: none (−), mild (+, up to one third of cells stained), moderate (++, one third to two thirds of cells stained), or heavy (+++, two thirds to all cells stained). The degree of staining of the scavenger receptors was determined by comparing the relative number of positively stained cells to all cells in the section. For each cell marker, the degree of staining was determined by comparing the relative number of double-stained cells that appeared yellow to all cells positively stained with each scavenger receptor antibody.

**Figure 1.** Detection of oxidized lipoproteins in choroidal neovascular (CNV) membranes. Photomicrographs of sections from CNV membrane that was incubated with a monoclonal antibody against oxidized lipoprotein (DLH3) (A) and nonimmune mouse IgM as a negative control (B). Immunostaining of oxidized lipoprotein (red) exists in and around the region of autofluorescent pigment granules in CNV membrane (arrows). Original magnification, ×20.

**Figure 2.** Double staining of oxidized lipoproteins and CD68 in CNV membranes. Immunoreactions with the anti-oxidized lipoprotein antibody (red) are colocalized with those with anti-CD68 antibody (blue: arrowbeads) as well as pigment granules (arrows). These results suggest that oxidized lipoproteins are present in macrophages and RPE cells. Bar, 10 μm. Original magnification, ×630.
Reverse Transcription–Polymerase Chain Reaction

Total cellular RNA was extracted from three CNV membranes (RNaseq Mini Kit; Qiagen, Valencia, CA), and the extracted RNA was reverse transcribed with random primers (Toyobo, Osaka, Japan). The transcribed cDNA was used for polymerase chain reaction amplification with specific primers for LOX-1, SR-PSOX, and GAPDH. The two specific primers used to amplify LOX-1 were 5'-TGCTGTCAGACACG-GCAAAGAGCA-3', and 5'-GGGATCCCGTGTCGTTTGAACG-3'; for SR-PSOX 5'-ACTCAGCCAGCAATGAGCAAC-3', and 5'-GGTATTAGGTCAAGTGCACAC-3'; and for GAPDH 5'-GGTGAAGGTCCGT-GAAGC-3' and 5'-CAAAGTTGCATTGATGACCC-3'. PCR amplification was performed by 35 cycles of denaturation, annealing, and elongation with polymerase (TagDNA; Toyobo). For positive control of each scavenger receptor, total cellular RNA was isolated from a cultured human monocyte cell line, THP-1.25

RESULTS

Oxidized Lipoproteins in CNV Membranes

Surgically excised CNV membranes were immunopositive to a monoclonal antibody against oxidized lipoprotein (Fig. 1). Although the surgically excised neovascular tissues contained no retinal components except for RPE cells and did not show the normal tissue structure, immunoreactivity to oxidized-lipoprotein was present mainly in and around the area of the autofluorescent pigment granules. Double staining revealed that immunostaining of oxidized lipoproteins colocalized with the CD68-positive cells as well as cells with pigment granules (Fig. 2), which suggested that oxidized lipoproteins are present on macrophages and RPE cells.

Scavenger Receptors for Oxidized Lipoproteins

SR-PSOX was detected in all CNV membranes. The peripheral regions of the CNV membranes were strongly immunopositive and the membrane stroma mildly immunopositive for SR-PSOX. The distribution of LOX-1 was similar to that of SR-PSOX, but the immunostaining was less intense and was observed in only 6 of 10 samples (Table 1). These results suggest that SR-PSOX was more prominent than LOX-1 in CNV membranes of eyes with AMD.

Cells Expressing Scavenger Receptors for Oxidized Lipoproteins

To identify the cells expressing the scavenger receptors in the CNV membranes, we performed double staining with the cell-marker antibodies CD68, pan cytokeratin, and vWF factor, which stain macrophages, RPE cells, and vascular endothelial cells, respectively.

Merging the double-stained images composed of a section stained with an anti-scavenger receptor antibody and anti-CD68 antibody (Fig. 3) demonstrated that almost all the CD68-positive cells (i.e., macrophages) were SR-PSOX positive, and 60% to 70% of these cells were also LOX-1 positive. In contrast, most SR-PSOX-positive cells and many LOX-1-positive cells were identical with CD68-positive cells (Table 1). These results indicate that most macrophages expressed both scavenger receptors, but predominantly SR-PSOX. Cells expressing these scavenger receptors were mainly macrophages, but CNV membranes had a minor population of LOX-1-positive cells other than macrophages.

In the merged images composed of sections stained with an antiscavenger receptor antibody and anti-pan cytokeratin antibody (Fig. 4), almost all pan-cytokeratin-positive cells (i.e., RPE cells) were SR-PSOX negative, and most were LOX-1 positive. In contrast, most SR-PSOX-positive cells were pan cytokeratin negative, and many LOX-1-positive cells were identical with pan cytokeratin-positive cells (Table 1). These results indicate that almost all RPE cells expressed only LOX-1. Cells expressing SR-PSOX were not the RPE cells, and there were cells other than RPE cells that expressed LOX-1.

Images composed of sections stained with an anti-scavenger receptor antibody and anti-vWF factor antibody demonstrated that almost all the vWF-positive cells (i.e., vascular endothelial cells), were not SR-PSOX-positive, that some of those cells were LOX-1 positive, and that most of the SR-PSOX-positive or LOX-1-positive cells were not vWF-positive (Fig. 5; Table 1). These results indicate that only a limited number of vascular endothelial cells expressed only LOX-1.

Summarizing the double-staining results, cells expressing SR-PSOX were almost exclusively macrophages, and cells expressing LOX-1 were macrophages and RPE cells, with a minority of vascular endothelial cells. Macrophages are the main cell type expressing scavenger receptors for oxidized lipoproteins.

mRNAs of Scavenger Receptors for Oxidized Lipoproteins Expressed in CNV Membrane

To determine whether the mRNAs of scavenger receptors are expressed in human CNV membranes, RT-PCR analyses were performed on total cellular RNA extracted from three surgically excised CNV membranes. The mRNAs of both SR-PSOX and LOX-1 were detected in human CNV membranes (Fig. 6).

The degree of staining of the scavenger receptors was determined by counting the relative number of positively stained cells among all cells in the section. For each cell marker, the degree of staining was determined by counting the relative number of double-stained cells that appeared yellow among all cells positively stained with each scavenger receptor antibody.

### Table 1. Immunohistochemical Staining for Scavenger Receptors and Rate of Cell Markers in CNV Associated with AMD

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**FIGURE 3.** Localization of scavenger receptors and macrophages. Images composed of a section stained with anti-SR-PSOX polyclonal antibody (red) and another with anti-CD68 monoclonal antibody (green), and a merged image (A, B) demonstrates that almost all CD68-positive cells were SR-PSOX positive. Serial sections of the CNV membrane were stained with anti-LOX-1 monoclonal antibody (red) and anti-CD68 monoclonal antibody (green), and a merged image (C, D) demonstrates that 60% to 70% of the CD68-positive cells were LOX-1 positive. Most, but not all SR-PSOX- or LOX-1-positive cells were identical with CD68-positive cells. Magnification: (A) ×7; (B, D) ×126; (C) ×14.

**FIGURE 4.** Localization of scavenger receptors and retinal pigment epithelium. Images composed of a section stained with anti-SR-PSOX polyclonal antibody (red) and anti-pan cytokeratin monoclonal antibody (green) and a merged image shows that almost all pan cytokeratin-positive cells were SR-PSOX negative. Serial sections of the CNV membrane stained with anti-LOX-1 monoclonal antibody (red) and anti-pan cytokeratin monoclonal antibody (green) and a merged image shows that almost all pan cytokeratin-positive cells were LOX-1 positive. Not all LOX-1-positive cells are identical with pan cytokeratin-positive cells. Magnification: (A, C) ×7; (B) ×20; (D) ×40.
Discussion

Because the histopathological changes in AMD are similar to those seen in atherosclerosis,9–12,16–19 we suspected that scavenger receptors for oxidized lipoproteins might be present in AMD lesions. Our results demonstrated that oxidized lipoproteins and the scavenger receptors for oxidized lipoproteins are colocalized in the CNV membranes of AMD eyes. We had already found that oxidized lipoproteins in the macula increase with age in normal eyes and in AMD eyes, compared with age-matched normal eyes.27 These findings support our hypothesis that pathogenesis of AMD has some similarities with the pathologic mechanisms of atherosclerosis in which macrophages accumulate to ingest the oxidized low-density lipoprotein by scavenger receptors specific for oxidized lipoproteins at the early stage.

Oxidized lipoproteins were observed in and around the area of the autofluorescent pigment granules and colocalized with RPE cells and macrophages. This finding suggests that oxidized lipoproteins may accumulate in RPE cells and Bruch’s membrane, which is consistent with the accumulation of cholesterol esters or phospholipid-containing debris in Bruch’s membrane.17,18 In addition, considering that the antioxidant lipoprotein antibody DLH3 detects foam cells, which take up Ox-LDL in early atherosclerotic lesions,24 the positive staining may in part indicate oxidized lipoprotein-laden cells, which are possibly RPE cells and macrophages.

Although previous studies demonstrated that macrophages accumulate in AMD lesions,9–12,16 it is not known why macrophages accumulate in these areas. van der Schaft et al.28 reported that immune reactions do not appear to be involved in attracting the macrophages in AMD, because distinct immune complexes have not been found in the basal laminar deposits or in drusen. In this study, we found accumulation of oxidized lipoproteins and macrophages that possessed scavenger receptors. Oxidized lipoproteins have been shown to cause inflammatory reactions resulting in the accumulation of macrophages29–31 in many studies on atherosclerosis, and the
histopathology of atherosclerosis is similar to that of AMD. Chang et al. reported that oxidized phosphatidylcholine binds to C-reactive protein, an opsonic molecule activating the classic complement pathway and Fc-$\gamma$ receptor endocytosis of macrophages.\textsuperscript{29,30} Complements have also been shown in atherosclerotic plaques together with oxidized lipoprotein, C-reactive proteins, and macrophages.\textsuperscript{31} In recent genetic investigations, Hageman et al.\textsuperscript{32} reported that a variation in the factor H gene increases the likelihood of development of AMD, and three other studies have demonstrated a linkage of the same gene to AMD.\textsuperscript{33–35} These findings suggest that complement–H gene increases the likelihood of development of AMD, and may reduce the risk of development of AMD.\textsuperscript{37} Taken together at the molecular level. Our findings support the suggestion that macrophages to AMD. Additional studies are needed to evaluate any evidence of a relationship between the LOX-1-positive RPE macrophages may function predominantly. We do not have almost exclusively macrophages, and cells expressing LOX-1 were macrophages and RPE cells, with a minority of vascular endothelial cells. A specificity of scavenger receptors may also exist in AMD lesions, which means that SR-PSOX expressed on macrophages may function predominantly. We do not have any evidence of a relationship between the LOX-1-positive RPE and SR-PSOX-positive macrophages.

In conclusion, our results provide new and significant information on the close link between oxidized lipoproteins and macrophages to AMD. Additional studies are needed to evaluate these changes more quantitatively and to clarify the results at the molecular level. Our findings support the suggestion that supplementation with antioxidants, vitamins, and minerals may reduce the risk of development of AMD.\textsuperscript{37} Taken together with the results of recent studies demonstrating that macrophage depletion reduced CNV formation in an animal model,\textsuperscript{14,15} our findings suggest that suppressing macrophage accumulation by controlling the macrophage responses to oxidative lipoproteins or suppressing phospholipid oxidation may be treatments for AMD.

References


