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Accumulation of LOX-1 Ligand in Plasma and Atherosclerotic Lesions of Watanabe Heritable Hyperlipidemic Rabbits: Identification by a Novel Enzyme Immunoassay

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LOX-1 (lectin-like oxidized LDL receptor) is a newly identified cell surface receptor for oxidized LDL mainly expressed in endothelial cells. Recombinant soluble LOX-1(LOX-Fc) was generated by fusing the extracellular domain of LOX-1 with the Fc portion of IgG. A novel sandwich enzyme immunoassay specific for LOX-1 ligand is designed, using LOX-Fc and anti-apoB antibody. This immunoassay was used to determine LOX-1 ligand activity in normal and Watanabe heritable hyperlipidemic (WHHL) rabbit plasma. LOX-Fc was further applied for staining of atherosclerotic lesions of WHHL rabbits. LOX-1 ligand levels were significantly elevated in the plasma of hyperlipidemic rabbits compared with controls. Furthermore, LOX-1 ligand activity was detected in the atherosclerotic lesions *in situ*. These results support the potential roles of LOX-1 interacting with its ligand in the pathogenesis of atherosclerosis, which is enhanced in hyperlipidemia. © 2001 Academic Press

Key Words: **lectin-like oxidized LDL receptor-1 (LOX-1); LOX-1 ligand; Watanabe heritable hyperlipidemic rabbit; atherosclerosis.**

An increasing body of evidence has demonstrated that oxidized low-density lipoprotein (OxLDL) plays a critical role in atherogenesis (1, 2). In response to the stimulation of OxLDL, endothelial cells reduce the release of nitric oxide, express adhesion molecules, and secret chemoattractant and growth factors (3–5). Subsequently, OxLDL was avidly ingested by macrophages, resulting in foam cell formation. OxLDL was also involved in inducing smooth muscle cells migration, proliferation, and transformation (6).

We have initially identified a lectin-like oxidized low density lipoprotein (LOX-1) from aortic endothelial cells which is distinct from the classical scavenger receptor in macrophages. LOX-1 is characterized as a cell surface receptor that structurally belongs to the C-type lectin family and is highly expressed in vascular endothelium and in vascular-rich organs. Recent studies have demonstrated that LOX-1 is expressed by an inducible manner not only in vascular endothelial cells, but also in monocyte-derived macrophages and smooth muscle cells (7, 8). The expression of LOX-1 is induced by many proinflammatory cytokines and oxidized LDL (7, 9). *In vivo,* LOX-1 exhibited an enhanced expression in many proatherogenic circumstances such as hypertension and hyperlipidemia, and indeed accumulated in atherosclerotic lesions (10, 11).

The aim of the present study is to further analyze the role of LOX-1 in the pathogenesis of atherosclerosis, especially to investigate its ligand. Applying the novel endothelial receptor for oxidized LDL, we designed a novel method to detect the modified LDL in the circulation via the mechanisms of specific binding to LOX-1. We demonstrate the LOX-1 ligand was accumulated in the hyperlipidemic circulation, and present *in situ* in the atherosclerotic lesions of WHHL rabbits.

MATERIALS AND METHODS

Preparation of lipoproteins. LDL (d = 1.019–1.063) of human and Japanese white rabbits were isolated by sequential ultracentrifugation from plasma, as described previously (12). For the preparation of OxLDL for the standard of enzyme immunoassay, LDL was oxidatively modified at the concentration of 3 mg protein/ml in PBS by the exposure to 7.5 μM $CuSO_4$ for 16 h at 37°C (12). Oxidation was

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evaluated based on the amount of thiobarbituric acid-reactive substances (TBARS) and by the relative electrophoretic mobility (REM) in agarose gel compared with native LDL. TBARS values for OxLDL of human and rabbit were 10.7 nmol/mg protein and 5.8 nmol/mg protein, respectively. REM values for OxLDL from human and rabbit were 3.25 and 2.25, respectively.

Lipoprotein with different degree of oxidation was prepared at the concentration of 200 μg protein/ml by the exposure to 5 μM $CuSO_4$ for 1, 3, 6, 12, and 24 h at 37°C. The degree of modification was monitored by the measurement of TBARS, agarose gel electrophoresis to see the increase of negative charge, and SDS–polyacrylamide electrophoresis to see degradation of apoB.

Malondialdehyde-LDL (MDA-LDL) and acetyl LDL were prepared as described previously (13, 14).

Preparation of LOX-Fc. LOX-Fc chimeric protein of the extracellular domain of LOX-1 and the Fc portion of human IgG was prepared as described previously (15).

Agarose-gel electrophoresis and Western blot analyses. Two μg of lipoprotein was electrophoretically separated in 0.45% agarose at 40 V for 3 h in 50 mM barbiturate buffer (pH 8.6). Then, the lipoproteins were transferred to a nylon membrane (Immobilon, Millipore) by electrophoresis also in 50 mM barbiturate buffer (pH 8.6) at 140 mA for 1 h. Lipids on the membrane were visualized by incubating with 0.03%(w/v) Fat Red 7B (Nacalai tesque, Kyoto, Japan) dissolved in 80%(v/v) methanol. For detection of apoB, the membranes were incubated overnight at 4°C with Block Ace solution (Snow Brand, Japan) for blocking. Then, the membrane was incubated for 1 h at room temperature with peroxidase-conjugated purified sheep anti-human apoB polyclonal antibody (The Binding Site, Birmingham, UK) diluted 1000-times with phosphate-buffered saline (PBS) supplemented with 0.1%(w/v) bovine serum albumin (BSA) and 1%(v/v) normal goat serum. After three washes with PBS, the membranes were visualized by a peroxidase reaction with Konica Immunostain kit (Konica, Japan).

For the detection of LOX-1 ligand activity, the membranes were incubated overnight at 4°C with Block Ace solution for blocking, and then with 5 μg/ml LOX-Fc dissolved in PBS containing 20%(v/v) newborn calf serum (NBCS, Gibco) for 1h at room temperature. After three washes with PBS, the membranes were incubated for 1 h at room temperature with biotinylated goat antibody against the Fc portion of human IgG (Vector) diluted 200-times with PBS, 0.1%(w/v) BSA, and 1%(v/v) normal goat serum. After three washes with PBS, the membrane was visualized by peroxidase reaction with Vectastain Elite kit (Vector) and Konica Immunostain kit.

Enzyme immunoassay (EIA) for LOX-1 ligand activity. LOX-Fc or human IgG (negative control) (0.5 μg/well) was immobilized on 96-well plates (Maxisorp, Nunc) by incubating overnight at 4°C in 0.1 ml of PBS. After two washes with PBS, the plates were blocked with 0.32 ml of 25%(v/v) Block Ace at room temperature overnight. After three more washes with PBS, the plates were incubated overnight at 4°C with 0.1 ml of the standard OxLDL or the plasma diluted 100-times with PBS containing 0.5%(v/v) NBCS and 2%(w/v) BSA. Then, the plates were washed 3 times with PBS, and incubated for 2 h at room temperature with the peroxidase-conjugated sheep anti-human apoB polyclonal antibody diluted 1000 times with PBS containing 1%(w/v) BSA. After six washes with PBS, peroxidase reaction was initiated by incubating the plates for 20 min at room temperature with 0.1 ml of 0.1 M sodium citrate (pH 5.5), 0.1 mg/ml *o*-phenylenediamine (Sigma), and 0.02%(w/v) hydrogen peroxide. The reaction was terminated with 0.1 ml of 2 M sulfuric acid. The peroxidase activity was determined by the measurement of absorbance at 490 nm. The specific binding to LOX-Fc was determined by the subtraction of the peroxidase activity obtained with human IgG from that with LOX-Fc.

Enzyme-linked immunosolvent assay (ELISA) for human apo B. The lipoproteins were immobilized on 96-well plates (Maxisorp, Nunc) by incubating overnight at 4°C in 0.1 ml of PBS. After three washes with PBS, the plates were incubated with 0.32 ml of 25%(v/v) Block Ace (Snow Brand, Japan) at room temperature for at least 6 h. After three more washes with PBS, the plates were incubated for 2 h at room temperature with the peroxidase-conjugated sheep anti-human apoB polyclonal antibody diluted 1000 times with PBS containing 1%(w/v) BSA. The plates were washed again with PBS, six times, and the peroxidase activity bound to the plates was determined as above.

Plasma samples. Blood samples collected from WHHL and Japanese White rabbits (12 weeks old, 5 males and 5 females) were heparinized (10 U/ml) and centrifuged at 3000 rpm for 15 min at 4°C. Immediately the plasma samples were measured by EIA.

Immunohistochemical staining with LOX-Fc. Thoracic aortas were excised from 12-week-old WHHL rabbits and Japanese White rabbits which were euthanized by overdose of sodium pentobarbital (50 mg/kg, iv) according to an experimental protocol approved by the animal research committee at National Cardiovascular Center Research Institute. They were immediately submerged in PBS containing 20 μM butylated hydroxytoluene (Sigma) and 100 μM EDTA to be protected from oxidation. The aortas were snap-frozen on dry ice and stored at −80°C until use. These samples were sectioned serially at 6 μm thickness. Every first and second section was stained with hematoxylin and eosin (H-E) and Oil red O, respectively; the other sections were used for immunohistochemical staining. The cellular components were analyzed by use of monoclonal antibodies against smooth muscle cells (1A4, Dako A/S), and macrophages (RAM11, Dako A/S). LOX-Fc was biotinylated with EZ-Link Sulfo-NHS-LC-Biotinylation Kit (Pierce). Sections were incubated with 1A4, RAM11, anti-human apoB antibody, and biotinylated LOX-Fc, respectively, for 1 h at room temperature. The sections were subjected to a two or three step staining procedure with the use of streptavidin-biotin complex with horseradish peroxidase for color detection. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole, and the sections were counterstained faintly with hematoxylin. Moreover, to identify cell types that stain positive for LOX-Fc, immunodouble staining with biotinylated LOX-Fc and RAM11 was also performed, according to procedures previously reported (16). The specificity and results obtained with biotinylated LOX-Fc were checked by use of a biotinylated nonimmune human IgG-Fc as negative control.

RESULTS

Specificity for the Recognition by LOX-Fc Fusion Protein

LOX-1 recognizes oxidized LDL but not native LDL(12). To utilize this property of LOX-1, we prepared recombinant soluble LOX-1, which consists of the extracellular domain of LOX-1 and IgG-Fc (LOX-Fc). First, we examined whether LOX-Fc retained this ligand specificity after modification to the soluble form. LDL and OxLDL, prepared by standard procedures, were separated by agarose-gel electrophoresis and transferred to a nylon membrane. Both native LDL and OxLDL were clearly detected by Fat Red 7B and Anti-apoB polyclonal antibody, while LOX-Fc only recognized OxLDL, but not native LDL (Fig. 1). These results suggest that the recombinant LOX-Fc keeps the ligand binding activity of LOX-1 that specifically recognizes OxLDL.

Enzyme Immunoassay System for LOX-1 Ligand

By the use of LOX-Fc, we developed an enzyme immunoassay to quantify LOX-1 ligand activity. Two binding sites within modified LDL were included for the recognition by this system, one was detected by anti-human apoB polyclonal antibody, and the other was by LOX-Fc. Then, we characterized the specificity

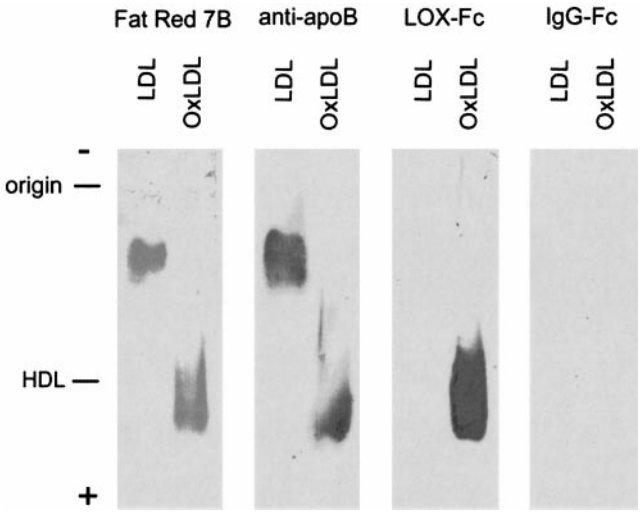

FIG. 1. Western blot analysis of human LDL with or without oxidation by copper ions. LDL(3 mg protein/ml) was oxidized with 7.5 μM $CuSO_4$ for 16 h at 37°C, separated in a 0.45% agarose gel, and transferred to a nylon membrane. The membrane was visualized with Fat Red 7B, anti-apoB antibody, LOX-Fc, or human IgG-Fc.

for several types of modified LDL. The affinity rank order of LOX-1 for modified LDL was OxLDL > acetyl LDL > MDA-LDL ≫ native LDL (Fig. 2). This is consistent with our previous report using the cell line expressing LOX-1 (14, 15).

We further analyzed the relationships between the degree of oxidation of OxLDL and the reactivity in the EIA system. We have prepared OxLDL with different degrees of oxidation depending on the time of incubation with copper ion (Fig. 3A). LDL oxidized for 6h (TBARS: 4.87) exhibited higher reactivity than more or less oxidized LDL (Fig. 3B). Since apoB protein was degraded during LDL oxidation, we confirmed apoB proteins equally react with the anti-apoB polyclonal antibody among the different preparations of OxLDL. As shown in Fig. 3C, the apoB proteins in the OxLDL

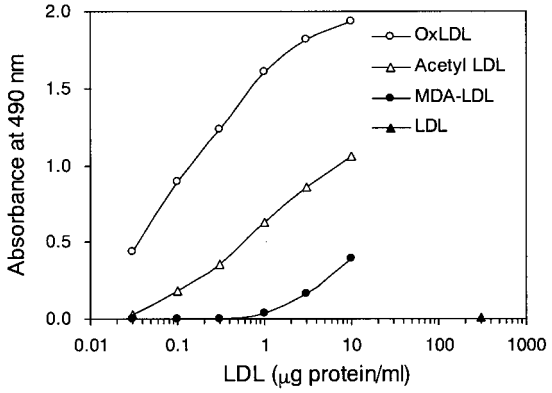

FIG. 2. Specificity of the EIA system for modified LDL. The reactivity to OxLDL, acetyl LDL, MDA-LDL, and native LDL in the EIA system are indicated.

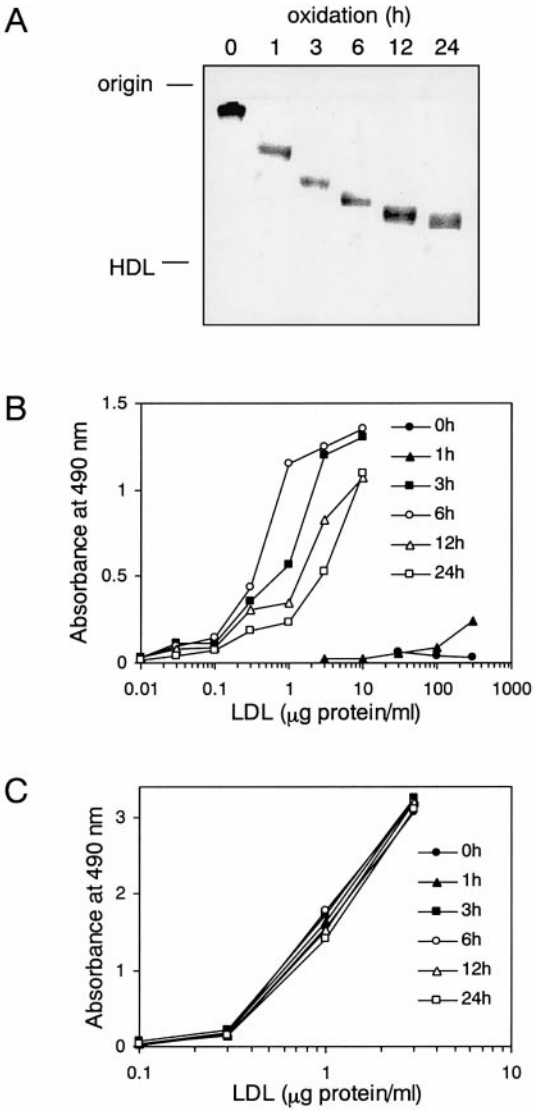

FIG. 3. Recognition of LDL oxidized to different extents by the EIA system. (A) Agarose gel electrophoresis of the OxLDL preparations. (B) Reactivity of the preparations of OxLDL in the EIA system. (C) Reactivity of the preparations of OxLDL to the anti-apoB polyclonal antibody used in the present study. No changes in reactivity to anti-apoB antibody was observed, suggesting the changes of reactivity observed in B are due to the change in the reactivity to LOX-1.

preparations showed virtually the same reactivity to the anti-apoB antibody. This may be explained by the polyclonality of the antibody that efficiently recognizes even the degraded apoB proteins. These results further confirmed the specificity of LOX-1 toward mildly oxidized LDL.

Detection of LOX-1 Ligand Activity in the Plasma of WHHL Rabbits

Since the EIA system could measure rabbit OxLDL (Fig. 4), we applied it to quantify LOX-1 ligand activity in WHHL rabbits that are good model animal for ath-

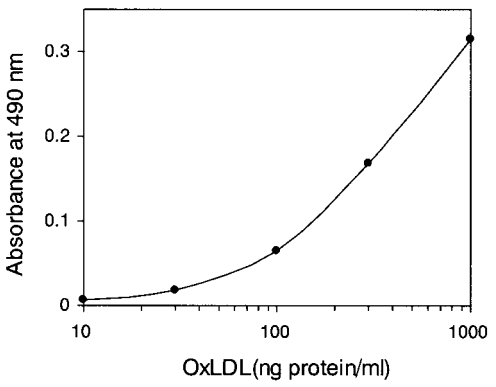

FIG. 4. Typical standard curve of EIA for rabbit OxLDL.

erosclerosis. The cholesterol levels for WHHL and Japanese White rabbits were 782.7 ± 181.1 and 33.3 ± 5.44 mg/dl, respectively. LOX-1 ligand activity was detectable in the plasma of WHHL as 4.00 ± 0.52 μg/ml, while it was not detectable in the case of Japanese White rabbits.

Immunohistochemical Detection of LOX-1 Ligand Activity in Atheroma

We further applied LOX-Fc to detect LOX-1 ligand in the vasculature *in situ* by histological methods. In the aorta of Japanese White rabbits, the medial smooth muscle cells and endothelial cells were negative for LOX-1 ligand staining. In contrast, in the aorta of WHHL rabbits, focal intimal lesions were found. These intimal lesions were always stained positive with Oil red O (Fig. 5A). Moreover, aortic areas, which were not associated with intimal thickening, occasionally showed positive stain with Oil red O (Fig. 5A). Immunohistochemically, the lesions with intimal thickening were characterized by an accumulation of macrophages with a foamy appearance (Fig. 5B), and these intimal lesions showed distinct positivity for LOX-1 ligand (Figs. 5C and 5D). LOX-1 ligand staining was also observed occasionally in endothelial cells at some sites of the aortic wall without intimal thickening (Fig. 5E). Immunodouble staining for macrophages and LOX-1 ligand revealed that in the intimal lesions, both macrophages and endothelial cells were positive for LOX-1 ligand (Fig. 5F). Immunostaining of the lesion with anti-apoB antibody showed similar staining pattern, supporting that the LOX-1 ligand in the atheroma detected by the present method would consist of apoB containing lipoproteins (Fig. 5G).

DISCUSSION

Much evidence supports the presence of OxLDL *in vivo*. The increased levels of OxLDL antigen and autoantibodies against OxLDL appear to be positively correlated with atherosclerosis (18, 19). After our identification of LOX-1, a lectin-like endothelial receptor for OxLDL, the following studies revealed that it is inducibly expressed in culture cells that are present in atheroma, particularly high in endothelial cells (7, 14, 20). It has recently been shown that LOX-1 is enhanced by many proatherogenic circumstances, and it is indeed accumulated in atherosclerotic lesions (11, 21).

LOX-1 is a type II membrane protein which structurally belongs to C-type lectin family. The extracellular lectin-like domain of LOX-1 protein among several species is highly conserved. We found lectin-like domain is the functional domain mediating OxLDL binding (Chen *et al.* unpublished data). Therefore, we designed a recombinant protein to fuse the extracellular domain of LOX-1 to IgG Fc region. LOX-Fc was further characterized possessing the activity of binding OxLDL *in vitro*.

In this study, we develop a detection system for LOX-1 ligand by the use of LOX-Fc. The sandwich enzyme immunoassay enables us to determine the specific ligand for LOX-1 *in vivo*. It has been established that severe oxidation of LDL is unlikely to occur within plasma. As shown in Fig. 3, mild degree of modification of OxLDL (TBARS: 4.87) is sufficient for the binding to LOX-Fc. Therefore, LOX-1 may recognize some subtle changes of LDL in the circulation. Actually, we found that LOX-1 ligand activity was significantly elevated in the plasma of WHHL rabbits hyperlipidemic which is genetically deficient in LDL receptor and exhibits severe hypercholesterolemia.

When LOX-Fc was used to detect the atherosclerotic lesions of WHHL rabbits, large amount of staining was widely distributed in both endothelial cells and the subendothelial macrophages. These findings further indicated that LOX-1 ligand was deposited in the atherosclerotic lesions *in situ*. As LOX-1 can actively bind apoptotic cells and activated platelets (15, 22), we can not exclude the possibility that some apoptotic cells in the atherosclerotic lesions are shown as a part of positive staining by LOX-Fc. However, it is not likely that all the signals detected by LOX-Fc are from these kinds of cells, since LOX-1 ligand activity was detected in most of the cells in atheroma that is mostly consisted of live cells. The higher concentration of LOX-1 ligand together with the high expression of LOX-1 provides a molecular basis for linking OxLDL to endothelial cells, and resultant cellular activation, dysfunction, and injury. The close coordination of OxLDL/LOX-1 system on endothelial cells activation will initiate the early stages of atherogenesis. The binding of OxLDL to LOX-1 in endothelial cells induces the production of reactive oxygen species resulting in activation of NF-κB (23), the expression of monocyte chemoattractant protein-1 and LOX-1 itself (20, 24), and further induces apoptosis (25).

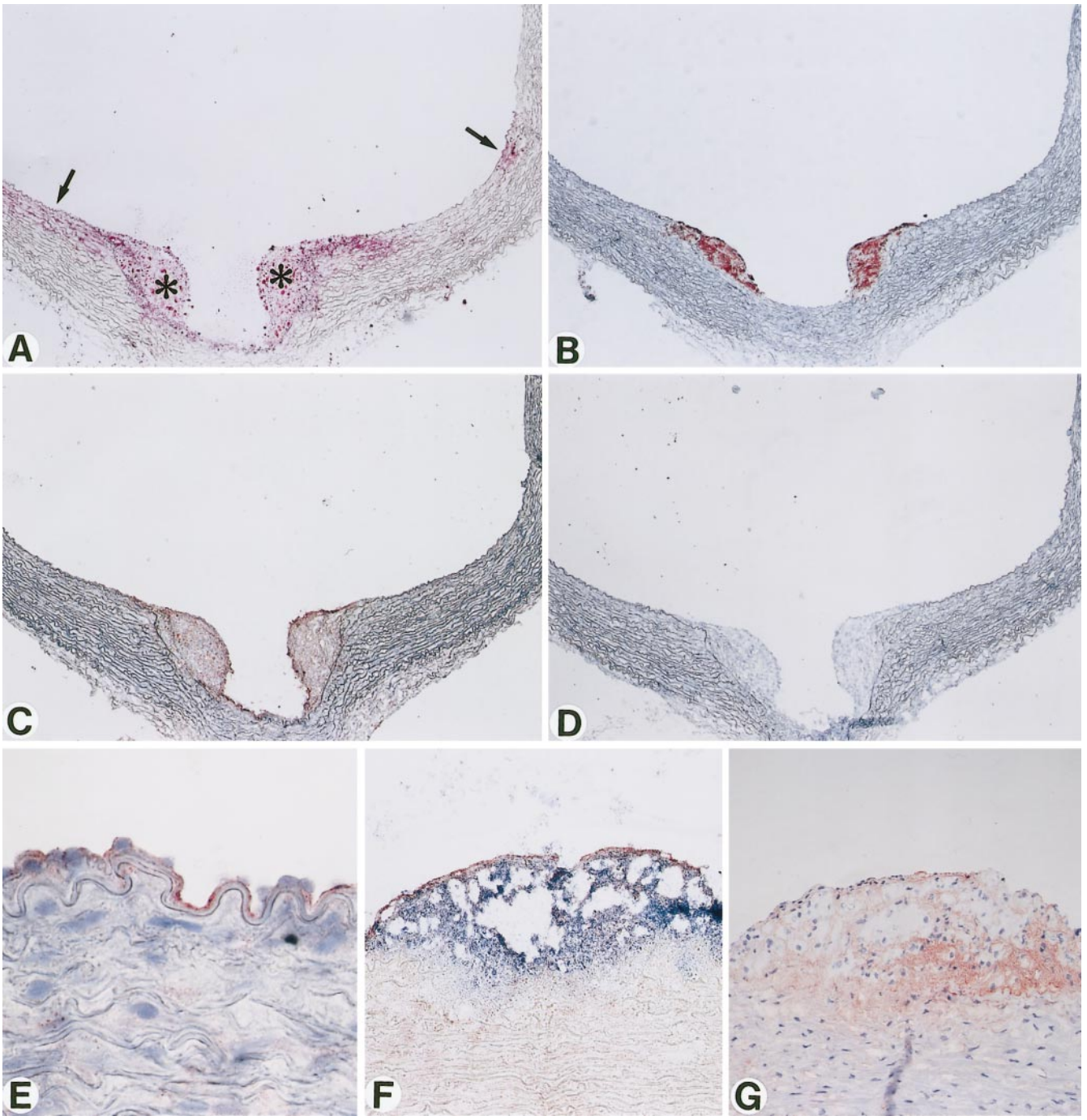

FIG. 5. Sections of the aorta in a WHHL rabbit (12 weeks old). (A) Oil red O stain. Intimal lesions indicated by asterisks are positive with Oil red O. Some areas (arrows) of the aortic wall without intimal thickening are also positive with Oil red O. (B) Staining for macrophages. The intimal lesion contains abundant macrophages with a foamy appearance. (C) Staining with LOX-Fc. LOX-1 ligand positivity is detected in the intimal lesion. (D) Adjacent section treated with nonimmune human IgG-Fc is negative. (E) Staining with LOX-Fc. Some endothelial cells in an area without intimal thickening are stained positive for LOX-Fc. (F) Immunodouble staining for macrophages (blue) and LOX-1 ligand (red). Macrophages in the intima show double staining (purple), indicating LOX-1 ligand positivity. Endothelial cells are also positive for LOX-1 ligand (red). (G) Staining for apoB. Distribution of apoB positivity is similar to that of LOX-1 ligand. Original magnification: A–D, ×60; E, ×698; F and G, ×179.

As LOX-1 is also expressed in macrophage and smooth muscle cells in the atherosclerotic lesions, thus it might be involved in the form cell formation. The persistent accumulation of LOX-1 ligand in the lesions indicates a long-term effect of modified LDL in the progression of atherosclerosis. Taken together, we sug-

gest that the enhanced interactions of modified LDL with LOX-1 might be implicated in both the initiation and progression of atherosclerosis.

In conclusion, we demonstrated the presence of LOX-1 ligand *in vivo*, which is significantly increased by hyperlipidemia and accumulated in atherosclerotic lesions. These findings support the hypothesis that LOX-1 ligand-receptor interactions play a critical role in the hyperlipidemia-based atherogenesis. Additionally, the LOX-1 ligand level would be a good indicator for the diagnosis and the evaluation of therapeutic interventions of atherosclerosis.

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