Low Density Lipoprotein Receptor-related Protein Is Required for Macrophage-mediated Oxidation of Low Density Lipoprotein by 12/15-Lipoxygenase*

Wanpeng Xu‡, Yoshitaka Takahashi‡, Toshiki Sakashita§, Tadao Iwasaki†, Hiroaki Hattori‡, and Tanihiro Yoshimoto¶

From the Departments of ‡Molecular Pharmacology and §Molecular Genetics of Cardiovascular Disorders, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan and †Research Department, R&D Center, BML, Inc., Saitama 350-1101, Japan

The oxidative modification of low density lipoprotein (LDL) has been implicated in the early stage of atherosclerosis through multiple potential pathways, and 12/15-lipoxygenase is suggested to be involved in this oxidation process. We demonstrated previously that the 12/15-lipoxygenase overexpressed in mouse macrophage-like J774A.1 cells was required for the cell-mediated LDL oxidation. However, the mechanism of the oxidation of extracellular LDL by the intracellular 12/15-lipoxygenase has not yet been elucidated. In the present study, we found that not only the LDL receptor but also LDL receptor-related protein (LRP), both of which are cell surface native LDL-binding receptors, were down-regulated by the preincubation of the cells with cholesterol or LDL and up-regulated by lipoprotein-deficient serum. Moreover, 12/15-lipoxygenase-expressing cell-mediated LDL oxidation was decreased by the preincubation of the cells with LDL or cholesterol and increased by the preincubation with lipoprotein-deficient serum. Heparin-binding protein 44, an antagonist of the LDP receptor family, also suppressed the cell-mediated LDL oxidation in a dose-dependent manner. The cell-mediated LDL oxidation was dose-dependently blocked by an anti-LRP antibody but not by an anti-LDL receptor antibody. Furthermore, antisense oligodeoxyribonucleotides against LRP reduced the cell-mediated LDL oxidation under the conditions in which the expression of LRP was decreased. The results taken together indicate that LRP was involved essentially for the cell-mediated LDL oxidation by 12/15-lipoxygenase expressed in J774A.1 cells, suggesting an important pathophysiological role of this receptor-enzyme system as the initial trigger of the progression of atherosclerosis.

Lipoxygenases are a class of enzymes that incorporate one molecular oxygen into unsaturated fatty acids giving rise to their hydroperoxy derivatives. There are 5-, 8-, 12-, and 15-lipoxygenases in mammalian tissues, named according to the number of carbon atoms of arachidonic acid to be oxygenated (1–4). The 12-lipoxygenase subfamily includes leukocyte, platelet, and epidermal isoforms. 15-Lipoxygenase-1 was first isolated from reticulocytes, and 15-lipoxygenase-2 was cloned from hair follicles (3). Because the leukocyte 12-lipoxygenase and 15-lipoxygenase-1 are highly related in their primary structures and enzymological properties and are abundant in various tissues of many species, these enzymes are called collectively 12/15-lipoxygenases (2, 5). Although the pathophysiological functions of the 12/15-lipoxygenases are still a subject of investigation and discussion, recent research progress has revealed the involvement of the enzymes in the development of atherosclerosis (6–8).

The oxidative modification of low density lipoprotein (LDL) has been implicated in the early stage of atherosclerosis (9, 10). Macrophages are likely candidates to mediate in vivo LDL oxidation, because they are accumulated in the atherosclerosis lesions and are capable of in vitro LDL oxidation in culture medium free of metal ion additives (11). A number of evidences suggest that the 12/15-lipoxygenase present in monocyte-macrophage contributes to the cell-mediated LDL oxidation (12, 13). Incubation of LDL with 12/15-lipoxygenase led to significant oxidation of LDL (14, 15). The 12/15-lipoxygenase and oxidized fatty acids colocalized with oxidized LDL in fatty streak lesions (16–18). A disruption of the 12/15-lipoxygenase gene diminished atherosclerosis in apoE-deficient mice (19), and overexpression of 12/15-lipoxygenase facilitated atherosclerosis in the LDL receptor-deficient mice (20).

We previously demonstrated that 12/15-lipoxygenase overexpressed in mouse macrophage-like J774A.1 cells was involved essentially in the oxidation of LDL based upon the stereospecific oxygenation of esterified unsaturated fatty acid in LDL (21). This fact suggests direct interaction of the enzyme with LDL or the transfer of cellular lipids oxygenated by the enzyme to LDL. Secretion or leakage of the 12/15-lipoxygenase to the medium was ruled out (21). As the mechanism of the cell-mediated oxidation of extracellular LDL, we postulate that binding of native LDL to cell surface receptors is the first step in the 12/15-lipoxygenase-expressing cells. Among such receptors, the LDL receptor plays an important role in LDL metabolism in liver and steroidogenic tissues. However, the LDL receptor is not expressed in the intima of normal or atherosclerotic arteries (22). Importantly, the LDL receptor is not required in the cell-mediated LDL oxidation as shown by in vitro experiments (23) and LDL receptor-deficient mice studies (20, 24). The native LDL also binds to LDL receptor-related protein

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1 To whom correspondence should be addressed. Tel.: 81-76-265-2186; Fax: 81-76-234-4227; E-mail: yoshimot@med.kanazawa-u.ac.jp.

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EDTA was removed from the LDL by dialysis against phosphate-buffered saline, was conjugated to keyhole limpet hemocyanin (Calbiochem, H11032), and was added to LRP-expressing cells (21). As determined by Western blotting using a membrane fraction from the manufacturer protocol. Specificity of the anti-LDL receptor antibody was confirmed in the control experiments using a mouse monoclonal antibody to the LDL receptor, which was labeled with [125I]iodoacetamide using the MegaPrime DNA labeling system. After washing the membrane, radioactivity was analyzed by a Fujix BAS1000 imaging analyzer (Tokyo, Japan). Nucleotide sequences of the LDL receptor, LRP, scavenger receptor BI, GAPDH, and heparin-binding protein 44 (see below) were determined by dRhodamine terminator cycle sequencing kit using an automated DNA sequencing ABI PRISM 310 (PerkinElmer Life Sciences).

**Expression of Heparin-binding Protein 44**—The full-length cDNA for heparan sulfate- and heparin-binding protein 44 (32) was prepared with total RNA from J774A.1 cells by RT-PCR using the following primers: upstream, 5'-TCTAGAATGGGGGTTCTCATTGCTCATGTTGAGGCGAAGCC-3', and downstream, 5'-TCTAGATCATGCTGGTTGGGAGCTGC-3'. The cDNA was subcloned in pCR2.1 and then ligated to the pEF-BOS vector. The resultant plasmid was introduced into COS-7 cells by the DEAE-dextran method (33). After incubation for 48 h, the cells were harvested and sonicated on ice in 50 mM Tris-HCl buffer, pH 7.4, 1.5 mM MgCl2, 1 mM CaCl2, and 1 mM phenylmethylsulfonyl fluoride. The supernatant was subjected to nickel-nitritroleic acid agarose column chromatography using manufacturer instructions. The histidine-tagged heparin-binding protein 44 was eluted with 0.2 M histidine in phosphate-buffered saline at pH 7.4.

**Materials**—Dulbecco’s modified Eagle’s medium (DMEM) was obtained from Nissui (Tokyo, Japan), fetal bovine serum from JRH Biologicals (Southwestern Medical Center), fetal bovine serum from JRH Biologicals (Southwestern Medical Center), fetal bovine serum from JRH Biologicals (Southwestern Medical Center), and fetal bovine serum from JRH Biologicals (Southwestern Medical Center). Human LDL (relative density 1.019–1.063) was isolated from the plasma of healthy volunteers by sequential flotation procedures and dialysis against phosphate-buffered saline. LDL was transferred to a nylon membrane and fixed by UV irradiation. The membrane was hybridized in QuikHyb hybridization solution with cDNA for the LDL receptor, LRP, scavenger receptor BI, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The cDNA was synthesized using oligo(dT)12-18 as a primer and SuperScript II reverse transcriptase. cDNA was carried out with ExTaq DNA polymerase and primers as shown in Table 1 under the following conditions: denaturation at 94°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min. An equal amount of aliquots from 15 and 20 thermocycles was electrophoresed in 2% agarose gels. The amplified DNAs were transferred to a nylon membrane and fixed by UV irradiation. The membrane was hybridized in QuikHyb hybridization solution with cDNA for the LDL receptor, LRP, scavenger receptor BI, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which had been labeled with [32P]dCTP using the MegaPrime DNA labeling system. After washing the membrane, radioactivity was analyzed by a Fujix BAS1000 imaging analyzer (Tokyo, Japan). Nucleotide sequences of the LDL receptor, LRP, scavenger receptor BI, GAPDH, and heparin-binding protein 44 (see below) were determined by dRhodamine terminator cycle sequencing kit using an automated DNA sequencing ABI PRISM 310 (PerkinElmer Life Sciences).

**RESULTS**

Effect of Up-regulation and Down-regulation of LDL-binding Receptors on LDL Oxidation—We previously reported that the 12/15-lipoxygenase of porcine leukocyte overexpressed in macrophage-like J774A.1 cells was responsible for oxidative modification of LDL in the medium (21). As shown in the control of Fig. 1, the oxidation of LDL by the 12/15-lipoxygenase-expressing cells was 3.7 times higher than that by mock-transfected cells as determined by TBARS assay. To explore mechanisms of the LDL oxidation caused by intracellular 12/15-lipoxygenase of porcine leukocytes overexpressed in macrophage-like J774A.1 cells, we examined the possible involvement of the LDL receptor expressed on the surface of 12/15-lipoxygenase-expressing cells. It is known that expression of the LDL receptor is down-regulated by the incubation of the cells with LDL or cholesterol and up-regulated by the incubation of lipoprotein-deficient serum (36). In fact, as shown in Fig. 2, incubation of the 12/15-lipoxygenase-expressing cells with LDL or cholesterol decreased the mRNA level of the LDL receptor by 77 and...
LDL receptor family including the LDL receptor and LRP and other hand, scavenger receptor BI mRNA expression was decreased by preincubation with LDL and expressed in J774A.1 cells (22, 37). We then examined whether expression levels of these receptors would be altered by 12/15-lipoxygenase-expressing cells preincubated with LDL, cholesterol, or lipoprotein-deficient serum (LPDS). Washed cells were then incubated with 400 µg/ml LDL in serum-free DMEM for 12 h, and TBARS in the medium was measured. The data are shown as mean ± S.E. of eight experiments after subtraction of no-cell control. MDA, malondialdehyde.

58%, respectively, as determined by RT-PCR using each set of primers shown in Table I. In contrast, incubation of the cells with lipoprotein-deficient serum increased the mRNA of the LDL receptor by 2.6 times under our experimental conditions. We examined whether the LDL oxidation would be changed by the above-mentioned preincubations. As shown in Fig. 1, TBARS generation by the 12/15-lipoxygenase-expressing cells was reduced by 39 and 19% after the preincubation of the cells with LDL and cholesterol, respectively. On the other hand, an ~2-fold increase in TBARS generation was observed after the preincubation of the cells with lipoprotein-deficient serum. There were no apparent changes of TBARS generation in mock-transfected cells preincubated with LDL, cholesterol, or lipoprotein-deficient serum (Fig. 1, open bars). These results suggest that the LDL oxidation by the 12/15-lipoxygenase-expressing cells is mediated by either the LDL receptor or other cell surface proteins that bind to native LDL and are down- and up-regulated by the culture conditions described above. It was reported that LRP and scavenger receptor BI bind to native LDL and expressed in J774A.1 cells (22, 37). We then examined whether expression levels of these receptors would be altered by the different culture conditions. RT-PCR was carried out to analyze the mRNA level of these receptors in the 12/15-lipoxygenase-expressing cells preincubated with LDL, cholesterol, or lipoprotein-deficient serum. Primers for GAPDH were also included as internal control for RNA quantity and integrity. As shown in Fig. 2A, aliquots from 15 and 20 cycles were analyzed by agarose gel electrophoresis to verify the linearity of PCR amplification. The LRP mRNA level of the 12/15-lipoxygenase-expressing cells was decreased by preincubation with LDL and cholesterol to 65 and 62% of the control, respectively. The LRP expression was up-regulated 1.3-fold after the preincubation of the cells with lipoprotein-deficient serum (Fig. 2B). On the other hand, scavenger receptor BI mRNA expression apparently did not change under the same preincubation conditions.

Effect of Heparin-binding Protein 44 on LDL Oxidation—Heparin-binding protein 44, a mouse homologue of human LRP receptor-associated protein, is a universal antagonist of the LDL receptor family including the LDL receptor and LRP and inhibits the binding of LDL (38–40). To examine the effect of heparin-binding protein 44 on the 12/15-lipoxygenase-mediated LDL oxidation, the hexahistidine-tagged heparin-binding protein 44 was expressed in COS-7 cells and purified by affinity chromatography. The 12/15-lipoxygenase-expressing or mock-transfected cells were incubated with LDL in the presence of the purified heparin-binding protein 44. As shown in Fig. 3A, heparin-binding protein 44 inhibited TBARS generation in a concentration-dependent fashion, and a maximal inhibition was observed at a concentration as low as 2 µg/ml. These results suggest that the LDL receptor and/or LRP are involved in the LDL oxidation by intracellular 12/15-lipoxygenase.

Effect of Antibody against the LDL Receptor or LRP—To examine whether the LDL receptor and LRP are involved in the cell-mediated LDL oxidation, we employed antibodies against the LDL receptor (this study) and LRP (29), both of which blocked the LDL binding to the cells. The LDL binding to the LDL receptor was inhibited almost completely by 10 µg/ml IgG against the LDL receptor (data not shown). The 12/15-lipoxygenase-expressing cells and mock-transfected cells then were incubated with 400 µg/ml LDL in the serum-free DMEM in the presence of the anti-LDL receptor IgG at 37 °C for 12 h. As shown in Fig. 3B, the 12/15-lipoxygenase-mediated TBARS generation was not inhibited by the antibody at concentrations up to 50 µg/ml. It should be noted that the anti-LRP antibody suppressed TBARS generation by the 12/15-lipoxygenase-expressing cells in a dose-dependent manner (Fig. 3C). The TBARS generation was inhibited by more than 90% with 10 µg/ml antisera. The anti-LRP antibody had no effects on the LDL oxidation by CuSO₄ or mock-transfected cells. On the other hand, the nonimmunized rabbit serum was without effect on the TBARS generation. The results strongly suggest that LRP is at least one of the receptors required for the LDL oxidation by 12/15-lipoxygenase-expressing cells.

Effect of Antisense Oligodeoxyribonucleotides—To confirm the role of LRP in cell-mediated LDL oxidation, antisense oligodeoxyribonucleotides complementary to the 5’ region of the mouse LRP, LDL receptor, and scavenger receptor BI mRNA containing the initiator AUG codon were synthesized as well as the corresponding sense oligodeoxyribonucleotides (Table II). After incubation of the 12/15-lipoxygenase-expressing cells with the antisense or sense oligodeoxyribonucleotides for 7 days, RT-PCR analysis was carried out. As shown in Fig. 4A, the antisense oligodeoxyribonucleotides inhibited the mRNA expression of respective receptors, whereas the sense oligodeoxyribonucleotides did not inhibit the receptor expression. We performed Western blotting for LRP and the LDL receptor, but the specific bands were not observed probably because of the low expression of these receptors. We then examined the TBARS generation by the 12/15-lipoxygenase cells after the treatment with the antisense or sense oligodeoxyribonucleotides. As shown in Fig. 4B, the antisense oligodeoxyribonucleotides against LRP suppressed TBARS generation in the culture medium by the 12/15-lipoxygenase cells by 67% as compared with control incubation, whereas sense oligodeoxyribonucleotides did not inhibit the TBARS generation. As anticipated from the data in Fig. 3B, antisense oligodeoxyribonucleotides against the LDL receptor or scavenger receptor BI did not affect the TBARS generation by the 12/15-lipoxygenase cells. These results taken together indicate that LRP is responsible for the 12/15-lipoxygenase cell-mediated LDL oxidation, and neither the LDL receptor nor scavenger receptor BI is involved in this process.

Specific Binding of LDL—To confirm the specific binding of native LDL to LRP, fluorescence-labeled LDL, DiI-LDL, was incubated with the 12/15-lipoxygenase-expressing cells. After a 2-h incubation, bright fluorescence was observed in most of the cells (Fig. 5A), whereas little or no fluorescence in the presence of a 100-fold excess of unlabeled LDL (Fig. 5D). When the LRP (Fig. 5B) or the LDL receptor (Fig. 5C) was blocked by incubation of the antibodies with DiI-LDL, much less fluorescence was observed in the 12/15-lipoxygenase-expressing cells.
as compared with the control incubation. The means of the fluorescence intensity as determined by the flow-cytometric analysis was decreased by 39, 41, and 78% in the presence of anti-LRP antibody at 10 μg/ml, anti-LDL receptor IgG at 50 μg/ml, and a 100-fold excess of unlabeled LDL, respectively. This result indicates that LRP as well as the LDL receptor is responsible for the specific binding of LDL to the 12/15-lipoxygenase-expressing cells.

**TABLE I**

Primer Sequences Length of PCR product
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GAPDH-1F 5’-GAGAAGCGGGAGCTTGTGCATCAATGG-3’ base pairs 339
GAPDH-2R 5’-AGTTGAGTCTCTCTCAGCATACAAAG-3’
LDL receptor-1F 5’-GAAGACTCATGCAAGGAAACG-3’ 468
LDL receptor-2R 5’-CTCATCAGGAGCCTCAACACAG-3’
LRP-1F 5’-GTATCTCAAAGGCGCTGCGAG-3’ 619
LRP-2R 5’-TGACACCACAGCTACGGC-3’
Scavenger receptor BI-1F 5’-CTGGCGTTGTGCATGATCCTCAT-3’ 912
Scavenger receptor BI-2R 5’-GCACAGCTCAGCATGGC-3’

**DISCUSSION**

LRP was shown to be expressed in the 12/15-lipoxygenase-expressing J774A.1 cells and responsible for cell-mediated LDL oxidation by these cells. The LRP expression was down-regulated by incubations with LDL or cholesterol and up-regulated by lipoprotein-deficient serum (Fig. 2). A previous study showed that LRP expression was not changed significantly by cholesterol loading in the LDL receptor-deficient human fibro-
Role of LRP in Macrophage-mediated LDL Oxidation

The LDL oxidation as determined by TBARS of 1 × 10⁵ peritoneal macrophages was 0.39 ± 0.03 (n = 4) nmol of malondialdehyde/mg of LDL, and that of 2 × 10⁵ 12/15-lipoxygenaseexpressing J774A.1 cells was 0.26 ± 0.07 (n = 4) nmol of malondialdehyde/mg of LDL. When the 12/15-lipoxygenase activity of these cells was determined using 25 μM exogenous arachidonic acid as substrate (21), it was 74.8 and 37.2 nmol/10 min/mg of protein, respectively. Our results taken together suggest that LRP, at least in part, mediates the LDL oxidation not only by 12/15-lipoxygenase-expressing J774A.1 cells but also by normal macrophages, which express 12/15-lipoxygenase at a high level and accumulate in atherosclerotic lesions (16, 22). The very low level of LDL oxidation by mock-transfected cells that hardly expressed 12/15-lipoxygenase was not affected by heparin-binding protein 44 or an anti-LRP antibody, suggesting 12/15-lipoxygenase-independent LDL oxidation, if any, was not mediated by LRP.

We could not detect specific bands of LRP or the LDL receptor by Western blotting, suggesting that the expression level of these receptors in the 12/15-lipoxygenase-expressing cells was not high. However, antibodies against these two receptors significantly inhibited binding of Dil-LDL to these cells as assessed by flow-cytometric analysis, indicating that functional receptors were definitely expressed in these cells. Small fluorescence was still observed even when both antibodies were added at the same time to the cells incubated with Dil-LDL (data not shown). The results agree with the expression of scavenger receptor BI in these cells and also suggest the presence of other LDL-binding receptors.

The LDL receptor did not seem to be involved in the cell-mediated LDL oxidation (Figs. 3B and 4B). This is in good agreement with the previous reports showing that macrophages prepared from the LDL receptor-deficient mouse could oxidize LDL to the same extent as the wild-type mouse (24). In fact, the LDL receptor processes native LDL via receptor-mediated endocytosis in which the LDL particle is delivered to lysosomes, in which cholesteryl ester is hydrolyzed to free cholesterol for use by the cells (43). LRP is a multiligand receptor, and the binding of ligands to the receptor is usually followed by receptor-mediated endocytosis and degradation of LDL.
the ligands in lysosomes (26). However, a recent paper reported that LRP also mediated the selective uptake of cholesteryl ester in LDL, which is transferred to the plasma membrane without internalization and degradation of LDL particles (44). It is possible that the cholesteryl ester in the plasma membrane is oxygenated directly by the intracellular 12/15-lipoxygenase followed by reincorporation to the LDL particles (45, 46).

Scavenger receptor BI is an 82-kDa protein that binds high density lipoprotein, LDL, modified LDL, and very low density lipoprotein (47). The receptor is expressed on the surface of macrophage in atherosclerotic lesions (22). It was reported that scavenger receptor BI, similar to LRP, mediates the selective uptake of cholesteryl ester in LDL and high density lipoprotein (48–50). However, mRNA expression of scavenger receptor BI was not altered by the preincubation with LDL, cholesteryl, or lipoprotein-deficient serum under our experimental conditions (Fig. 2). Furthermore, no inhibition of LDL oxidation by anti-sense oligodeoxyribonucleotides against scavenger receptor BI suggests a trivial role of this receptor (Fig. 4). The reason that scavenger receptor BI is not responsible for the cell-mediated LDL oxidation is not known, but this receptor could be involved in the LDL oxidation in the other experimental systems (48).

LRP was reported to bind to a variety of ligands including LDL, α2-macroglobulin, very low density lipoprotein remnants, plasminogen activator, and so on (41). Thus, the receptor has been postulated to participate in a number of diverse physiological and pathological processes such as the homeostasis of plasma lipoproteins and atherosclerosis, fibrinolysis, and neuronal regeneration (41). A recent study revealed the role of the receptor in clearance of chylomicron remnants by inducible disruption of “hepatic” LRP in mice (51). Our study implicates a novel function of “macrophase” LRP in the 12/15-lipoxygenase-mediated LDL oxidation as the initial trigger of the progression of atherosclerosis. Further investigations are needed to explore the LRP-mediated LDL oxidation in detail in relation to other receptors and cellular factors.

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