Deletion of Macrophage LDL Receptor–Related Protein Increases Atherogenesis in the Mouse

Cheryl D. Overton, Patricia G. Yancey, Amy S. Major, MacRae F. Linton, Sergio Fazio

Abstract—Macrophage low-density lipoprotein receptor–related protein (LRP) mediates internalization of remnant lipoproteins, and it is generally thought that blocking lipoprotein internalization will reduce foam cell formation and atherogenesis. Therefore, our study examined the function of macrophage LRP in atherogenesis. We generated transgenic mice that specifically lack macrophage LRP through Cre/lox recombination. Transplantation of macrophage LRP−/− bone marrow into lethally irradiated female LDLR−/− recipient mice resulted in a 40% increase in atherosclerosis. The difference in atherosclerosis was not caused by altered serum lipoprotein levels. Furthermore, deletion of macrophage LRP decreased uptake of 125I–very-low-density lipoprotein compared with wild-type cells in vitro. The increase in atherosclerosis was accompanied by increases in monocyte chemoattractant protein type-1, tumor necrosis factor-α, and proximal aorta macrophage cellularity. We also found that deletion of macrophage LRP increases matrix metalloproteinase-9. This increase in matrix metalloproteinase-9 was associated with a higher frequency of breaks in the elastic lamina. Contrary to what was found with other lipoprotein receptors, deletion of LRP increases atherogenesis in hypercholesterolemic mice. Our data support the hypothesis that macrophage LRP modulates atherogenesis through regulation of inflammatory responses. (Circ Res. 2007;100:670-677.)

Key Words: low-density lipoprotein receptor–related protein • atherosclerosis • lipoproteins • matrix metalloproteinase • macrophage

First defined as a complex for removal of α2-macroglobulin,1,2 and then later identified as the low-density lipoprotein receptor (LDLR)-related protein (LRP),3 LRP is a 600-kDa membrane receptor linked to numerous cellular functions and intracellular signaling events.4 It has multiple extracellular ligands, including apolipoprotein E (apoE), lipoprotein lipase, plasma proteases (urokinase-type and tissue plasminogen activators), fibrinolytic factors (IXa and VIIIa), thrombospondin 1 and 2, and chaperone proteins receptor associated protein and heat shock protein-96 (re-viewed elsewhere5). The cytoplasmic tail of LRP binds to multiple intracellular adapter and scaffold proteins including disabled-1 and FE65.6,7 LRP is present in numerous cell types, including macrophages and hepatocytes, and its systemic expression is essential for embryonic development.8 A fundamental role for hepatic LRP in the clearance of plasma remnants has been demonstrated, as conditional hepatic LRP deletion results in increased plasma triglyceride and chylomicron levels, particularly in the absence of the LDLR.9 Furthermore, decreased expression of hepatic LRP causes delayed chylomicron remnant clearance, supporting a protective effect of hepatic LRP on atherogenesis via reduced plasma lipoprotein burden.10 Besides the effect on lipoprotein remnants, hepatic LRP may provide additional vascular protection by mediating the clearance of other proinflammatory ligands including matrix metalloproteinases (MMPs).11

Macrophage lipoprotein receptors and membrane lipid transporters can modulate atherosclerosis development and progression by facilitating uptake of native and oxidized LDL and mediating efflux of excess cellular cholesterol to high-density lipoprotein (HDL). The deletion of macrophage receptors, including scavenger receptor type A, CD36, and LDLR, has resulted in decreased atherosclerosis in hypercholesterolemic mice, maybe as a consequence of reduced lipoprotein internalization and delayed foam cell formation.12–15 Similar to other lipoprotein receptors, LRP recognizes numerous extracellular ligands and has an important role in intracellular signaling pathways through tyrosine phosphorylation of its cytoplasmic tail.4,5 Interestingly, deletion of LRP from vascular smooth muscle cells surprisingly increases atherosclerosis via mechanisms related to the disruption of complex formation with platelet-derived growth factor receptor.16 The influence of macrophage LRP on atherosclerotic lesion development may be more complex than that of other receptors because of the multiple ligands for this receptor and its signaling effects.

To study the effects of macrophage LRP in vivo, we developed a Cre/lox-based approach to generate macrophage-specific LRP-deficient mice. Through bone marrow trans-
plantation (BMT), we demonstrate that despite decreased macrophage remnant uptake, the deletion of macrophage LRP in the LDLR-deficient mouse (LDLR−/−) model results in enhanced atherosclerosis development. Our studies suggest that the effects of macrophage LRP on the vessel wall are likely attributable in part to macrophage LRP expression limiting the inflammatory response.

Materials and Methods

Generation of Macrophage LRP-Deficient Mice by Cre/lox Recombination

Mice expressing floxed loxP sites flanking the LRP gene (provided by Dr Joachim Hertz (University of Texas Southwestern Medical Center, Dallas) were crossed with mice expressing Cre recombinase, under the control of the macrophage-specific lysozyme M promoter (provided by Dr Irmgard Forster, Cologne, Germany),17 resulting in macrophage LRP-deficient mice (M0LRP−/−).18 The mice were backcrossed >10 times on a C57BL6 background. All offspring were genotyped by PCR using primers 5′-GCAAGTCTCGTGTCAGACC-3′ and 5′-CATACTATTCTCATAACACCCCTTC-3′ for loxp to obtain homozygous floxed band size of 350 bp, and 5′-GCAATTGTCGTTCAGGT1-3′ and 5′-CCTCAGGCTCCGCTGATAAC-3′ to obtain 300-bp bands specific for Cre. All experiments were conducted according to the guidelines, and under approval of, the Vanderbilt University Institutional Animal Care and Usage Committee.

Bone Marrow Transplantation

Recipient LDLR−/− mice were lethally irradiated (9 Gy) using a cesium γ source and transplanted with 5×106 bone marrow cells from C57BL6 (wild-type [WT]) and M0LRP−/− mice through injection in the retro-orbital venous plexus, as previously described.18,19 Four weeks post-BMT, the mice were placed on a Western-type diet (WD) for 8 weeks.

Aorta Lesion Analysis

Proximal arterial lesion area was measured 12 weeks after BMT. The extent of atherosclerosis was examined both in oil red O-stained cross-sections of the proximal aorta (15 alternate, 10-μm cryosections) and by en face analysis with quantitation using the KS300 imaging system (Kontron Elektronik GmbH), as described previously.19,20

Lipid and Lipoprotein Analysis

Total serum cholesterol and triglyceride levels were determined by enzymatic colorimetric assays (Raichem, San Diego, Calif) from mice fasted for 4 hours. Lipoprotein profiles of recipient mice were determined by separation of serum by fast-performance liquid chromatography using a well-established method as previously described.19

Cell Membrane Isolation for Western Blotting

Peritoneal macrophages were collected 4 days after 2×106 thioglycolate injection. Cellular membranes were isolated according to previously published methods with minor modifications.21 Extracted proteins were analyzed with polyclonal rabbit anti mouse LRP antibodies (Dr Joachim Hertz, University of Texas Southwestern) and a horseradish peroxidase–conjugated goat anti-rabbit secondary antibody (Sigma).

125I-VLDL

Human very-low-density lipoprotein (VLDL) was isolated at d<1.019 g/mL from a healthy, fasted donor, as described previously.22 The VLDL was iodinated by the Iodogen method (Pierce Chemicals, Rockford, Ill) using 125I (IMS-30) from Amersham Pharmacia Biosciences (Piscataway, NJ). The 125I-VLDL (10 μg) was incubated with the macrophages for 4 hours at 37°C. The total uptake of VLDL was determined by the cell-associated and trichlo-
membranes. In contrast, LRP was not detected in macrophage membranes from MφLRP+/− mice. Consistent with this observation, WT macrophages had 9-fold greater LRP mRNA levels compared with LRP−/− cells (Figure 1A). The deletion of LRP was specific for macrophages as LRP protein levels were similar in hepatic membranes isolated from WT and MφLRP−/− mice (Figure 1B). We next examined the effect of LRP deletion on 125I-VLDL uptake by macrophages. The absolute mass of degraded 125I-VLDL by LRP−/− macrophages was reduced by 5-fold compared with control (Figure 2A). In addition, the absolute mass of LRP-deficient cell associated 125I-VLDL was decreased 2.5-fold compared with WT macrophages (Figure 2B). Therefore expression of Cre recombinase resulted in macrophage specific LRP deletion and consequently decreased remnant uptake.

**Effects of Macrophage LRP Deletion on Atherosclerosis and Plasma Lipids**

To investigate the influence of macrophage LRP on plasma lipids and atherogenesis, 6- to 8-week-old female LDLR−/− mice (n=14 to 15 per group) were lethally irradiated and transplanted with bone marrow from female WT or MφLRP−/− donor mice. Four weeks post-BMT, recipient mice were placed on a Western diet to raise plasma cholesterol levels and induce atherogenesis. The recipient mice were euthanized 12 weeks post-BMT (8 weeks on Western diet). Deletion of macrophage LRP did not alter plasma cholesterol or triglyceride levels (Table) or plasma lipoprotein profiles (fast-performance liquid chromatographic analyses; not shown), demonstrating no significant contribution of macrophage LRP-mediated remnant clearance in influencing plasma lipoprotein levels in vivo. Quantitation of proximal aorta lesions by oil red O showed a 40% increase in the cross-sectional atherosclerosis lesion area in the MφLRP−/− marrow recipient mice compared with the control recipients (Figure 3), demonstrating that macrophage LRP has a protective effect on atherogenesis. En face analysis of the distal aorta showed no significant differences between groups, likely attributable to the paucity of lesions (<1%) produced by our short-term experiments (data not shown).

### Mechanisms of the Atheroprotective Effects of Macrophage LRP

The integrity of the extracellular matrix (ECM) is highly dependent on its degradation by MMPs and has long been linked to the progression of vulnerable atherosclerotic plaques.24,25 Because some MMPs are ligands for LRP,26–28 we examined the effects of macrophage LRP deletion on secreted MMPs and their activity by gelatin zymography (Figure 4A). Compared with WT macrophages, the absence of macrophage LRP increased medium MMP-9 activity by 10-fold. In addition, stimulation with the liver X receptor (LXR) agonist T090317 decreased overall enzyme activity in both WT and LRP−/− macrophages. However, even with LXR stimulation, the MMP-9 activity in LRP-deficient macrophages was 2.8-fold higher compared with WT cells. Interestingly, this increased activity of secreted MMPs was in part attributable to increased synthesis of the enzyme, as real-time RT-PCR demonstrated that in the absence of LRP, MMP-9 mRNA levels were 3-fold higher than those of WT macrophages (Figure 4B). Histochemical analyses of proximal aortas showed a 42% increase in the number of breaks in the elastic lamina of MφLRP−/− compared with control mice (Figure 4C and 4D). These data suggest that macrophage LRP participates in maintaining the integrity of the atherosclerotic plaque.

Consistent with the idea that early lesions are comprised almost exclusively macrophages, MOMA-2 staining within
the atherosclerotic lesions showed that nearly two-thirds (71% to 74%) of the cross-sections consisted of macrophages in both study groups (data not shown). However, compared with lesions of WT recipient mice, atherosclerotic lesions of MφLRP−/- marrow recipient mice had an absolute increase (31%) in MOMA-2–positive area (Figure 5A and 5B), suggesting that LRP deletion affects macrophage cellularity in the proximal aorta. We examined the possibility that deletion of macrophage LRP results in enhanced MCP-1 secretion and monocyte accumulation in the arterial wall (Figure 5). We measured MCP-1 levels in media from LPS-stimulated, as well as TNF-α and PMA (data not shown), WT and MφLRP−/- macrophages (Figure 5C). Compared with WT macrophages, LRP−/- macrophages secreted 1.7- to 2.7-fold more MCP-1 during 24 hours of incubation (P<0.05), with similar results using TNF-α and PMA stimulation (data not shown). Similar to the MMPs, this increased secretion was in part caused by increased MCP-1 mRNA synthesis (Figure 1 in the online data supplement, available at http://circres.ahajournals.org). Consistent with the increased number of macrophages present in the lesion area, we found that deletion of macrophage LRP increased cell migration in response to MCP-1 (Figure 5D). We investigated whether LRP deletion affects TNF-α secretion. We measured TNF-α levels in media and mRNA levels from LPS-stimulated WT and LRP−/- macrophages. Compared with WT macrophages, LRP deletion increased secretion 1.7- to 13-fold and increased mRNA levels 1.5- to 50-fold over control macrophages (Figure 6). Therefore these data suggest that LRP deletion initiates a proinflammatory response through increased TNF-α and MCP-1 secretion.

**Discussion**

In this article, we report that specific deletion of LRP increases atherogenesis in fat-fed LDLR-deficient mice. Using Cre/lox recombination and BMT, we developed a macrophage LRP-deficient mouse model to show that (1) macrophage LRP deletion in a high cholesterol environment significantly increases atherosclerosis without altering serum lipoproteins; (2) macrophage LRP regulates matrix MMP secretion, which may impact plaque vulnerability; and (3) deletion of LRP in macrophages compromises cellular inflammatory responses through increases in cytokine production. These studies provide definitive evidence for the atheroprotective functions of macrophage LRP.

In this study, we established a well-defined model system to investigate the specific contribution of macrophage LRP in atherogenesis. Because deletion of LRP is lethal in embryonic development, we used Cre/lox recombination to specifically delete macrophage-expressed LRP.29 Specificity of macrophage LRP deletion was demonstrated by Western blot of macrophage and liver cellular membranes. Macrophage LRP deletion was confirmed by real-time RT-PCR, resulting in >90% deletion of macrophage LRP mRNA synthesis (Figure 1).

Macrophage lipoprotein receptors may have overall atherogenic effects by accelerating the uptake of lipids into plaque phagocytes and accelerating the expansion of the foam cell lesion. Indeed, previous studies have demonstrated that the deletion of scavenger receptor type A, CD36, or LDLR results in decreased atherosclerosis.12–15 Also, data have shown that scavenger receptor type A, along with mitogen-activated protein kinases, in endoplasmic reticulum stress–induced apoptosis, leading to lesional necrosis and plaque instability.30 However, opposite results have also been presented,31,32 suggesting the possibility of an antiatherogenic role for scavenger receptor type A. Although LRP is a member of the LDLR superfamily, we hypothesized that LRP can modulate atherogenesis through multiple pathways via signaling mechanisms in addition to its effect as a lipoprotein internalizing receptor. Earlier investigations using the approach of homologous recombination in the mouse have shown that hepatic LRP is implicated in the uptake of

<table>
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<td>MφLRP+/+</td>
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<td>154±16</td>
<td>659±35</td>
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<td>MφLRP−/-</td>
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Values are expressed as mean±SD mg/dL±SD. There were no significant differences among BMT groups at any time point, as determined by Student’s t test.
remnant lipoproteins from the plasma compartment, an effect that can obviously reduce the atherogenic burden. However, we show that macrophage-specific deletion of LRP does not alter plasma levels of serum lipoproteins (Table). Consistent with previous studies showing that LRP mediates the removal of apoE-containing lipoproteins, we show that macrophage LRP deletion significantly decreases the uptake of VLDL in vitro (Figure 2). Despite this, LDLR mice harboring LRP-deficient macrophages had significantly increased atherosclerosis (Figure 3). These data indicate that although the absence of macrophage LRP leads to reduced cellular lipoprotein accumulation, the loss of LRP signaling produces proinflammatory and proatherogenic changes that overrule the benefits of decreased lipid entry.

The in vivo modulation of vessel wall remodeling mediated by MMPs is a key event in the progression of atherosclerosis. Specific MMPs degrade collagen and elastin fibers in the vessel wall, leading to altered migration of vascular smooth muscle cells and plaque stability. Smooth muscle cell and fibroblast LRP modulates synthesis and secretion of MMP-9 and pro-MMP-2.

**Figure 4.** The effect of LRP deletion on MMP secretion and ECM. A, Gelatin zymography of MMP-9 and pro-MMP-2 activity in media from PMA-induced (100 ng/mL) WT and MγLRP−/− macrophages stimulated with T090317 (5 μmol/L) at 24 hours. B, Real-time RT-PCR analysis of MMP-9 synthesis in LPS-stimulated (200 ng/mL) peritoneal macrophages at 24 hours. C, Representative proximal aorta atherosclerotic lesions stained with Movat’s pentachrome. D, Quantitation of the mean cross-sectional lesion area of elastic lamina breaks. Significance was determined by Student’s t test.

**Figure 5.** Macrophage LRP deletion increases inflammatory responses. A, Representation of proximal aorta atherosclerotic lesions stained for MOMA-2. B, Quantitation of the mean positive MOMA-2 stain in proximal aorta. C, MCP-1 ELISA analysis of conditioned media from LPS-stimulated (200 ng/mL) peritoneal macrophages at indicated time points. D, Quantitation of MCP-1 mediated macrophage migration across a 5-μm porous membrane (*). Significance was determined by Student’s t test.
several active MMPs that have also been localized in human coronary arteries that have undergone vascular remodeling.\(^{28,34–36}\) Very recently, Hu and colleagues have reported increased atherosclerosis in apoE\(^{-/-}\)/LDLR\(^{-/-}\) mice lacking macrophage LRP.\(^{37}\) Even though their main reported outcome is in line with our current results, they attribute their results to increased collagen content in the lesion and did not find any differences in immunostaining for MMP-9 in the lesion. Conversely, our in vitro analyses show that macrophage LRP deletion increased MMP-9 enzyme activity and MMP-9 mRNA by 10- and 3-fold, respectively. Possibly related to this is our observation that the proximal aortas of M\(^{-/-}\) mice lacking macrophage LRP.\(^{37}\) Our studies support the idea that macrophage LRP can significantly alter lesion monocyte content,\(^{42}\) and the receptor for MCP-1, CCR2, is expressed by circulating monocytes.\(^{45,46}\) Consistent with the hypothesis that macrophage LRP deletion results in increased atherosclerosis in M\(^{-/-}\) bone marrow recipient mice is attributable to enhanced monocyte recruitment, MCP-1 secretion was markedly increased (1.7- to 2.7-fold) at various time points in the absence of macrophage LRP (Figure 5C). This increased secretion was accompanied by a 2-fold increase in MCP-1 mRNA synthesis (supplemental Figure I); however, we speculate that MCP-1 is a possible ligand for LRP thus preventing the reuptake of MCP-1, leading to increased monocyte accumulation in the media. Alternatively, MCP-1 can be endocytosed via LRP through complex formation with glycosaminoglycans.\(^{47}\) Consistent with this possibility, macrophage LRP deletion resulted in enhanced cell migration in response to exogenous MCP-1 during short incubations (Figure 5D). Taken together, it is likely that increased secretion of MCP-1 and increased monocyte cellular content in the arterial wall contribute to the enhanced atherosclerosis in M\(^{-/-}\) bone marrow recipient mice. Furthermore, the increased macrophage content of lesions in M\(^{-/-}\) bone marrow recipient mice could also be attributable in part to decreased migration of macrophages out the arterial wall. Recent studies of Cao et al demonstrated that deletion of macrophage LRP inhibited macrophage detachment and migration via loss of endocytosis and recycling of the Mac-1 integrin/fibrin/tissue plasminogen activator adhesion complex.\(^{48}\) This detachment process required plasminogen activator inhibitor-1 as a ligand of LRP in forming a bridge with the adhesion complex.

\[ \alpha_\text{v}\beta_\text{3}\]-Macroglobulin is secreted by monocytes and macrophages,\(^{49,50}\) and, once secreted, it is cleaved by proteases and the resulting complex is then recognized and catabolized by LRP.\(^{51}\) Interestingly, native and activated \(\alpha_\text{v}\beta_\text{3}\)-macroglobulin can bind to TNF-\(\alpha\) and can then be internalized via LRP.\(^{52}\) Our data show that macrophage LRP deletion results in 1.7- to 13-fold increases in TNF-\(\alpha\) secretion. Furthermore evidence have shown that MCP-1 secretion is induced by TNF-\(\alpha\).\(^{53}\) Taken together, these observations allow us to speculate that TNF-\(\alpha\) bound to \(\alpha_\text{v}\beta_\text{3}\)-macroglobulin may not be properly cleared by LRP\(^{-/-}\) macrophages, thus leading to increased TNF-\(\alpha\) media accumulation and consequently increased MCP-1 secretion.

In conclusion, we found that the deletion of macrophage LRP results in increased atherosclerosis in fat-fed LDLR\(^{-/-}\) mice. Our studies indicate that the potential atheroprotective
effects of macrophage LRP include monocyte recruitment, regulation of inflammatory responses, and MMP activity.

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Disclosures

None.

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Supplemental Figure 1. Real time RT PCR analysis of MCP-1 mRNA synthesis in peritoneal macrophages. Macrophage RNA was isolated after 24 hr stimulation with 200 ng/mL of LPS. Significance was determined by student’s t-test.