Overexpression of Angiopoietin-Like Protein 4 Protects Against Atherosclerosis Development

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Objective—Macrophage foam cells play a crucial role in several pathologies including multiple sclerosis, glomerulosclerosis, and atherosclerosis. Angiopoietin-like protein 4 (Angptl4) was previously shown to inhibit chyle-induced foam cell formation in mesenteric lymph nodes. Here we characterized the regulation of Angptl4 expression in macrophages and examined the impact of Angptl4 on atherosclerosis development.

Approach and Results—Macrophage activation elicited by pathogen-recognition receptor agonists decreased Angptl4 expression, whereas lipid loading by intralipid and oxidized low-density lipoprotein increased Angptl4 expression. Consistent with an antilipotoxic role of Angptl4, recombinant Angptl4 significantly decreased uptake of oxidized low-density lipoprotein by macrophages, via lipolysis-dependent and -independent mechanisms. Angptl4 protein was detectable in human atherosclerotic lesions and localized to macrophages. Transgenic overexpression of Angptl4 in atherosclerosis-prone apolipoprotein E*3-Leiden mice did not significantly alter plasma cholesterol and triglyceride levels. Nevertheless, Angptl4 overexpression reduced lesion area by 34% ($P<0.05$). In addition, Angptl4 overexpression decreased macrophage content (−41%; $P<0.05$) and numbers of monocytes adhering to the endothelium wall (−37%; $P<0.01$). Finally, plasma Angptl4 was independently and negatively associated with carotid artery sclerosis measured by 3-T MRI in subjects with metabolic syndrome and low-grade systemic inflammation.

Conclusions—Angptl4 suppresses foam cell formation to reduce atherosclerosis development. Stimulation of Angptl4 in macrophages by oxidized low-density lipoprotein may protect against lipid overload. (Arterioscler Thromb Vasc Biol. 2013;33:1529-1537.)

Key Words: atherosclerosis ■ inflammation ■ lipoprotein lipase ■ lipoproteins ■ macrophages

Macrophages are an important component of the innate immune system. Via phagocytosis of foreign pathogens, macrophages play a critical role in the body’s first line of defense. In addition, macrophages are involved in removal of cellular debris and clearance of postapoptotic cells. The ability of macrophages to secrete various cytokines allows them to communicate with other immune cells and orchestrate the inflammatory response.

Besides cellular debris and foreign particles, macrophages can, under certain conditions, also engulf lipid particles via the expression of various scavenger receptors, thereby becoming foam cells. Foam cell formation has been studied primarily in the context of atherosclerosis characterized by accumulation of foam cells in the atherosclerotic plaque.1-2 However, foam cells also participate in other pathologies including multiple sclerosis,3 obesity,4 nonalcoholic steatohepatitis,5 and glomerulosclerosis.5,7

Recently, we described the accumulation of macrophage foam cells in mesenteric lymph nodes of mice fed a highly saturated fat diet.8 In particular, foam cell formation specifically occurred in mice lacking angiopoietin-like protein 4 (Angptl4), an endogenous inhibitor of the triglyceride-hydrolyzing enzyme lipoprotein lipase (LPL) that catalyzes uptake of circulating lipids into tissues.9 Angptl4 irreversibly inhibits LPL activity by converting active LPL dimers into inactive monomers.10,11 Consequently, overexpression of Angptl4 leads to hypertriglycerideremia and reduced fatty acid uptake in tissues, whereas Angptl4 deletion causes lowering of circulating triglyceride levels.12-15
Angptl4 is produced by various tissues including adipose tissue, liver, skeletal muscle, and intestine. In addition, Angptl4 as well as LPL are expressed at high levels in macrophages. Other than a role of lipids and lipid-activated transcription factors peroxisome proliferator activated receptor (PPAR) β/δ and PPARγ, currently little is known about specific stimuli impacting Angptl4 expression in macrophages. Taking into account the importance of foam cells in atherosclerosis, and given the facilitative role of LPL in macrophage foam cell formation, we hypothesized that changes in Angptl4 expression may influence atherosclerosis development. Accordingly, the present study was aimed at better characterizing the regulation of Angptl4 expression in macrophages and examining the potential impact of Angptl4 on atherosclerosis development in a well-established model for human-like lipoprotein metabolism.

Materials and Methods

Materials and Methods are available in the online-only Supplement.

Results

Macrophage Angptl4 Is Regulated by Lipid Emulsion and Toll-Like Receptors 3 and 4 Agonists

First, we characterized the regulation of Angptl4 expression in cultured macrophages. Previous studies have shown that Angptl4 expression is highly upregulated by chylomicrons and fatty acids in peritoneal macrophages. In line with these data we observed that incubation of mouse RAW 264.7 macrophages with a triglyceride emulsion, which causes foam cell formation, increases Angptl4 mRNA expression as well as expression of inflammatory markers (ie, Ptgs2, Cxcl2) and endoplasmic reticulum stress-marker Ddit3 (Figure 1A). Similar results were obtained in mouse bone marrow–derived macrophages (Figure 1B).

To investigate whether activation of macrophages independent of foam cell formation may also alter Angptl4 expression, macrophages were treated with various pattern-recognition ligands of Toll-like receptors (TLRs). Macrophages were treated for 4 hours with lipopolysaccharide (LPS; 1 μg/mL) and various TLR agonists (Figure 1C). These treatments increased Angptl4 mRNA expression, with the greatest effect observed after treatment with Pam2CGDPPKHPKSF (Figure 1D).
receptor agonists. In contrast to the lipid emulsion, several pattern-recognition receptor agonists, including lipopolysaccharide (LPS; Toll-like receptor 4 [TLR4] agonist), Pam2CGDPKPKSF-1 (TLR2/6 agonist), and polyI:C (TLR3 agonist), markedly reduced Angptl4 mRNA levels in mouse RAW 264.7 macrophages while inducing inflammatory markers Ptgs2 and Cxcl2 (Figure 1C). Downregulation of Angptl4 mRNA by various pattern-recognition receptor agonists in BMDMs could be partially relieved by an inhibitor of the nuclear factor-κB pathway, which effectively blunted induction of inflammatory markers (Figure II in the online-only Data Supplement). However, the major portion of the effect of pattern-recognition receptor agonists on Angptl4 seemed to be independent of nuclear factor-κB activation. Downregulation of Angptl4 by LPS was confirmed in mouse BMDMs and peritoneal macrophages (Figure 1D). Taken together, these data show that expression of Angptl4 in macrophages is differentially regulated by lipid emulsions and LPS.

**Angptl4 Decreases the Uptake of Oxidized Low-Density Lipoprotein in Macrophages**

Oxidized low-density lipoprotein (oxLDL) is often used as substrate to induce macrophage foam cell formation and mimic the events in the atherosclerotic plaque. Treatment of mouse BMDMs (Figure 2A) and human THP-1 macrophages (Figure 2B) with oxLDL significantly increased expression of Angptl4 mRNA. Induction of ANGPTL4 mRNA in THP-1 cells was paralleled by a marked increase in ANGPTL4 protein secretion (Figure 2C). Similar results were obtained for native LDL.

The PPAR transcription factors were previously shown to at least partially mediate the effects of oxLDL on gene expression in macrophages. Synthetic agonists for PPARγ (rosiglitazone) and especially PPARδ (GW501516) markedly induced ANGPTL4 mRNA levels in THP-1 macrophages (Figure 2D). Induction of ANGPTL4 by GW501516 was paralleled by a significant increase in ANGPTL4 protein in cell lysate and medium (Figure 2E). Accordingly, PPARδ and possibly PPARγ may be suspected to mediate the effect of oxLDL on ANGPTL4 gene transcription.

By inhibiting LPL activity, Angptl4 was previously shown to reduce macrophage uptake of triglycerides-derived fatty acids and impair macrophage activation, which may indirectly lead to decreased uptake of oxLDL. Using THP-1 macrophages, we confirmed that human recombinant Angptl4 as well as the LPL inhibitor orlistat markedly decreased the uptake of triglycerides from glycerol tri[3H]oleate-labeled very–low-density lipoprotein (VLDL). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [3H]cholesteryl oleoyl ether (COEth)–labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2F). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [3H]cholesteryl oleoyl ether (COEth)–labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2F). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [3H]cholesteryl oleoyl ether (COEth)–labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2F). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [3H]cholesteryl oleoyl ether (COEth)–labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2F). We subsequently preincubated THP-1 cells with unlabeled VLDL in the presence or absence of recombinant Angptl4 for 24 hours followed by a wash and treatment of the cells with [3H]cholesteryl oleoyl ether (COEth)–labeled oxLDL for 6 hours. Consistent with our expectation, Angptl4 and orlistat significantly decreased the uptake of oxLDL (Figure 2F).
After 2 hours, VLDL was washed away and cells were treated with [3H]COEth-labeled oxLDL or [3H]COEth-labeled LDL in the presence or absence of human recombinant Angptl4 or orlistat for 6 hours. Recombinant Angptl4 significantly reduced uptake of oxLDL by 50%, compared with 30% for orlistat. Recombinant Angptl4 had no effect on uptake of native LDL (Figure 2H). Because orlistat specifically inhibits the lipolytic activity of LPL, these data suggest that Angptl4 reduced oxLDL uptake via a combination of lipolysis-dependent and independent mechanisms.

Subsequently, we assessed the impact of Angptl4 overexpression on oxidized LDL uptake and consequent formation of foam cells, using BMDMs from wild-type (WT) and Angptl4Tg mice. Angptl4Tg mice moderately overexpress Angptl4 in a variety of tissues including macrophages (Figure 3A). In contrast to native LDL, oxLDL efficiently promoted foam cell formation in BMDMs of both WT and Angptl4Tg mice (Figure 3B). To quantitatively assess macrophage uptake of oxLDL, we incubated BMDMs from WT and Angptl4Tg mice with [3H]COEth-labeled oxLDL or [3H]COEth-labeled LDL for 48 hours. Remarkably, uptake of oxLDL was significantly lower in Angptl4Tg compared with that in WT macrophages, whereas uptake of LDL was unaltered (Figure 3C). Consistent with previous data, oxLDL significantly induced expression of its receptor Cd36 and the cellular cholesterol exporter Abca1 in BMDMs (Figure 3D).21,26 Interestingly, Abca1 expression was significantly higher in Angptl4Tg compared with WT macrophages, whereas no difference was observed for Cd36. Expression of Abcg1 and Msr1 (scavenger receptor class A member 1) were also not different between WT and Angptl4Tg BMDMs (Figure III in the online-only Data Supplement).

Overall, these data indicate that endogenous and exogenous Angptl4 suppress oxLDL uptake in macrophages.

**Impact of Angptl4 Overexpression in Apolipoprotein E*3-Leiden Mice on Plasma Cholesterol and Triglycerides**

To study the potential role of Angptl4 in atherosclerosis, we first ascertained the presence of ANGPTL4 protein in human atherosclerotic plaques. Mouse plaques could not be studied because of lack of suitable antibody. Staining of serial sections from human carotid tissue with antibodies against CD68 and ANGPTL4 revealed colocalization of ANGPTL4 with CD68, suggesting ANGPTL4 is present in macrophages (Figure IV in the online-only Data Supplement).

To explore the role of Angptl4 in atherosclerosis development, we crossbred Angptl4Tg mice with apolipoprotein E (ApoE)*3-Leiden (E3L) mice. E3L mice represent a unique human-like model for atherosclerosis characterized by plasma cholesterol levels that are proportional to dietary cholesterol content and development of diet-induced atherosclerosis in the presence of the endogenous LDL receptor and ApoE.27 Both Angptl4Tg.E3L mice and control E3L mice were fed a Western-type diet containing 0.4% cholesterol for 24 weeks. Weight gain was equal between the 2 groups (Figure 4A). In contrast, Angptl4Tg.E3L mice ate slightly less than E3L mice (Figure 4B). After 4 weeks of being fed the Western-type diet, all animals were hypercholesterolemic and plasma cholesterol levels remained high until the end of the study. Importantly, plasma cholesterol levels were not significantly different between the 2 groups (Figure 4C). Whereas plasma triglycerides were increased in Angptl4Tg.E3L mice up to week 4, triglycerides subsequently dropped to levels that were not significantly different from the E3L group (Figure 4D). Elevated plasma cholesterol levels in Angptl4Tg.E3L and E3L mice could be attributed to elevated VLDL/LDL levels, as determined by fast liquid protein chromatography (Figure 4E and 4F). Quantitative polymerase chain reaction analysis...
verified that after 24 weeks of feeding Angptl4 mRNA expression was significantly elevated in liver, white adipose tissue, and aorta of Angptl4Tg.E3L mice compared with E3L mice (Figure VA and VB in the online-only Data Supplement).

**Angptl4Tg.E3L Mice Develop Smaller Lesions Than E3L Mice**

After 24 weeks of Western-type diet, we investigated atherosclerosis development in the aortic root by measuring the lesion area and lesion types based on cellular composition of the plaques (see Materials and Methods). Whereas Angptl4Tg.E3L mice developed on average an equal number of plaques per cross section compared with E3L mice (Figure 5A), average lesion size expressed as total lesion area was reduced by 34% in Angptl4Tg.E3L mice (P<0.05; Figure 5B and 5C). We next classified lesion types and determined the distribution of lesions according to severity in the 2 groups. Angptl4Tg.E3L mice showed a tendency toward development of less severe lesions (no lesion, type 1 and type 2) compared with E3L mice (Figure 5D), although the difference did not reach statistical significance.

We next evaluated the effect of Angptl4 overexpression on monocyte recruitment and lesion composition with respect to macrophage content, collagen content, and smooth vascular muscle cell content. Angptl4 overexpression decreased the number of monocytes adhering to the vessel wall compared with E3L mice by 37% (P<0.05; Figure 6A). The decrease in adhering monocytes was accompanied by a 41% decrease in macrophage content in the intima of Angptl4Tg.E3L mice (P<0.05; Figure 6B). The collagen area tended to be reduced (~27%; P=0.07) in Angptl4Tg.E3L mice compared with E3L mice (Figure 6C), and vascular smooth muscle cell area was not different between the 2 groups (Figure 6D). Thus, overexpression of Angptl4 in E3L mice reduces lesion size and leads to a less inflammatory lesion phenotype characterized by decreased monocyte recruitment and macrophage accumulation. Interestingly, no major differences in expression of relevant genes were observed between aortas of E3L and Angptl4Tg.E3L mice, including adhesion molecule...
Vcam1, metalloproteinase Mmp2, macrophage marker Cd68, chemokine Ccl2 (monocyte chemoattractant protein 1) and its receptor Ccr2, proapoptotic Bax, and antiapoptotic Bcl2 (Figure VB in the online-only Data Supplement).

Consistent with an inhibitory effect of Angptl4 on foam cell formation in E3L mice, unelicited monocytes/macrophages isolated from the peritoneal cavity of Angptl4Tg.E3L mice after 24 weeks of Western-type diet showed virtually no Oil Red O droplets, in contrast to extensive Oil Red O droplets in monocytes/macrophages isolated from E3L mice (Figure 6E).

In the early stages of atherogenesis, monocytes/macrophages are recruited to the vessel wall in response to chemokines such as monocyte chemoattractant protein 1 (Ccl2) produced by the inflamed endothelium. To further investigate the effect of Angptl4 on the chemotactic recruitment of macrophages, we performed an in vitro macrophage migration assay. Strikingly, BMDMs from Angptl4Tg mice were unable to migrate toward the chemotactic signal monocyte chemoattractant protein 1 compared with WT macrophages (Figure 6F). These results suggest a suppressive effect of Angptl4 on macrophage migration and chemotaxis. Gene-expression analysis of unstimulated and oxLDL-stimulated BMDMs from WT and Angptl4Tg mice showed that oxLDL mildly induced Ccl2 and markedly suppressed Ccr2 and Il1b expression (Figure VI in the online-only Data Supplement), consistent with a previous study. No major differences in gene expression were observed between WT and Angptl4Tg macrophages. A trend toward reduced expression of Tnf was observed in Angptl4Tg macrophages.

Angptl4 and MRI-Derived Measurements of Atherosclerosis

Finally, to evaluate the potential role of Angptl4 in atherosclerosis development in humans we determined whether plasma ANGPTL4 levels are associated with carotid atherosclerosis as measured by MRI. Clinical characteristics and MRI results are given in Table II in the online-only Data Supplement. The median plasma ANGPTL4 level was 6.75 ng/mL and ranged from 3.30 to 13.40 ng/mL. Median (inter quartile range) common maximal vessel-wall thickness was 1.50 mm and median (inter quartile range) common vessel-wall area was 1.68 cm².

Remarkably, plasma ANGPTL4 showed a significant negative correlation with an early marker of the degree of focal plaque formation measured by common maximal vessel-wall thickness ($r=-0.242$, $P=0.041$; Figure VII in the online-only Data Supplement). Multiple regression analysis with common maximal vessel-wall thickness as dependent variable showed that the association with ANGPTL4 was still significant ($\beta=-0.226$; $P=0.045$) after correcting for age, smoking, blood pressure, waist circumference, glucose, high-density lipoprotein cholesterol, triglycerides, and hsCRP. The model explained 26% of variance of the common maximal vessel-wall thickness. No relation was observed between plasma ANGPTL4 and common vessel-wall area ($r=-0.126$; $P=0.292$).

Discussion

The data presented show that Angptl4 reduces foam cell formation and decreases atherosclerosis in atherosclerosis-prone E3L mice. This effect was not caused by reduction of plasma cholesterol and triglycerides, because levels were similar between the 2 groups. Importantly, Angptl4Tg.E3L mice exhibited a less proinflammatory phenotype, with decreased accumulation of monocytes/macrophages in the atherosclerotic plaque, suggesting an anti-inflammatory role of Angptl4 in atherosclerosis development. Finally, we found that plasma Angptl4 is negatively associated with carotid artery sclerosis measured by 3-T MRI in subjects with the metabolic syndrome and low-grade systemic inflammation.

The impact of Angptl4 on atherosclerosis has been previously investigated. In that study Angptl4−/− mice on an ApoE−/− background developed less atherosclerotic lesions on a chow diet compared with control mice. It should be realized
that ApoE−/− mice are characterized by a severe deficiency in clearance of VLDL remnants, develop severe atherosclerosis from birth, and have impaired innate immunity. In contrast, E3L mice only develop hyperlipoproteinemia after being fed a diet rich in fat and cholesterol, which we believe better mimics the lifestyle-dependent development of atherosclerosis in humans. Angptl4−/− mice on ApoE−/− background exhibited a decrease in circulating LDL-cholesterol and triglyceride levels, which very likely accounted for the improvement in atherosclerosis in that model. In our study on ad libitum fed mice, plasma VLDL/LDL-cholesterol, total cholesterol, and plasma triglycerides were minimally affected in Angptl4Tg. E3L mice compared with E3L mice after several weeks of feeding the atherogenic diet. The minor elevation of levels of plasma triglycerides and cholesterol in Angptl4Tg.E3L mice is consistent with the relative minor effect of Angptl4 overexpression on plasma triglyceride and cholesterol levels in fed mice, as opposed to fasted mice. Interestingly, the reduction in hypertriglyceridemia in Angptl4Tg.E3L mice on starting the Western-type diet coincided with the
development of hypercholesterolemia. Recently, it was shown that triglyceride-rich lipoproteins may interfere with the ability of Angptl4 to inhibit LPL, a property that may also extend to LDL. Accordingly, the hypercholesterolemia in the ApoE3Leiden transgenic model may interfere with the effect of Angptl4 on circulating triglycerides levels. In contrast, such a mechanism may be expected to have minimal effect on macrophage Angptl4 action in the atherosclerotic plaque.

Because the slight increase in VLDL levels would be expected to increase rather than decrease atherosclerosis, Angptl4 overexpression apparently reduces atherosclerosis via a mechanism independent of its effect on plasma lipid levels. Previously, we have shown that Angptl4 dramatically reduced foam cell formation in peritoneal macrophages incubated with triglyceride-rich lipoproteins. The present study indicates Angptl4 also inhibits oxLDL uptake by macrophages, presumably via a direct and an indirect mechanism: (1) by directly inhibiting oxLDL uptake by macrophages; and (2) by inhibiting lipid loading from triglyceride-rich lipoproteins and associated activation of macrophages, leading to downregulation of subsequent oxLDL uptake. Previously, it was found that externally added and endogenously produced LPL enhance binding and uptake of oxLDL in macrophages. Accordingly, it is plausible that Angptl4 inhibits oxLDL uptake via its effect on LPL. Supporting a stimulatory effect of macrophage LPL on atherosclerosis in vivo, macrophage-specific overexpression of LPL stimulated the formation of atherosclerotic lesions and accumulation of macrophage-derived foam cells, which occurred in the absence of any changes in circulating lipoproteins. Whereas the opposite was observed in macrophage-specific LPL knockout mice. Transgenic mice expressing catalytically active or inactive LPL were used to show that the noncatalytic bridging function of LPL is sufficient for its proatherogenic effect. Whereas Angptl4 was shown to potently inhibit macrophage LPL catalytic activity, it is unclear whether Angptl4 inhibits the bridging function of LPL. In the present study we found that Angptl4 more effectively reduced uptake of oxLDL compared with orlistat, which suggests that Angptl4 reduces oxLDL uptake via a combination of lipolysis-dependent and -independent mechanisms. Expression of oxLDL receptors Cd36 and Msr1 was not different between Angptl4Tg and WT macrophages. Interestingly, expression of Abca1 was higher in Angptl4Tg and WT macrophages, indicating a potential effect of Angptl4 on cholesterol efflux. However, additional studies are required to better delineate the mechanisms of Angptl4 action in macrophages.

Besides lowering oxLDL uptake, Angptl4 may reduce atherosclerosis by reducing chemotaxis. Specifically, we found that Angptl4 overexpression led to decreased accumulation of monocytes/macrophages in the atherosclerotic plaque. Additionally, Angptl4Tg macrophages exhibited a decreased chemotactic response in an in vitro migration assay. No major differences in expression of chemotactic and inflammatory genes were observed between WT and Angptl4Tg macrophages at baseline or on stimulation with oxLDL. In addition, no in vivo effect of Angptl4 overexpression could be observed on expression of chemotaxis and adhesion-related genes in aortas. Angptl4 thus might influence chemotaxis independently of any changes in gene expression.

Evidence has been provided that oxLDL may trigger inflammatory signaling in mouse macrophages via a mechanism involving TLR4. Because TLR4 activation by LPS reduced Angptl4 expression in mouse macrophages, it is unlikely that the stimulatory effect of oxLDL and lipid emulsion on Angptl4 expression is mediated by TLR4. Instead, induction of Angptl4 by oxLDL may occur via PPARs, which are potent activators of Angptl4 expression in macrophages and are activated by oxidized lipoproteins or their component oxidized lipids. We found that activation of especially PPARδ led to a dramatic induction of ANGPTL4 protein and mRNA in THP-1 macrophages. At the functional level, induction of Angptl4 in intimal macrophages by oxidized lipoproteins may be a protective mechanism to reduce foam cell formation and mitigate anti-inflammatory responses.

Expression of Angptl4 in mouse RAW 264.7 macrophages was markedly reduced by TLR2/6, TLR3 and TLR4 agonists, as well as by the NOD2 agonist muramyl dipeptide. These results are consistent with a recent study showing suppression of Angptl4 mRNA by LPS, zymosan, polycl, and imiquimod. How TLR activation leads to downregulation of Angptl4 expression requires further study.

In conclusion, the present study reveals a protective role of Angptl4 in atherosclerosis development, which is independent of changes in levels of plasma lipoproteins. Furthermore, the study suggests an inhibitory effect of Angptl4 on macrophage oxLDL uptake and chemotaxis. We postulate that stimulation of Angptl4 gene expression in macrophages by oxLDL may be part of a protective feedback mechanism aimed at minimizing lipid overload.

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Disclosures
None.

References
Angiopoietin-like protein 4 (Angptl4) is a secreted factor that reduces lipid uptake in cells by inhibiting the enzyme lipoprotein lipase. Macrophages are immune cells that accumulate in atherosclerotic plaques and take up oxidized low-density lipoprotein to become foam cells. This article shows that oxidized low-density lipoprotein stimulates Angptl4 production by macrophages. Angptl4 protein was detectable in human atherosclerotic lesions and localized to macrophages. Overexpression of Angptl4 in an atherosclerosis-prone mouse model suppresses development of atherosclerosis. It is concluded that stimulation of Angptl4 in macrophages by oxidized low-density lipoprotein protects against excess lipid uptake and foam cell formation.
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MATERIAL and METHODS

Materials

Intralipid was purchased from Fresenius Kabi and used at a final concentration of 2 mM. Recombinant ANGPTL4 (full length 4487-AN) was from R&D Systems and used at a concentration of 1.5 μg/mL. Different TLR agonists were from Sigma, Fluka, Brunwich and InvivoGen and used at the following concentrations: Lipopolysaccharide (LPS, TLR4) 1 μg/mL; S-(2,3-bispalmitoyloxypropyl)-Cys-Gly-Asp-Pro-Lys-His-Pro-Lys-Ser-Phe, Pam2CGDPKHPKSF (FSL-1,TLR2/6) 1 μg/mL; Pam3-Cys-Ser-Lys4 (Pam3cys,TLR1/2) 1 μg/mL; Flagellin (Flag,TLR5) 10 ng/mL; Polyinosinic-polycytidylic acid (polyI:C,TLR3) 2 μg/mL; Muramyl dipeptide (MDP,NOD2); 10 μg/mL; Lipoteichoic acid (LTA,TLR2). Wy14643 was obtained from Eagle Picher Technologies laboratories. GW510516 and rosiglitazone were from Alexis Biochemicals. Orlistat was from Sigma.

Animals

Animal studies were done using wild-type (WT) and Angptl4Tg mice\textsuperscript{1,2} crossbred with ApoE\textsuperscript{*3}-Leiden (E3L) mice\textsuperscript{3}, all on a C57Bl/6J background, referred to as E3L and Angptl4Tg.E3L. Mice were housed under standard conditions with a 12 h light/dark cycle and free access to food and water. Female mice were fed a Western type diet with 15% w/w cacao butter (diet T, Hope Farms, Woerden, Netherlands) supplemented with 0.4% cholesterol, (Sigma-Aldrich, Zwijndrecht, Netherlands).

Plasma Lipids

Blood was collected from non-fasted mice in the middle of the light cycle into EDTA containing tubes. Blood samples were placed on ice and centrifuged at 4°C for 10 min at 10,000g. Plasma triglycerides and cholesterol were determined as described previously\textsuperscript{4}. 
**ANGPTL4 Elisa**

ANGPTL4 was measured exactly as detailed previously⁵.

**Lipoprotein Profiling**

Plasma lipoprotein profiling was carried out as described previously⁴.

**RNA isolation and qRT-PCR**

RNA isolation and qPCR were carried out as described previously⁶. The primers used are listed in Supplemental Table I.

**Oil red O Staining**

Oil red O stock solution was prepared by dissolving 0.5 g oil red O (Sigma) in 500 mL isopropanol. Oil red O working solution was prepared by mixing 30 mL oil red O stock with 20 mL dH₂O, followed by filtration. Attached cells were washed twice with PBS and fixed for 10 min and fixated for 10 min in formal calcium (4% formaldehyde, 1% CaCl₂). After that cells were washed twice with PBS and covered with Oil Red O working solution for 20 min, followed by two rinses with dH₂O.

**Lipoprotein Isolation and Radiolabelling**

Human VLDL and LDL were isolated at their respective densities after density gradient ultracentrifugation at the respective density at 40,000 rpm in a Ti-40 fixed-angle rotor (Beckman Instruments, Geneva, Switzerland) overnight at 4°C, followed by dialysis at 4°C overnight against PBS, pH 7.4. VLDL and LDL were isolated and radiolabeled with glycerol tri[^3]H]oleate ([^3]H]TO) and[^3]H]cholesteryl oleoyl ether ([^3]H]COEth), respectively. The
labelling protocol has been previously described. Protein concentrations in the lipoprotein fractions were determined with BCA Pierce assay using a BSA standard.

**LDL Oxidation**

LDL was oxidized with 20 μM CuSO₄ for 3 h at 37°C. Oxidation was terminated by cooling on ice and by adding 200 μM EDTA. EDTA and CuSO₄ were removed by overnight dialysis at 4°C against PBS, pH 7.4.

**Quantification of Cellular Uptake of Radiolabeled Lipoproteins**

After incubation of macrophages with radiolabeled lipoproteins, cells were washed twice with 500 μL PBS and cell lysates were obtained by adding 500 μL of 0.1 M NaOH, followed by gentle shaking for 15 min. 250 μL of cell lysates was used for quantification of ³H-radioactivity. Dpm values were normalised to the total amount of protein (mg) present in 250 μL of cell lysates. Protein concentration in cell lysates was quantified with BCA Pierce assay.

**Atherosclerosis quantification and plaque composition**

After 24 weeks on diet T with 0.4% cholesterol, final blood collection was drawn from the eye after the mice had been anaesthetized with injection of VDF (Vetranquil, Dormicum, Fentanyl). Mice were killed by CO₂ inhalation and perfused with ice-cold PBS via the heart. Hearts were isolated and later fixed in phosphate-buffered 4% formaldehyde, dehydrated, embedded in paraffin, and cross-sectioned (5 μm), throughout the aortic root area. Twelve sections per mouse with 50 μm intervals were used for atherosclerosis measurements. For further histological analysis, sections were stained with hematoxylin-phloxin-saffron (HPS). Lesions were categorized for severity according to the guidelines of the American Heart Association, adapted for mice. Briefly, the lesion criteria were: type 0 (no lesion), type 1
(early fatty streaks containing up to 10 foam cell macrophages in the intima), type 2 (early fatty streaks with more than 10 foam cell macrophages in the intima), type 3 (early fatty streak, containing foam cells in the intima, covered by a fibrous cap), type 4 (advanced lesions with foam cell macrophages in the intima, signs of fibrosis, lipid core, but no disruption of the media), and type 5 (advanced lesions containing foam cells in the media, presence of fibrosis, cholesterol clefts, mineralization, and/or necrosis). Lesion area was determined with Leica Qwin image analysis software (Image J). AIA 31240 antiserum (1:3000, Accurate Chemical and Scientific, Westbury, NY) was used to quantify the macrophage area and the number of monocytes adhering to the endothelium. In each segment used for total lesion area, the number of monocytes adhering to the endothelium in the aortic root area was counted. Sirius Red was used to quantify the collagen area and M0851 (1:800, Dako) against smooth muscle cell actin to quantify the smooth muscle cell area. Quantification of the slides was performed by an investigator unbeknownst with their identity. Human atherosclerotic plaques were collected from 12 patients eligible for surgical carotid endarterectomy (CEA) during surgery and immediately transported to the laboratory, where they were dissected into segments representing the core of the plaque (defined as the site where the specimen is the thickest), the shoulder of the plaque, and adjacent tissue. The study was approved by the Medical Ethical Committee of the hospital and all patients gave a written informed consent. Sections from human carotid arteries embedded in paraffin, were cross sectioned (5 µm) and used for histological analysis. A general hematoxylin-eosin (HE) staining, to visualize the structure of the tissue was performed. Human Antibody for CD68 staining was purchased from AbD Serotec. The antibody against human ANGPTL4 was raised in rabbit and previously used successfully to visualize ANGPTL4 in the human heart\(^1\). The first day, sections were left overnight at 37°C. The second day, deparafinization of the sections was performed, followed by inhibition of endogenous peroxidases for 30 min using
10% H₂O₂ in PBS. Antibody for CD68 and for ANGPTL4 was used in dilution 1:100. Staining with primary antibody was performed overnight. Negative control was not incubated with primary antibody. Secondary antibody was used in dilution 1:200 and it was applied on the sections for 45min. Visualization of the complex was done with DAB staining. Hematoxylin staining was performed as well. Areas of positive staining obtained a brown colour.

**Migration assay**

QCM™ Chemotaxis 96 well (5 mM) Cell Migration Assay Fluorimetric assay from Millipore was used. Boyden chambers with filters of 5 µm pore size were used. Mouse bone marrow cells were differentiated into macrophages for 9 days in Dulbecco’s modified eagle medium (DMEM), containing 10% heat inactivated fetal calf serum (FCS) and 20% L929 conditioned medium. Medium was changed every day after day 9 and cells were used at day 11. Before the migration assay cells were starved from FCS for 24h (medium containing only 1.5% of FCS derived from L929 medium). At the upper part of the filter we added 2x10³ cells in 100 µL serum-free DMEM with additional 1% BSA. At the bottom part of the filter, serum free DMEM with additional 1% BSA was added, with or without mouse recombinant MCP-1 (5 µg/mL). Migration was stopped after 3 h. Cells attached at the bottom of the filter were collected after incubation with a detachment buffer provided with the kit and pooled with cells that migrated towards the MCP-1 containing medium at the bottom chamber. A mix of lysis buffer/DNA binding fluorescent dye (CyQuant GR Dye) was added on the migrated cells for 15 min at RT. Fluorescence was measured at 480/520 nm.
Cell Culture

Bone marrow cells from WT and Angptl4Tg mice were grown in DMEM containing 10% heat-inactivated FCS and 1% penicillin/streptomycin (P/S) with additional 30% L929 conditioned medium to stimulate macrophage differentiation. Differentiation was allowed up to 7-9 days. During that period fresh medium was added to the cells without removing the old medium. After that period medium was renewed every day. Bone marrow derived macrophages (BMDMs) were used for experiments between day 7 and 11.

Human THP-1 monocytes were grown in RPMI medium containing 10% heat-inactivated FCS and 1% P/S for 10-15 passages. They were differentiated into macrophages after 2 days incubation by adding 100 ng/mL phorbol-12-myristate-13-acetate (PMA). After 2 days, PMA was removed by washing with PBS and macrophages were kept in complete medium without PMA for 2 additional days and then used for experiments. In one experiment, THP-1 macrophages were treated with agonists for PPARα (Wy14643, 1 μM), PPARδ (GW501516, 0.5 μM), and PPARγ (rosiglitazone, 0.5 μM) for 6h.

Mouse RAW 264.7 cells were grown in DMEM supplemented with 10% FCS and 1% P/S.

Mouse peritoneal macrophages were elicited in C57Bl/6 mice by intraperitoneal injection with 1 mL 4% thioglycolic acid. After 3 days, mice were killed with CO₂. The macrophages were collected from the peritoneal cavity by washing with 10 mL DMEM supplemented with 1% P/S. Erythrocytes were removed by incubating the cell pellets with red blood cell lysis buffer (RBC) for 5 min on ice. After removal of the RBCs, the cells were suspended in warm DMEM with 10% heat-inactivated FCS and seeded at a density of 3x10^5 cells/cm² and incubated at 37°C in a humidified 5% CO₂ incubator. 3 hours post plating, the cells were
washed twice with warm PBS to remove non-adherent cells. Attached macrophages were kept always in fresh medium for two more days, before the experiments. Mouse peritoneal macrophages isolated after 24 weeks of Western type diet feeding from E3L and Angptl4Tg.E3L mice were unelicited.

**Human Study Design and Subjects**

We studied 72 male subjects (mean age 57±5 years) with the metabolic syndrome [defined using International Diabetes Federation criteria] and low grade inflammation, defined as moderately elevated high sensitive C-reactive protein levels (1.8 mg/L ≤ hsCRP < 15 mg/L). The subjects for this study partly overlap with participants in a previous study describing the relation between CRP levels and atherosclerosis. Exclusion criteria were: diabetes mellitus (fasting glucose >7 mmol/L), manifest cardiovascular disease, the use of lipid lowering medication or non-steroidal anti-inflammatory drugs, alcohol abuse, contra indications for MRI and a BMI > 40 kg/m². All subjects gave informed consent and the study protocol was approved by the institutional review committee. Further information on anthropometric and laboratory measurements, magnetic resonance imaging of carotid atherosclerosis and statistical analysis of plasma ANGPTL4 levels and MRI derived measurements of atherosclerosis are provided in extended methods.

**Human Anthropometric and Laboratory Assessments**

Medical history and physical examination were performed on the same day. Blood samples were drawn after an overnight fast and chemical lab assessments were performed in the hospital laboratory. Blood pressure was measured using an automatic blood pressure monitor (Omron 705IT, Hoofddorp, Netherlands). The average of three readings was used to calculate blood pressure. The high-sensitive CRP assay was performed with the Tina Quant C-reactive
protein (latex) high sensitive assay (Roche, Basel, Switzerland). Subjects with hsCRP levels >15 mg/L were regarded as having an inter-current infection and were not included.

**Magnetic Resonance Imaging of Carotid Atherosclerosis**

Imaging of the left carotid vessel wall was performed on a 3-T MRI (Philips, Achieva, Best, The Netherlands) as previously described\(^{10}\). VesselMASS software package developed at our institution was used to analyze images of the carotid artery. Ten images, covering 2 cm of the left common carotid artery and bulbus were produced. Atherosclerosis was assessed by the following derived parameters: maximal vessel wall thickness (MVT), calculated by the mean of the thickest of 6 equal segments per image for common carotid artery (commonMVT). Moreover, vessel wall area (VWA) of the common carotid artery (commonVWA).

**Statistical Analysis**

Data are expressed as means±standard deviation or as median (interquartile range). Plasma ANGPTL4 levels and MRI derived measurements of atherosclerosis were not normally distributed (Kolmogorov-Smirnov \(p>0.05\)). The correlation between VWA and MVT and plasma ANGPTL4 was tested by Spearman’s correlation analysis. To investigate the possible confounding effect of age, waist circumference, smoking, triglycerides and high density lipoprotein cholesterol, multivariable regression analysis was performed for the association between ANGPTL4 and MRI derived measurements of atherosclerosis. Logarithmically transformed MRI derived parameters as dependent variables were applied in this analysis. We used SPSS for Windows (version 17.0; SPSS, Chicago, Illinois, USA) for statistical analysis. \(P < 0.05\) was considered statistically significant.
References


Supplemental Table 1: Sequences of primers used for qRT-PCR.

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<th>Primer Name</th>
<th>Forward sequence</th>
<th>Reverse sequence</th>
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<td>ANGPTL4</td>
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<td>CYCLOPHILIN</td>
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**Supplemental Table II:** Clinical and MRI parameters in male (n=72) with the metabolic syndrome and low grade inflammation. Values represent mean ± standard deviation or median (interquartile range).

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
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<tbody>
<tr>
<td>Age, y</td>
<td>57 ± 5</td>
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<tr>
<td>Waist circumference, cm</td>
<td>108 ± 8</td>
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<tr>
<td>Body mass index, kg/m²</td>
<td>29.4 ± 2.9</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>148 ± 5</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
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<tr>
<td>Fasting blood glucose, mmol/l</td>
<td>5.3 ± 0.8</td>
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<tr>
<td>Total cholesterol, mmol/l</td>
<td>5.81 ± 0.92</td>
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<tr>
<td>Low density lipoprotein-cholesterol, mmol/l</td>
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<tr>
<td>High density lipoprotein-cholesterol, mmol/l</td>
<td>1.26 (1.04-1.48)</td>
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<td>Triglycerides, mmol/l</td>
<td>1.78 (1.37-2.42)</td>
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<tr>
<td>hsCRP, mg/l</td>
<td>2.70 (10.95-1.40)</td>
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<tr>
<td>ANGPTL4 ng/ml</td>
<td>6.75 (5.30-8.10)</td>
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</table>

**MRI derived measurements of atherosclerosis**

<table>
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<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CommonVWA, cm²</td>
<td>1.68 (1.45-1.97)</td>
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<tr>
<td>CommonMVT, mm</td>
<td>1.50 (1.38-1.69)</td>
</tr>
</tbody>
</table>

Abbreviations; hsCRP: high sensitive C-reactive protein, MVT: maximum vessel thickness, VWA: vessel wall area.
Supplemental figures

**Supplemental Figure I: Lipid loading of macrophages by intralipid.** (A) Oil red O staining of RAW 264.7 mouse macrophages treated with 2 mM intralipid for 6h. (B) Oil red O staining of mouse bone-marrow derived macrophages treated with 2 mM intralipid for 6h.

**Supplemental Figure II: Angptl4 suppression by PPR agonists is only partially mediated via NF-κB.** (A) Effect of various PRR agonists on mRNA expression of Angptl4, Tnf, and Il1b in mouse BMDMs in presence and absence of NF-κB inhibitor JSH-23. (B) Effect of various PRR agonists on IL-6 release in mouse BMDMs in presence and absence of NF-κB inhibitor JSH-23. BMDMs were pre-incubated with 30 μM JSH-23 for 4h, followed by 0.1 μg/mL LPS, 1 μg/mL FSL-1 or 1 μg/mL Pam3Cys for 18h. Error bars represent SD. Differences between Vehicle and JSH-23 were evaluated by Student’s t-test (* indicates P-value < 0.05).
Supplemental Figure III: Expression of selected genes in BMDM treated with oxLDL. (A) mRNA expression of Abcg1 and Msr1 in BMDMs from WT and Angptl4-Tg mice treated with 25 μg/mL oxLDL for 24h. Error bars represent SEM. Differences between control and oxLDL-treated BMDMs were evaluated by Student’s t-test (* indicates P-value<0.05).

Supplemental Figure IV: ANGPTL4 protein localizes with macrophages in the human arterial wall. Staining of serial sections of human carotid artery. Top left, top middle: Hematoxilin and eosin (HE) staining of sections of human carotid artery to inspect the structural morphology of the arterial wall. Arrows indicate lipid-loaded macrophages (foam cells). Top right: Staining with antibody against human CD68. Bottom right: Staining with antibody against human ANGPTL4. Bottom left: negative control (only second antibody).
Supplemental Figure V: expression of Angptl4 and selected genes in E3L and Angptl4Tg.E3L mice. (A) qPCR analysis of Angptl4 mRNA in liver and gonadal adipose tissue of E3L and Angptl4Tg.E3L mice fed Western diet containing 0.4% cholesterol for 24 weeks. (B) qPCR analysis of selected genes in aortas of E3L and Angptl4Tg.E3L mice fed Western diet containing 0.4% cholesterol for 24 weeks. Error bars represent SEM. Differences between E3L and Angptl4Tg.E3L mice were evaluated by Student’s t-test (* indicates P-value<0.05).

Supplemental Figure VI: Expression of selected genes in BMDM treated with oxLDL. (A) mRNA expression of selected genes in BMDM from WT and Angptl4-Tg mice treated with 25 μg/mL oxLDL for 24h. Error bars represent SEM. Differences between control and oxLDL-treated BMDM were evaluated by Student’s t-test (* indicates P-value<0.05).
Supplemental Figure VII: Plasma ANGPTL4 levels are associated with carotid atherosclerosis as measured by magnetic resonance imaging (MRI). Relation between plasma ANGPTL4 and mean vessel wall thickness of the common carotid artery (r= -0.242, P=0.041).