The interplay between cholesterol and inflammation in the evolution of atherosclerosis

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Chapter 9

Summary & General Discussion

Atherosclerosis is a complex multifactorial disease of the large arteries and the leading cause of morbidity and mortality in industrialized nations.[1] There is ample evidence that hypercholesterolemia (i.e. elevated plasma levels of VLDL and LDL) is a major causative factor in atherogenesis.[2, 3] However, despite the success of statins in effectively lowering cholesterol levels and reducing cardiovascular causes of death, two thirds of the statin-treated patients still experience adverse coronary events.[4] This suggests that factors other than dyslipidemia play a role in the development of atherosclerotic diseases. Besides 'classical' risk factors such as hypertriglyceridemia, low high-density lipoprotein (HDL) and hypertension, inflammation has recently been recognized as a major driving force of lesion development.[3, 5] While the lipid component that contributes to the development of atherosclerosis is relatively well-understood, the origin and exact contribution of the inflammatory component remains largely unknown.

This thesis defines the origin of the inflammatory component under hypercholesterolemic conditions and delineates the role of inflammation in atherosclerotic disease progression and regression. Evidence is provided that the liver is an important source of inflammation; quenching of the inflammatory component of the disease has an additional beneficial effect and reduces atherosclerosis beyond the effect achieved by lipid-lowering alone.

Dietary cholesterol is an inducer of the inflammatory component of atherosclerotic disease

Recent observations suggest that the liver plays a key role in the inflammatory response that is evoked by dietary constituents and that contributes to atherosclerotic disease.[6, 7] For example, consumption of cholesterol-rich diets can result in elevated plasma levels of liver-derived inflammatory markers such as C-reactive protein (CRP) and Serum amyloid A (SAA).[7] These inflammatory markers are not only associated with future cardiovascular risk but also can participate actively in pathological processes in the vasculature.[8, 9] In **chapter 2** we investigated the effect of increasing doses of dietary cholesterol on liver responses, using a humanized mouse model for atherosclerosis, ApoE*3Leiden (E3L) mice. Our data showed that feeding E3L mice a Western-type diet containing a low dose of cholesterol (0.25% w/w; LC-group) mainly increased plasma lipid levels and only slightly induced atherosclerosis after 10 weeks of treatment. At a higher dose of dietary cholesterol (1.0% w/w; HC-group), plasma lipid levels further increased, an effect that was accompanied by a significant increase in plasma inflammation markers and enhanced atherosclerosis.

Since liver-derived inflammation markers were elevated with HC but not LC feeding, we investigated the response of the liver to dietary cholesterol in more detail and in a comprehensive way by applying metabolomics/lipidomics and genome-wide transcriptomics technology. Our data demonstrated that the liver absorbs moderately increased doses of dietary cholesterol primarily by adjusting the expression level of genes involved in lipid metabolism. At high doses of dietary cholesterol, an inflammatory stress response is elicited. This hepatic inflammatory response is characterized by activation of inflammatory pathways and an up-regulation of pro-atherogenic candidate genes, among which cytokines and chemokines. The pro-atherogenic nature of some of these hepatic inflammatory factors has been demonstrated in animal models for atherosclerosis by others.[10-12]

A refined bioinformatical analysis revealed that four specific inflammatory pathways were switched on by the HC diet and that inflammation and cholesterol metabolism were molecularly linked via specific node points, e.g. transcription factors that regulate both cholesterol- and inflammation-associated genes. The interrelationship between cholesterol and inflammation was confirmed by the observation that intervention in the inflammatory component of the disease (blockage of NF-κB activation) not only reduced plasma levels of SAA but also the ones of cholesterol.

Taken together, chapter 2 demonstrates a) that dietary cholesterol is not only a lipid risk factor but also a trigger of hepatic inflammation and, as such, involved in the evolution of the inflammatory arm of atherosclerotic disease and b) that cholesterol metabolism and inflammation are interlinked processes.

In the following chapters, different approaches were applied to investigate the extent to which inflammatory processes contribute to the atherogenic process. First, anti-atherosclerotic compounds were screened in human-CRP transgenic (huCRPtg) mice to evaluate their anti-inflammatory potential. In a second step, compounds with marked anti-inflammatory effects were further examined in a humanized atherosclerosis model (E3L mice). In this model it was possible to analyze whether the anti-inflammatory property of a compound would contribute to a reduction of atherogenesis., In a last step, the impact of inflammation in cardiovascular and metabolic diseases was studied a) by genetically depleting a single pro-inflammatory factor (the cytokine macrophage migration inhibitory factor, MIF) in Ldlr-/- mice and b) by analyzing the involvement of MIF in human aortic biopsies of atherosclerotic and abdominal aneurismal disease.

Anti-atherogenic compounds with anti-inflammatory potential

The most sensitive inflammation marker to predict future cardiovascular risk in humans is C-reactive protein (CRP). CRP is expressed predominantly in liver and integrates and stabilizes the inflammatory signals of the relative unstable cytokines IL-1β, IL-6 and TNFα.[13-15] The use of huCRPtg mice allowed us to identify compounds with anti-inflammatory potential relevant for cardiovascular disease (CVD). Various compounds, among which HMG-CoA reductase inhibitors (statins), ligands of the nuclear hormone receptor PPARα and LXR, were tested, and their capacity to lower basal and cytokine-stimulated inflammation was assessed (**chapter 3**).

Our data provided evidence that statins such as atorvastatin and simvastatin, PPARα activators such as Wy14643 and fenofibrate, and a synthetic LXR-agonist such as T0901317 can reduce basal and IL-1β-induced huCRP gene expression in huCRPtg mice, independent of their lipid-modulating effect.

A subsequent analysis of the underlying molecular mechanism revealed that the anti-inflammatory action of the compounds had a common basis: all compounds up-regulated the cytosolic inhibitor of NF-κB, IκB, and thereby trapped p50-NF-κB in the cytosol. As a consequence, diminished nuclear translocation of p50-NF-κB occurred, which resulted in less nuclear p50-NFκB~C/EBPβ complexes and thereby in reduced huCRP gene transcription.[13]

The observations that cholesterol-lowering statins, PPARα activators and an LXR-agonist could reduce inflammation independently of their lipid-modulating effects, suggests that these compounds may reduce atherosclerosis more than can be expected by their lipid-modulating effect alone and that they would have added value in subjects with high grade of inflammation. Indeed, investigators in the CARE trial of secondary prevention found that the benefit of statins was greater among subjects with elevated hs-CRP levels.[16] It is also conceivable that the anti-inflammatory potential of statins may contribute to the observed reduction of CV events in normocholesterolemic patients, a finding that cannot be fully explained by the cholesterol-lowering properties of the drugs.[17, 18] The recently started JUPITER trial will address this topic in further detail and will assess whether statin treatment of normolipidemic patients is beneficial in primary prevention. In this trial, subjects with low levels of LDL-cholesterol who may be at risk because of a high inflammatory status (elevated hs-CRP levels) will be treated with a statin and hard endpoints (number of cardiovascular events) will be determined in the following years.[19]

The mouse studies described in chapters 4, 5 and 6 of this thesis address comparable research questions as the JUPITER trial, using a humanized mouse model for atherosclerosis, ApoE3Leiden mice (E3L). E3L mice display a lipoprotein profile comparable to that of patients suffering from dysbetalipoproteinemia, i.e. plasma cholesterol and triglycerides are mainly confined to the very-low density lipoprotein (VLDL)/LDL fraction, and respond to hypolipidemic drugs in a similar way as humans (reviewed in [20]). We investigated whether compounds with anti-inflammatory properties identified in chapter 3 would reduce atherosclerosis more than can be expected by their lipid-modulating activities alone. The E3L model allowed us to study a potential additional anti-atherosclerotic effect under experimental conditions of lesion progression and lesion regression.

Impact of reducing inflammation on top of lowering plasma lipids

The E3L mouse model is unique in its human-like sensitivity to hypolipidemic drugs, a feature that distincts this model from the classical mouse atherosclerosis models (Ldlr-/- and ApoE-/- mice). In E3L mice, plasma cholesterol levels can be titrated to a desired level by adjusting their dietary cholesterol intake. This possibility of the model enabled us to compare drug-treated animals with a cholesterol-matched control group and thereby assess putative additional effects of the test compound on top of cholesterol-lowering. Three test compounds (with different mode of action) were studied: a fibrate (PPARα agonist), a statin (HMG-CoA reductase inhibitor) and an LXR-agonist.

Fibrates are potent lipid-lowering drugs that are used to treat hypertriglyceridemia and mixed dyslipidemia.[21] They exert their activity partly via activation of the nuclear receptor peroxisome proliferator-activated receptor α (PPARα). Independent of their lipid-lowering activity, PPARα-agonists can also act as a negative regulator of pro-inflammatory genes via antagonizing the activity of inflammatory transcription factors.[13, 22, 23] We showed **(chapter 4)** that fenofibrate, applied at a dose at which strong anti-inflammatory effects were observed in huCRPtg mice, reduces atherosclerosis more than can be explained by its cholesterol-lowering effect alone. The anti-atherogenic effects of fenofibrate that contributed to the additional reduction of atherosclerosis involved a reduction of local aortic inflammation (e.g. reduced vascular expression of NF-κB, monocyte adherence and macrophage content), a reduction of liver-derived inflammatory factors (e.g. SAA,

fibrinogen), an up-regulation of plasma ApoE levels, and an enhanced vascular expression of genes involved in reverse cholesterol transport (e.g. ABCA1 and SR-B1).
A recent human study using fenofibrate at a dose comparable to the dose used in this mouse study demonstrated that the observed reduction of plasma inflammation can also be relevant for the human situation. [23] Fenofibrate-treated patients displayed reduced plasma levels of inflammatory markers CRP, SAA, fibrinogen, plasminogen and α2-macroglobulin when compared to placebo control.

Statins are inhibitors of HMG-CoA reductase that effectively lower LDL cholesterol levels and that reduce the risk of cardiovascular events significantly.[24] In the past few years, it has become clear that statins can also exert pleiotropic effects, i.e. effects that are not directly related to the reduction of LDL cholesterol levels. It is unclear whether these effects may contribute to reduction of atherosclerosis in humans. Pleiotropic effects have been reported in mice using high statin concentrations, i.e. in cases where very strong reductions of plasma cholesterol and inflammation were achieved.[25] In **chapter 5** we investigated whether atorvastatin, when applied at a relatively low dose (equaling 10 mg/day in humans), exerts pleiotropic effects and whether these may contribute to a reduction of already established atherosclerotic lesions. We demonstrate that a moderate reduction of plasma cholesterol (about 20 %) and a strong reduction of the inflammation marker SAA (about 50%) by atorvastatin, results in an anti-atherogenic effect in the aortic root (i.e. the part of the aorta that represents the most advanced stage of the disease).[26] The effect in the aortic root could fully be ascribed to atorvastatin's cholesterol-lowering effect. Analysis of the aortic arch, the part of the aorta which represents the early stage of lesion development, revealed that atorvastatin reduced atherosclerosis more than can be explained by its cholesterol-lowering effect. An important process in early lesion development is the adhesion and migration of monocytes into the vessel wall.[26, 27] A subsequent analysis of factors that mediate monocyte recruitment showed that the additional effect of atorvastatin in the aortic arch may at least partly be explained by a reduced expression of vascular cell adhesion molecule-1 (VCAM-1).

Agonists for the Liver-X-Receptor (LXR) are relatively new pharmaceutical compounds. Genes that are controlled by LXRs encode for proteins that regulate intestinal cholesterol absorption, hepatic bile acid synthesis, cholesterol efflux and foam cell formation (through

up-regulation of ABCA1 cholesterol transporter and apoE in macrophages), and the inflammatory response.[28]

Experiments with huCRPtg mice treated with an LXR-agonist (T0901317) or not revealed that activation of LXR can result in a strong decrease of basal CRP and IL-1β–induced CRP stimulation **(chapter 3)**. Subsequent longitudinal analysis of the effects of T0901317 in a series of atherosclerosis experiments in E3L mice showed that LXR activation by T0901317 inhibited the initiation and progression of atherosclerotic plaques and promoted regression of lipid- and macrophage-rich existing plaques **(chapter 6)**. Importantly, T0901317 exerted these anti-atherosclerotic effects in absence of lowering cholesterol and despite a substantial increase in plasma triglycerides.

T0901317 blocked atherosclerosis progression at the initial stage and this was, at least partially, due to a local anti-inflammatory activity in the vasculature (e.g. suppression of adhesion molecules and NF-κB). Under regressive conditions, T0901317 induced lesional macrophage disappearance. A refined molecular analysis showed that T0901317 may exert its anti-atherogenic effects by increasing the expression of the chemokine receptor CCR7, a factor functionally required for lesion regression, and by stimulating macrophage apoptosis. Our data support the view that specific targeting of local inflammatory processes in the vasculature may be a useful treatment strategy for atherosclerosis.

An undesirable effect of LXR activation is the up-regulation of genes that participate in lipogenesis[29, 30] leading to increased hepatic and plasma triglyceride levels.[31, 32] The LXR activator used in our experiments (T0901317) indeed induced the lipogenic pathway and increased plasma cholesterol and triglyceride levels resulting in an adverse lipoprotein profile (high levels of ApoB-containing lipoproteins). The lipogenic activity of LXR agonists represents a significant obstacle to the further development of these compounds for clinical applications. LXRα is the predominant LXR isoform expressed in the liver, and the ability of LXR agonists to stimulate hepatic lipogenesis is thought to result primarily from LXRα induction of SREBP1c and fatty acid synthase (FAS) expression.[33, 34] This suggests that partial or gene-specific LXR agonists which only increase reverse cholesterol transport, but not hepatic lipogenesis, would be a better therapeutics. A recent study demonstrates that separate activation of LXRα and LXRβ yields distinctive lipid outcomes *in vivo*. For example, a specific agonist of LXRβ increases ABCA1 expression in macrophages while keeping deleterious hepatic gene inductions low.[35] These results lend support to the idea that LXRβ-selective agonists may raise HDL-cholesterol and stimulate macrophage

cholesterol efflux without causing liver triglyceride accumulation, providing in vivo evidence for drug development strategies targeting LXRβ.

Taken together, the above experiments clearly demonstrated that it is possible to reduce atherosclerotic disease development beyond the effect that can be achieved by lowering cholesterol alone. Quenching of inflammation on top of lowering cholesterol is also relevant for reducing a further progression of already existing lesions and in atherosclerosis regression. To achieve an additional beneficial effect, compounds have to be employed at doses at which they exhibit anti-inflammatory effects. For identification of such compounds and their optimal dose, the human CRP mouse model may be very suitable in the future.

Impact of the pro-inflammatory cytokine MIF in cardiovascular and metabolic diseases

In the above chapters, compounds with specific molecular targets (PPARα, HMG-CoA reductase, LXR) were applied to asses the role of inflammation in atherogenesis. In **Chapter 7** we used another experimental approach, namely genetic deletion of a pro-inflammatory cytokine (Macrophage migration Inhibitory Factor; MIF) to reduce the inflammatory arm of the disease. MIF is known as a pleiotropic macrophage and T-cell cytokine[36] and endocrine factor[37] that is involved in the regulation of many inflammatory processes[38, 39] and diseases.[40] To investigate the role of inflammation in atherosclerosis development, mice deficient for MIF were cross-bred with mice deficient for the LDLreceptor (Ldlr-/-), resulting in MIF and Ldlr double knockout mice (Mif-/-Ldlr-/-). Ldlr-/- mice display a modestly elevated plasma cholesterol level and develop atherosclerosis when maintained on a regular chow diet. An additional feature of the Ldlr-/- mouse is the ability to develop insulin resistance in combination with atherosclerosis.[41] Therefore this model allowed us to extend our findings to another disease area in which chronic inflammation plays an important role, insulin resistance/type 2 diabetes.

Our data showed that genetic depletion of MIF did not affect plasma cholesterol and triglycerides levels when compared with their control littermates. Analysis of the inflammation status showed that the basal plasma level of SAA, a marker for (chronic) inflammation, was decreased in Mif-/-Ldlr-/- mice. In addition, when challenged with an inflammatory stimulator (IL-1β), the Mif-/-Ldlr-/- mice also displayed a strongly decreased inflammatory response. MIF thus controls the 'set point' and magnitude of the inflammatory response.

We further demonstrate that MIF enhances the influx of monocytes/macrophages in adipose tissue leading to an inflamed adipose tissue thereby promoting the development of insulin resistance. Remarkable parallels were found for the Mif-controlled processes regulating macrophage influx in adipose tissue and the Mif-controlled processes leading to macrophage recruitment in the vasculature.

Our data provide first evidenced for a role of MIF in the development of insulin resistance, glucose intolerance and associated atherosclerotic disease.

Importance of MIF in the human situation

Abdominal aortic aneurysms (AAA) are characterized by a massive accumulation of inflammatory cells and presence of inflammation-sustaining factors in the vascular wall.[42] Aneurysmal regions can dilate and eventually rupture as a result of extensive breakdown of structural proteins of the arterial wall by matrix metalloproteinases (MMPs).[43, 44] The molecular processes that contribute to evolution and rupture of AAA however are only poorly understood. As for atherosclerosis, the pro-inflammatory factor MIF may contribute to the pathogenesis of the disease, and elevated plasma MIF levels were found in patients suffering from AAA.[45] In **chapter 8**, we compared human biopsies of aortic atherosclerotic wall and AAA to gain insight into the role of MIF in disease evolution. When compared to healthy control aorta, mRNA and protein expression of MIF was up-regulated in human atherosclerotic aortic wall. MIF expression was also up-regulated in stable AAA and intensified in ruptured AAA. Immunohistochemical co-localization analyses provided evidence for an involvement of MIF in both pathologies and point to an inflammation-promoting effect of MIF on macrophages and MMP expression.

Conclusions

The studies of this thesis show that, besides plasma cholesterol, inflammation contributes to a substantial extent to atherogenesis. The results obtained also demonstrate that dietary cholesterol promotes atherosclerosis development in two ways: First, dietary cholesterol results in elevated plasma cholesterol levels and elevated levels of the atherogenic lipoproteins VLDL and LDL. Second, dietary cholesterol can evoke (hepatic) inflammation, which accelerates disease development.

The experiments performed herein also demonstrate that intervention in the inflammatory component of the disease is an effective strategy to diminish atherosclerosis progression, independent of cholesterol-lowering. Also, anti-inflammatory strategies have additive value

in lesion regression, i.e. the reduction of already existing lesions. Intervention strategies directed at lowering apoB-containing lipoproteins *and* reducing inflammation may therefore be more effective than current lipid-lowering strategies. Direct experimental evidence for this assumption mainly comes from animal experiments as described in this thesis. Human intervention studies are necessary to evaluate whether these findings can also be translated to the human situation. An important aspect of a successful anti-inflammatory therapy will be the choice of the molecular target. For example, only a few pro-inflammatory cytokines show explicit and consistent pro-atherogenic effects independent from the experimental approach and conditions chosen, while many others have a dual character and show variable effects.[46] Also, several of the pro-atherogenic cytokines affect plasma cholesterol levels and intervention in their action may have an adverse effect on plasma cholesterol. The chronic nature of the atherosclerotic disease process and the pleiotropic effects of individual inflammatory mediators will demand high specificity of action and/or effective targeting to prevent the emergence of adverse side effects with clinical applications.

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