Vascular inflammation and activation: new targets for lipid lowering

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Inflammatory cells, including macrophages, in atheroma overexpress matrix metalloproteinases (MMPs) and tissue factor which contribute to plaque rupture and thrombosis. Activated smooth muscle cells (SMCs) in the plaque’s fibrous cap also express MMPs and tissue factor. Lipid lowering appears to reduce the incidence of acute coronary events in patients by stabilizing atherosclerotic plaques. To improve mechanistic understanding, we tested the hypothesis that experimental manipulation of cholesterol level improves features of atheroma related to their propensity to provoke acute thrombotic complications. In rabbits with established atheroma, dietary lipid lowering reduced accumulation of macrophages expressing MMPs and increased collagen, a key determinant of plaque stability. Lipid lowering also decreased expression of tissue factor and its inducer, CD40 ligand. SMCs in the fibrous cap of rabbit atheroma expressed less MMP and tissue factor after lipid lowering. We have recently found that treatment with an HMG-CoA reductase inhibitor, cerivastatin, retards macrophage accumulation in atheroma of Watanabe heritable hyperlipidaemic (WHHL) rabbits, probably in part by suppressing proliferation. Macrophage expression of MMPs and tissue factor also decreased with cerivastatin treatment in vivo and in vitro. These results support the view that lipid lowering reduces acute thrombotic complications of atherosclerosis in patients by attenuating vascular inflammation.

Key Words: Lipid lowering, atheroma, atherosclerosis.

Introduction

Atherosclerosis is a multifactorial inflammatory disease. Factors involved in its pathogenesis include the accumulation of inflammatory cells, such as macrophages, the activation of vascular smooth muscle cells (SMCs) and endothelial cells (ECs) and oxidative stress. All of these factors may contribute to formation of vulnerable atherosclerotic plaques prone to acute thrombotic complications such as unstable angina and myocardial infarction[1–3]. Recent clinical studies have established that lipid lowering can reduce atherosclerotic events and prolong life in many patients[4–7]. Although our current understanding is that lipid lowering probably acts by stabilizing vulnerable plaques qualitatively or functionally, the precise cellular and molecular mechanisms remain unclear (Fig. 1). This article outlines the experimental evidence and suggests mechanisms by which lipid lowering improves vascular inflammation and activation responsible for plaque instability and thrombogenicity.

Macrophage accumulation, collagen loss and plaque vulnerability

Accumulating evidence makes it clear that coronary arteries with less severe angiographic stenosis (<50%) frequently precipitate acute coronary syndromes[1,2,8–10]. Pathological investigations have suggested that rupture of atherosclerotic plaques plays a key role in the pathogenesis of acute coronary events[11]. Plaques that rupture usually have a large, lipid-rich atheromatous core underlying a thin, collagen-poor fibrous cap (Fig. 1)[12]. The tensile strength of the plaque’s protective fibrous cap derives mainly from interstitial collagen fibrils[13]. Circumferential mechanical stress may cause rupture in the thinnest portion of the plaque’s fibrous cap with a low collagen content[13]. Rekhter et al. have recently reported that hypercholesterolaemia reduces mechanical strength in atheroma in rabbits, probably due to decreased collagen content[14]. Thrombus formation due to rupture of such vulnerable plaques with a thin fibrous cap is the most common cause of fatal acute myocardial infarction[1,15]. Thrombosis may also result from erosion of the atherosclerotic intima[16,17]. Changes in the expression of enzymes that degrade the extracellular matrix including interstitial collagen can influence the integrity of the fibrous cap[18]. Several
studies have shown that coronary lesions prone to rupture often contain a prominent accumulation of inflammatory cells such as macrophages\cite{16,19}. Activated macrophages within the atheromatous lesions overexpress matrix-degrading enzymes such as matrix metalloproteinases (MMPs) which may weaken the fibrous cap and promote plaque rupture. Tissue factor produced by macrophages may accelerate thrombus formation at the sites of rupture. Activated smooth muscle cells in the fibrous cap also express MMPs and tissue factor. Activated endothelial cells induce monocyte recruitment and macrophage accumulation in the intima. Lipid lowering probably reduces the incidence of acute coronary events by influencing the nature of the vulnerable plaque qualitatively or functionally (stabilization) rather than by merely shrinking the lesion or decreasing the stenosis (regression).

**Prothrombotic potential in atheroma**

Culprit atheroma causing acute events may also have an increased potential to promote thrombosis when they rupture\cite{32,33}. Macrophages in human atherosclerotic plaques can express abundant tissue factor, a potent activator of blood coagulation\cite{54,59}. Many cells in atheroma, including macrophages, express CD40 ligand (CD40L or CD154), a proinflammatory mediator, and its receptor, CD40\cite{40}. CD40 ligation markedly enhances tissue factor expression and activity in human monocyte/macrophages\cite{41}.
Smooth muscle cell activation

Smooth muscle cells in the atherosclerotic intima exhibit an immature or activated state compared with apparently normal SMCs in the tunica media. In human coronary lesions, intimal SMCs have decreased expression of differentiation/maturation markers such as myosin heavy-chain isoforms. In addition to macrophage accumulation, SMC activation in the fibrous cap also probably contributes to extracellular matrix degradation and plaque vulnerability. Intimal SMCs express higher levels of matrix-degrading enzymes, including MMPs and cathepsins, than do medial SMCs. Activated SMCs in the intima also overexpress tissue factor. Superficial erosion of the intima, rather than frank rupture into the lipid core, may cause some fatal myocardial infarctions. Tissue factor expression by SMCs in the fibrous cap may contribute to thrombus formation at such sites of erosion.

Endothelial cell activation and oxidative stress

Endothelial cell (EC) activation or dysfunction contributes to infiltration of inflammatory cells in atheroma. ECs overlying atheroma express leukocyte adhesion molecule 1 (VCAM-1), and chemoattractant molecules, including monocyte chemoattractant protein 1 (MCP-1). The latter substances probably participate in recruitment of monocytes from circulating blood into the vascular wall. Oxidative stress also promotes the pathogenesis of inflammatory diseases including atherosclerosis. Atherosclerotic arteries produce reactive oxygen species (ROS) such as the superoxide anion, which can promote oxidative modification of low-density lipoprotein (LDL). Oxidized LDL (oxLDL) accumulates in both human and rabbit atheroma and can alter the functions of vascular wall cells including ECs. For example, oxLDL and its derivatives increase expression of VCAM-1 and MCP-1 by ECs.

Effects of lipid lowering on atherosclerotic plaque stabilization

Results from recent clinical trials have indicated that lipid lowering by HMG-CoA reductase inhibitors (statins) can reduce acute coronary events. Angiographic studies, however, showed only modest reduction in stenosis of fixed lesions despite the consistent and substantial improvement of clinical outcomes. These findings suggested to us that lipid lowering may reduce acute thrombotic complications by functional 'stabilization' of the atherosclerotic plaques rather than by merely decreasing the stenosis. However, the precise mechanisms of these clinical benefits still remain unclear. We recently explored the effects of lipid lowering on inflammation and vascular cell activation in rabbit atheroma as indicators of potential mechanisms of atherosclerotic plaque stabilization.

Dietary lipid lowering reduces macrophage accumulation, proteolytic activity and prothrombotic potential in rabbit atheroma

We first examined changes in hypercholesterolaemic rabbit atheroma with lipid-lowering treatment by diet alone. We created aortic lesions in New Zealand White rabbits by an initial balloon injury followed by 4 months of an atherogenic diet. Fifteen rabbits were sacrificed to determine the nature of the baseline lesions. Of the remaining animals, 10 were given a normal diet for 16 months to reduce cholesterol levels, while the other five continued on the atherogenic diet.

Atheroma in the rabbit aorta after 4 months on the atherogenic diet showed that these baseline lesions contained numerous macrophages. After 16 months of lipid lowering by diet, however, few macrophages remained in the intima of these rabbits. In addition to reduced numbers of macrophages, dietary lipid lowering also reduced expression of proteolytic molecules. In the baseline aortic lesions, the macrophages overexpressed matrix-degrading enzymes including collagenase-1/MMP-1. The baseline atheroma revealed low levels of collagen accumulation resembling vulnerable human atheroma. Sixteen months of lipid-lowering diet yielded a substantial reduction in expression of MMP-1 and proteolytic activity ascribable to MMP-2, MMP-3 and MMP-9, paralleled by a marked increase in interstitial collagen content, a key contributor to plaque stability. Results from magnetic resonance imaging were also consistent with conversion of lipid-rich, vulnerable plaques into more fibrous plaques in these rabbits. Kockx et al. reported a similar result regarding increased collagen in cholesterol-fed rabbit atheroma during dietary lipid lowering.

Dietary lipid lowering also reduced the prothrombotic potential in rabbit atheroma. Macrophages in rabbit atheroma overexpressed tissue factor, a key trigger to thrombus formation, and its stimulators CD40L and CD40 after 4 months on the atherogenic diet. Furthermore, the baseline lesions contained binding sites for blood coagulation factors VIIa and X. Dietary lipid lowering produced a marked reduction in tissue factor expression and activity associated with decreased macrophage accumulation expressing CD40L and CD40. Taken together, these results indicate that dietary lipid lowering reduces the accumulation of proteolytic and prothrombotic macrophages in atheroma, suggesting a potential mechanism for the stabilization of atherosclerotic plaques.
Dietary lipid lowering improves smooth muscle cell activation in the fibrous cap of rabbit atheroma

Activated SMCs in the fibrous cap may also contribute to the instability of the lesions, as mentioned earlier. Like human coronary atheroma, the fibrous cap of hypercholesterolaemic rabbit aorta primarily contained immature SMCs, as gauged by decreased expression of smooth muscle myosin heavy-chain isoforms. In addition, macrophages present in the baseline lesions overexpressed platelet-derived growth factor B (PDGF-B), known to suppress SMC differentiation. Reduction in lipid levels favoured normalization of SMC phenotypes in the fibrous cap of rabbit atheroma. The 16 months of dietary lipid lowering decreased macrophage expression of PDGF-B and promoted accumulation of more mature SMCs in the fibrous cap, as determined by increased smooth muscle myosin expression and by ultrastructural analysis using electron microscopy. The increased accumulation of mature SMCs accompanied reduction in MMP-3 and MMP-9 expression. In the baseline lesions, SMCs in the fibrous cap showed tissue factor expression. However, lipid lowering reduced SMC expression of this pro-coagulant as well. These changes in SMC function associated with dietary lipid lowering suggest additional mechanisms for plaque stabilization.

Dietary lipid lowering reduces endothelial cell activation and oxidative stress

This article has discussed reduced macrophage accumulation by lipid lowering which favours plaque stabilization. However, mechanisms by which lipid lowering decreases macrophages in atheroma remain speculative. The initial steps in atherogenesis involve the adhesion of monocytes to the activated ECs followed by migration into the arterial wall, leading to macrophage accumulation. This process involves a number of steps, including expression of adhesion molecules and chemoattractants. Oxidative stress induces EC activation. We have recently found that dietary lipid lowering in rabbits can reduce oxidative stress and endothelial cell activation, suggesting potential mechanisms of decreased accumulation of proteolytic and prothrombotic macrophages. Figure 2 summarizes the concept of plaque stabilization by lipid lowering.

Lipid lowering by statins reduces macrophage accumulation in rabbit atheroma

As described above, lipid lowering by statins may stabilize atheroma in human patients. However, a number of studies have suggested that statins may also have other effects independent of their lipid-lowering actions. Ridker et al. have recently provided evidence for lipid-independent, anti-inflammatory effects of statin treatment.

Shiomi et al. have shown that reduction in lipid levels with statins retards progression of atheroma and alters lesional cell composition in WHHL rabbits, a model of endogenous hypercholesterolaemia due to a LDL-receptor deficiency. WHHL rabbits develop atherosclerosis spontaneously without high-cholesterol feeding. Reduction in serum LDL-cholesterol levels with pravastatin treatment significantly reduced lesion size, macrophage accumulation and extracellular lipid deposits compared with placebo. Shiomi and Ito recently showed that 32 weeks of cerivastatin treatment in WHHL rabbits reduced total cholesterol levels by 39% compared with placebo and improved atherosclerosis, as judged by the wall thickness and luminal stenosis. Cervastatin treatment also reduced macrophage accumulation and extracellular lipid deposits.

Effects of statins on macrophage proliferation and activation in vivo and in vitro

In addition to enhanced monocyte recruitment, macrophage proliferation may play an important role in the...
development of vulnerable atherosclerotic plaques\textsuperscript{[71–73]}. In collaboration with Shiomi's group, we recently explored the effects of cerivastatin treatment on macrophage growth in WHHL rabbits\textsuperscript{[62]}. As previously reported, 32 weeks of cerivastatin treatment reduced total cholesterol levels and the number of macrophages in atheroma. In situ hybridization for histone mRNA expression, a sensitive marker for cell replication\textsuperscript{[74]}, revealed that lipid lowering by cerivastatin significantly reduced macrophage growth in atheroma of WHHL rabbits compared with the control group\textsuperscript{[62]}. Special stains used to measure DNA fragmentation characteristic of cell death, including apoptosis, (the TUNEL technique) showed no difference in macrophage death. OxLDL can induce macrophage growth in vitro\textsuperscript{[75,76]}. Decreased LDL levels may limit oxLDL accumulation in atheroma and, in turn, suppress macrophage proliferation. Moreover, cerivastatin might affect macrophage division independent of cholesterol lowering. Sakai \textit{et al.} reported that simvastatin (0.1 \textmu M) and pravastatin (80 \textmu M) suppress oxLDL-induced macrophage growth in vitro\textsuperscript{[77]}. We therefore examined a potential direct effect of cerivastatin on macrophage proliferation using human monocyte-derived macrophages in culture\textsuperscript{[62]}. Cerivastatin (\textgtrless 10 nM) dose-dependently reduced the number of cultured macrophages. Cerivastatin suppressed DNA replication in cultured macrophages induced by macrophage colony stimulating factor (M-CSF) as shown by bromodeoxyuridine (BrdU) uptake. Supplying mevalonate and other non-steroidal intermediates (farnesyl pyrophosphate and geranylgeranyl pyrophosphate) in the mevalonate pathway reversed this inhibition. These results suggest that

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\caption{Reduced accumulation of macrophages expressing MMP-1 (collagenase-1) and tissue factor in atheroma of WHHL rabbits by cerivastatin treatment. (A) Aortic intima of WHHL rabbits contained prominent macrophage accumulation detected by anti-rabbit CD11b antibody. Immunoreactive MMP-1 and tissue factor colocalized with macrophages. In the intima of the aortas from WHHL rabbits treated with cerivastatin for 8 months, the number of macrophages and amount of immunoreactive MMP-1 and tissue factor decreased. The arrows indicate the tunica media. Scale bar: 200 \textmu m. Original magnification: \times 100. (B) Quantitative analysis of immunohistochemistry for macrophages, MMP-1 and tissue factor. Data for CD11b-positive areas is reported as areas (mm\textsuperscript{2}) within the intima. MMP- or tissue factor-positive macrophages are reported as a percentage of MMP-1- or tissue factor-immunoreactive areas within macrophage-containing regions. Bars represent SEM. (Adapted with permission\textsuperscript{[62].})}
\end{figure}
cerivastatin reduces macrophage growth through specific inhibition of the mevalonate pathway, not by cell injury or toxicity. Furthermore, this inhibition of macrophage growth occurred at concentrations achievable in humans after a single administration of 0.3–0.8 mg cerivastatin, whereas a number of previous in vitro studies on statins have employed somewhat high doses[78].

In atheroma of WHHL rabbits, cerivastatin not only diminished macrophage numbers but also reduced the percentage of macrophages expressing MMPs and tissue factor protein[62]. CD40L (CD154), which can enhance MMP and tissue factor expression in vitro[41,79], was also decreased in atheroma of WHHL rabbits by cerivastatin treatment[62]. These results suggest in vivo treatment of cerivastatin can suppress macrophage functions related to proteolytic and prothrombic potential (Fig. 3). Bellosta et al. reported that fluvastatin and simvastatin can reduce macrophage expression of MMP-9 in vitro[80]. In human monocyte-derived macrophages in culture, cerivastatin also prevented the increase in proteolytic activity by MMP-9 during differentiation of monocytes into macrophages[82]. Cerivastatin treatment also decreased tissue factor expression in cultured human macrophages. This result agrees with the previous study by Colli et al. which showed that fluvastatin and simvastatin decrease tissue factor expression and activity in a dose-dependent manner[61]. The suppression of tissue factor was accompanied by decreased tissue factor mRNA levels, indicating that the statins act by reducing gene expression.

Effects of statins on smooth muscle cells

The direct effects of statins extend beyond macrophages. Several studies have shown that statins can reduce proliferation of vascular SMCs in vitro[86]. Two studies showed that cerivastatin inhibited the replication of arterial smooth muscle cells from a range of animals, including rat and human, in a concentration-dependent manner[82,83]. Similar results have been observed with fluvastatin and simvastatin[84]. Laufs et al. recently suggested that simvastatin can inhibit SMC proliferation via prevention of rho-GTPase-induced decrease of p27[85]. Guijarro et al. showed that lipophilic statins (atorvastatin and simvastatin), but not the hydrophilic pravastatin, induced programmed cell death (apoptosis) in cultured rat vascular SMCs[86].

Such direct effects of statin therapy may prove useful in preventing intimal thickening after vascular injury or organ transplantation. However, in human chronic atherosclerotic lesions, SMCs proliferate quite slowly and can also die by apoptosis[77,87]. Therefore, aggressive inhibition of SMC growth or induction of SMC death may not favour stabilization of established atherosclerotic plaques, as SMCs furnish extracellular matrix macromolecules such as collagen, a key contributor to the mechanical integrity of the plaques. It is interesting that Shiomi and Ito have reported that cerivastatin
treatment reduced accumulation of macrophages, but not of SMCs, in aortic atheroma of WHHL rabbits[85]. This evidence suggests that it may prove possible to use doses that reduce atherogenesis related to macrophage functions while avoiding the potentially adverse effects of inhibiting SMC functions.

Summary and unanswered questions

This article has discussed the effects of lipid lowering in hypercholesterolaemic rabbit atheroma either by diet or by statin treatment so as to shed light on mechanisms by which lipid lowering may improve outcomes in patients. First, we demonstrated evidence that lipid lowering by diet alone can improve a number of features of rabbit atheroma and can also die by apoptosis[77,87]. Therefore, aggressive inhibition of SMC growth or induction of SMC death may not favour stabilization of established atherosclerotic plaques, as SMCs furnish extracellular matrix macromolecules such as collagen, a key contributor to the mechanical integrity of the plaques. It is interesting that Shiomi and Ito have reported that cerivastatin...
stabilization apply to patients still remains speculative. Moreover, the relative contribution of a lipid-lowering effect itself and of other lipid-independent effects to plaque stabilization may vary between statins. We should also keep in mind the likelihood of future discussion about whether the concentration of statins used in vitro and in vivo studies could reasonably apply in the clinical situation. A more complete understanding of the potential mechanisms of atheroprotective effects of lipid lowering by statins in patients will require further careful study (Fig. 4).

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Moreover, the relative contribution of a lipid-lowering effect itself and of other lipid-independent effects to plaque stabilization apply to patients still remains speculative. A more complete understanding of the potential mechanisms of atheroprotective effects of lipid lowering by statins in patients will require further careful study (Fig. 4).

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