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Evolution and Stabilization of Vulnerable Atherosclerotic Plaques

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Human coronary arteries, unlike those in most other mammals, have resident smooth muscle cells in the tunica intima underneath the endothelial monolayer, even before atherosclerotic changes begin.[1,2] The thickness of the intimal layer increases progressively as humans age from fetus to child to young adult, and its complex structure probably favors the formation of atheroma. When atherogenesis begins, the artery wall becomes host to inflammatory cells, including macrophages and T lymphocytes,[3] and an excess of risk factors accelerates the pathological changes in the artery.

Presently, the most understood risk factor is low-density lipoprotein (LDL). Epidemiological and clinical studies suggest that elevated levels of plasma cholesterol, especially LDL particles, increase the risk of acute coronary events.[4,5] Beginning with Anitschkow and Chalatow's description in 1913 of a diet rich in cholesterol that induced atherosclerosis in rabbits,[6] a number of animal studies have linked hypercholesterolemia to atherosclerosis.[7–9] In vitro studies, however, suggested that native LDL itself does not induce vascular cell activation and foam cell formation, the features that are related to atherosclerosis. In the late 1980s, the 'oxidized LDL' hypothesis postulated the missing link between hypercholesterolemia and atherosclerosis;[10] that is, excess LDL in the artery wall can be modified, which instigates an inflammatory response on the endothelial surface of the artery.[11] Endothelial activation, in turn, causes an infiltration of macrophages, one of the hallmarks of the atherosclerotic lesion.[12–15] Macrophage-rich atheroma, which are prone to rupture and thrombus formation, result in the onset of acute coronary syndromes such as unstable angina and myocardial infarction.[16–19]

Recent clinical and preclinical studies have repeatedly suggested that lipid lowering can stabilize these vulnerable plaques and improve clinical outcomes.[20] This review will discuss the current understanding of the biology of vascular inflammation, the pathophysiolgy of acute thrombotic complications, and the likely mechanisms responsible for the effects of lipid-lowering therapy.

Pathogenetic Mediators of the Development of Atherosclerosis

We are now beginning to appreciate the underlying molecular mechanisms associated with the presence of inflammatory cells, including macrophages, in the atheroma. Leukocyte attachment to the endothelium is the first step in recruiting mononuclear cells into the artery wall. Various leukocyte adhesion molecules expressed on the surface of the endothelial cells mediate this leukocyte attachment by engaging cognate ligands on the various classes of leukocytes. A combination of careful observation and traditional biochemistry, morphology, and cell biology has led to the definition of candidates for the various ways in which macrophages are recruited and activated during atherogenesis. Work with experimental atherosclerosis has yielded information that (1) implicates vascular cell adhesion molecule-1 (VCAM-1) in the adhesion reaction, among other adhesion molecules; (2) shows that monocyte chemoattractant protein-1 (MCP-1) is a potent chemoattractant that causes the directed migration of leukocytes; and (3) shows that macrophage colony stimulating factor (M-CSF) is a co-mitogen and activator that can cause the expression of scavenger receptors on macrophages (Fig 1).

Mediators in Monocyte Adhesion and Penetration

Vascular cell adhesion molecule-1 is an adhesion molecule of particular interest in the context of the development of atherosclerosis because it binds only monocytes and T-lymphocytes present in the nascent lesion.[21] VCAM-1 is expressed by the endothelium of nascent fatty streaks and by microvessels in the mature human atherosclerotic plaque,[22] and so it is a putative molecular mediator of the leukocyte adhesion reaction. Li et al, from our laboratory, showed that although normal rabbit endothelium did not express detectable levels of VCAM-1, expression of this adhesion molecule increased after 1 week of a high-cholesterol diet,[23] and VCAM-1-expressing endothelial cells with adherent leukocytes were present at 3 weeks. Early atherosclerosis in genetically-altered hypercholesterolemic mice has also showed increased expression of VCAM-1.[24]

One candidate mediator for leukocyte penetration into the nascent atheroma is MCP-1, a potent chemokine produced by endothelial and smooth muscle cells and localized in human and experimental atherosclerotic plaques.[25–27] It is difficult, however, to prove that a molecule functions as a mediator. Genetically-engineered mice are the models used to test causality and prove whether a particular candidate molecule is important in the disease process. Deficiency of the LDL receptor or apolipoprotein E (apoE) gene renders mice hypercholesterolemic and prone to develop atherosclerosis. The absence of MCP-1 in such a mouse model plays an important role in atherogenesis, as shown by Gu et al in collaboration with us.[28] The aortic arch of the LDL-receptor-deficient, MCP-1-wild-type mice showed MCP-1 expression, lipid deposition and macrophage accumulation,

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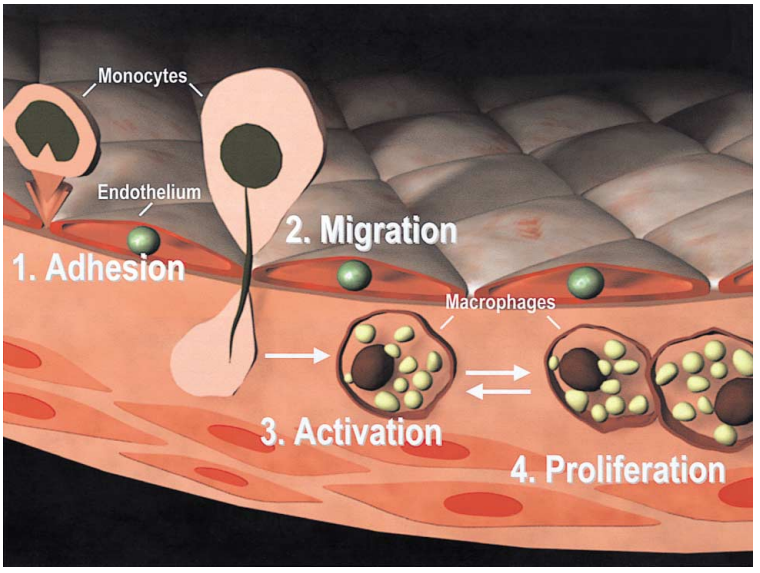

Fig 1. Mechanisms of macrophage accumulation in the arterial wall. (1) Monocyte adhesion to activated endothelial cells is an initial step of atherogenesis. Oxidative stress induces vascular endothelial cell activation and enhances expression of adhesion molecules such as vascular cell adhesion molecule-1 (VCAM-1), which binds blood monocytes. (2) Monocytes then migrate into the intima in response to chemokines, including monocyte chemoattractant protein 1 (MCP-1). (3) Monocytes differentiate into activated macrophages in the intima, induced by macrophage colony stimulating factor (M-CSF). Activated macrophages express a number of molecules related to atherogenesis, plaque instability and thrombogenicity. (4) Macrophage proliferation may play a role in formation of atheroma rich in this cell type. Oxidative stress can induce macrophage proliferation.

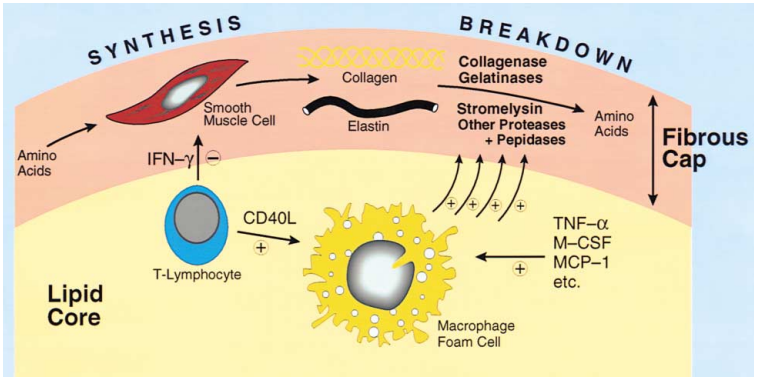

Fig 2. A schematic of matrix metabolism and integrity of the plaque's fibrous cap. When inflammation is present in the intima, an imbalance of collagen synthesis and degradation may occur and weaken the fibrous cap, resulting in plaque rupture. T-lymphocytes secrete interferon-γ (IFN-γ), which inhibits the biosynthesis of collagen by smooth muscle cells, and macrophages overexpress matrix-degrading proteinases, such as collagenases, which can cleave fibrillar collagen. Cytokines produced by the macrophages and T-lymphocytes further activate macrophages. TNF-α, tumor necrosis factor-alpha; M-CSF, macrophage colony stimulating factor; MCP-1, monocyte chemoattractant protein 1. (Modified from Libby[18] with permission.)

but there was a marked reduction in the lipid lesions in the double-knockout animals lacking the genes that encode for LDL receptor and MCP-1. Boring et al showed that genetically-altered mice that lack CCR2, a receptor for MCP-1, in apoE-deficient background had less atherosclerotic changes in the aorta.[29] These studies suggest that MCP-1 plays a causal role in the formation of atherosclerosis.

Mediators in Macrophage Activation and Proliferation

Once monocytes invade the atherosclerotic plaque, they are activated and change phenotype, becoming macrophages that express scavenger receptors and engulf modified lipoproteins to become foam cells. Although it is clear that the leukocytes become activated during atherosclerosis, the molecular mediator of this process remains speculative. Work from the 1980s and 1990s provides some candidates. Macrophage colony stimulator factor, a potent monocyte activator and co-mitogen, localizes in human and experimental atheroma,[30–32] and its causality in atherosclerotic macrophage function has been proven recently using a double knockout osteoperotic mouse.[33,34] There was a great deal of fatty lesion formation in the atherosclerosis-prone mice with a M-CSF-wild type background, but deficiency of functional M-CSF dramatically retarded lesion evolution.

In addition to monocyte recruitment into the arterial wall, macrophage proliferation in the atheroma may also participate in the formation of vulnerable atheroma rich in this cell type. Macrophages and smooth muscle cells replicate at similar levels in advanced human atherosclerotic plaque and in the atheroma of hypercholesterolemic rabbits.[35–38] M-CSF, granulocyte macrophage-CSF (GM-CSF), and oxidized LDL, each of which can usually be seen in atheroma, induce macrophage proliferation in vitro.[38–42]

Pathophysiology of the Acute Thrombotic Complications

The presence of a mature atherosclerotic lesion sets the stage for complications of atherosclerosis. Patients may present with symptoms from stenotic lesions or with thrombotic symptoms such as acute myocardial infarction or unstable angina that may occur without warning, as in chronic stable syndromes of atherosclerosis. We have learned much about the pathogenesis of the thrombotic complications of atherosclerosis.[3,43] The plaque's fibrous cap, of which collagen is the primary structural component,[18] protects the integrity of the atheroma and provides a shield between the thrombogenic material in the plaque's lipid core and the coagulation factors present in the circulating blood. When inflammation is present in the intima, the leukocytes can send signals to the smooth muscle cell to inhibit the biosynthesis of collagen, and can themselves overexpress collagen-degrading proteinases. The imbalance of collagen synthesis and degradation may weaken the

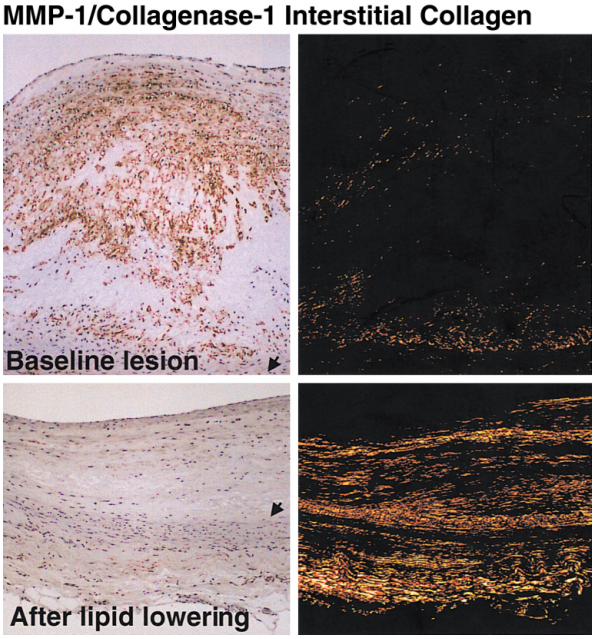

Fig 3. Inverse relationship between MMP-1/collagenase-1 expression and interstitial collagen content. (Top panels) The rabbit aortic lesion after 4 months on a high-cholesterol diet (Baseline lesion) had a high level of MMP-1 expression. Picrosirius red polarization barely detected accumulation of interstitial collagen on a serial section. (Bottom panels) 16 months of dietary lipid lowering decreased MMP-1/collagenase-1 expression and in parallel increased collagen content, a key determinant of plaque stability (original magnification: ×10). (From Aikawa et al[58] with permission.)

fibrous cap and lead to plaque rupture (Fig 2).[44,45] The leukocytes can also exchange signals to augment the production of the procoagulants that make it dangerous for the blood to enter the artery wall.[46] The thrombogenicity of the plaque's lipid core also plays a key role in the unstable coronary syndromes.[47] We will review experimental results that help to explain the features of the unstable atheroma.

Smooth Muscle Cells and Collagen Production

An experiment designed to measure collagen biosynthesis in human smooth muscle cells in culture showed that these cells incorporate proline, an amino-acid rich in collagen, in the basal state into newly synthesized collagen.[48] When the smooth muscle cells were exposed to platelet-derived growth factor or transforming growth factor-β, which are released during coagulation, the biosynthetis of collagen increased. This is very important for lesion healing. However, new collagen synthesis by smooth muscle cells was nearly inhibited when the cells were exposed to interferon-γ, a T-lymphocyte-derived cytokine.

We now recognize that there are many T cells in various regions of the plaque, particularly those areas that are prone to rupture.[16,49,50] Further, the cells in that region overexpressed HLA-DR, a transplantation antigen[16] defined by our laboratory more than a decade ago as a interferon-γ-inducible structure on the surface of the smooth muscle cell.[51] So the recent clinical finding that HLA-DR-positive smooth muscle cells are present at the sites of plaque rupture is strong evidence of interferon-γ action at the site of rupture of human atheroma.[52] Interferon-γ may weaken the fibrous cap by inhibiting biosynthesis of new collagen by the smooth muscle cell, thus impairing this cell type's ability to repair and maintain the plaque's fibrous cap.[48]

Expression of Matrix Degrading Enzymes

The level of collagen in the fibrous cap depends on the rate of synthesis as well as the rate of breakdown (Fig 2). The triple-helix collagen fiber ordinarily has a very strong, biochemically-resistant structure. Only a handful of enzymes are capable of attacking collagen, notably the interstitial collagenases.[53,54] The interstitial collagenases, members of the matrix metalloproteinase (MMP) family, can make an initial proteolytic cleavage and break the collagen fiber into three-quarter and one-quarter fragments. Fortunately, normal human arteries do not contain considerable levels of interstitial collagenase, but several studies, including our own, have shown that macrophages in the atherosclerotic plaque overexpress the collagen-degrading enzyme MMP-1 (collagenase-1).[55–58] Recent work by our laboratory shows that 2 enzymes expressed in the atherosclerotic plaque, MMP-1 (collagenase-1) and MMP-13 (collagenase-3), can break down collagen, and there is in-situ evidence of collagenolysis by MMP in the human atherosclerotic plaque.[59] Thus, inflammation places the collagen in the plaque's fibrous cap under the double attack of decreased synthesis and increased breakdown, setting the stage for plaque rupture. Potent endogenous tissue inhibitors of MMP (TIMPs) occur widely,[55,60–62] and our laboratory has described 3 of the 4 known TIMPs found in the atheroma. However, the precise molecular mechanisms regulating the balance of expression of MMPs and TIMPs remain unclear.

Prothrombotic Potential in Atheroma

For more than a decade it has been known that macrophages in the core of the human atherosclerotic plaque overexpress tissue factor, a potent procoagulant,[47,63–65] but until recently the molecular switch that turns on tissue factor expression remained elusive. In 1985, it was reported that activated T cells induce macrophage tissue factor by contact via an unknown signal,[66] and recent work from our laboratory has identified the missing link between T cells and macrophages, which exist side by side in ruptured human atherosclerotic plaques.[46] We studied a recently recognized signaling system known as CD154 (or CD40 ligand), a transmembrane protein. The usual soluble cytokines do not induce appreciable tissue factor activity, but recombinant CD154 and the membranes of activated T cells in a CD154-dependent manner briskly induce tissue factor gene expression. The same cells that express the receptor CD40, which binds CD154, contain tissue factor, the target gene product. Thus, we suggest that CD154 can turn on tissue factor expression when an inflammatory state prevails in the arterial intima.

Plaque Stabilization by Lipid-Lowering Therapy

Lipid-lowering therapy can decrease the onset of acute coronary events in patients despite only modest reduction in angiographic stenosis.[20,67] Thus it appears that lipid-lowering therapy changes the nature of established plaques qualitatively or functionally ('stabilization'), rather than merely decreasing the lesion ('regression').[18,68] A number of animal studies have addressed the possibility of lesion stabilization/regression by lipid-lowering treatment,[69–71] but the precise molecular and cellular mechanisms of plaque stabilization remain speculative. We therefore explored the

Table 1 Effects of Lipid-Lowering Therapy on Atheroma of Hypercholesterolemic Rabbits

	Results	*References*
Macrophage accumulation	*Decreased*	*38, 58, 73*
Macrophage proliferation	*Decreased*	*38*
Macrophage apoptosis	*No change*	*38*
MMP expression	*Decreased*	*38, 58, 76*
Collagen accumulation	*Increased*	*38, 58, 76*
Tissue factor expression	*Decreased*	*38, 73*
PAI-1 expression	*Decreased*	*77*
CD154 expression	*Decreased*	*58, 73*
PDGF-B expression	*Decreased*	*72*
SMC maturity	*Increased*	*72*
SMC proliferation	*Decreased*	*72*
OxLDL accumulation	*Decreased*	*75*
ROS production	*Decreased*	*75*
VCAM-1 expression	*Decreased*	*75*
MCP-1 expression	*Decreased*	*75*
eNOS expression	*Increased*	*75*
Microvessels	*Decreased*	*75*

MMP, matrix metalloproteinase; PAI, plasminogen activator inhibitor; PDGF, platelet-derived growth factor; SMC, smooth muscle cell; OxLDL, oxidatively-modified low density lipoprotein; ROS, reactive oxygen species; VCAM, vascular cell adhesion molecule; MCP, monocyte chemoattractant protein; eNOS, endothelial nitrous oxide species.

effects of lipid-lowering therapy on atheroma biology in rabbit models of atherosclerosis (Table 1).[38,58,72–77]

Lipid Lowering by Diet Reduces Vascular Inflammation and Proteolytic and Prothrombotic Potential

We first examined how lipid lowering by diet alone affects the rabbit atheroma in order to determine the effects of lipid lowering independent of the action of drugs.[58,72–75] We chose cholesterol-fed rabbits as a model because rabbit atheroma resembles 'vulnerable' plaques of the human coronary artery, especially when high-cholesterol feeding is combined with mechanical injury.[9] The balloon-injured aorta of New Zealand White rabbits after 4 months on an atherogenic diet contained a prominent macrophage accumulation underlying a thin smooth muscle layer that resembles the fibrous cap.[58] These macrophages in the baseline lesion contained high levels of MMPs, including the potent collagenolytic MMP-1 commonly seen in human atheroma.[55,57–59] Lesional macrophages also contained expression and activity of tissue factor and its inducers, CD154 and CD40.[38,46,63,64,73,78]

Some animals remained on the high cholesterol diet for 16 months, a period of time similar to that needed to accrue clinical benefit from statins in the mega trials. Other animals were shifted to a low cholesterol diet, during which time their cholesterolemia gradually declined towards normal for a rabbit. With the continued high cholesterol diet, many macrophages expressing MMPs and tissue factor remained. However, 16 months on dietary lipid-lowering therapy decreased the accumulation of macrophages and the expression and activity of MMPs, and dramatically increased collagen content, a key determinant of plaque stability.[58] This conversion of lipid-rich lesions into more fibrous plaques were detected by surface magnetic resonance imaging as well.[74] Kockx et al demonstrated a similar finding of decreased inflammation and increased collagen in rabbit atheroma during dietary lipid lowering.[79] Tissue factor expression and activity also declined strikingly in association with reduced CD154 and CD40.[73] This series of experimental observations suggest that in rabbits with diet-induced atherosclerosis, reduced cholesterol consumption could limit inflammation and improve those features of plaque that are associated with instability and thrombogenicity in humans. These observations provides a mechanistic explanation for the clinical benefit of lipid-lowering therapy.

Dietary Lipid Lowering Reduces Smooth Muscle and Endothelial Cell Activation

In addition to macrophage accumulation, phenotypic modulation of intimal smooth muscle cells toward the immature or activated state is a typical feature of human atherosclerosis.[80–82] Such smooth muscle cell activation in the plaque's fibrous cap likely plays another important role in plaque vulnerability through increased expression of proteolytic and prothrombotic molecules.[55,63,83] Smooth muscle cells in the fibrous cap of rabbit atheroma after 4 months on the atherogenic diet showed an immature phenotype based on decreased expression of smooth muscle cell-specific myosin isoforms and overexpressed MMP-3, MMP-9, and tissue factor.[58,73] However, dietary lipid-lowering therapy promoted accumulation of more mature smooth muscle cells expressing less MMP and tissue factor.

Endothelial cell activation contributes to infiltration of inflammatory cells in atheroma.[14,15] Oxidative stress may also play an important role in the pathogenesis of inflammatory diseases, including atherosclerosis,[84] and we have recently found that dietary lipid lowering can reduce oxidative stress and endothelial cell activation in rabbit atheroma.[75] Such findings on endothelial and smooth muscle cell activation should improve our understanding of mechanisms underlying the clinical outcomes observed with lipid-lowering therapy.

Plaque Stabilization by Statin Treatment

HMG-CoA reductase inhibitors (statins), potent lipid lowering agents first developed in Japan, have been widely used in the treatment of hypercholesterolemia in patients.[85] Accumulating evidence suggests that the lipid lowering by statins can prevent the onset of acute thrombotic complications,[18,20,67,68] and recent preclinical and clinical studies have also indicated that statins have anti-inflammatory effects independent of their lipid-lowering action.[86,87]

Statin Treatment Improves Vascular Inflammation

Shiomi et al have extensively studied in vivo the effects of lipid lowering by statins on the atheroma of Watanabe heritable hyperlipidemic (WHHL) rabbits, a model of endogenous hypercholesterolemia caused by LDL-receptor deficiency.[88–91] They have found that lipid lowering by statins including pravastatin, fluvastatin and cerivastatin, can reduce lesion size, extracellular lipid deposition, and macrophage accumulation. Aikawa et al, in collaboration with Shiomi's group, have recently demonstrated that cerivastatin treatment decreases accumulation of macrophages, probably in part by suppressing macrophage proliferation, in the atheroma of WHHL rabbits.[38] Their study also suggested that cerivastatin can reduce macrophage activation, as assessed by increased expression of MMPs and tissue factor. More recently, Fukumoto et al from our group have found that cerivastatin treatment reduces plasminogen activator inhibitor 1 (PAI-1), predominantly expressed by macrophages in the atheroma of WHHL rabbits.[77]

A number of in vitro experiments furthermore showed lipid-independent effects of statins on vascular cells.[86] Aikawa et al recently demonstrated that cerivastatin treatment with therapeutic doses reduced M-CSF-induced survival of and expression of MMP and tissue factor by human monocyte/macrophages.[38] Some other previous studies also demonstrated in vitro effects of statins on macrophage proliferation and activation.[92–94] These results suggest direct effects of statin treatment on plaque vulnerability and thrombogenicity in atheroma. However, there is controversy regarding whether the concentrations of statins used in in-vitro studies could apply in the clinical situation.

Effects of Statins on Vascular Smooth Muscle Cells

A number of in-vitro and in-vivo studies have repeatedly suggested that statins inhibit smooth muscle cell proliferation,[86,95–97] and some lipophilic statins can induce smooth muscle cell apoptosis in vitro.[98] Our understanding is that smooth muscle cells proliferate slowly in chronic human atherosclerosis,[35,37] unlike the neointima after mechanical vascular injury in animal models. In the plaque's fibrous cap especially, smooth muscle cells even undergo cell death, including apoptosis, which can cause collagen loss.[99,100] Lesional macrophages not only promote the proteolytic and prothrombotic potential of plaques, but also smooth muscle cell apoptosis.[101,102] These findings raise the question of whether inhibition of the growth/survival of macrophages, but not of smooth muscle cells, favors plaque stabilization.

Fukumoto et al from our group recently demonstrated differential effects of statins on interstitial collagen accumulation in the atheroma of WHHL rabbits.[76] Pravastatin (hydrophilic) and fluvastatin (lipophilic) decreased total cholesterol levels and suppressed the macrophage expression of MMPs in atheroma to a similar extent. Atheroma in pravastatin-treated WHHL rabbits contained more smooth muscle cells and collagen than atheroma in animals treated with fluvastatin. However, such results should not be extrapolated directly to the use of these 2 statins in patients, although the study clearly supports the hypothesis that decreased numbers of smooth muscle cells result in collagen loss in the fibrous cap, and further suggests that potential direct effects on vascular cells vary among statins.

Conclusions and Future Directions

We have long known the risk of elevated cholesterol levels in ischemic heart disease in humans. Considerable advances in vascular biology have recently revealed molecular mechanisms by which hypercholesterolemia induces inflammation and cell activation in the arterial wall. Discovery of a number of molecular mediators involved in such processes has improved the mechanistic understanding of this disease. Better understanding of its pathogenesis should also result in innovative therapeutic strategies. We have found that lipid-lowering therapy, both dietary and chemical, in experimental animals can modify expression of those inflammatory mediators in vivo. The combination of such information from modern biology and the accumulating knowledge from clinical medicine has recently suggested that lipid-lowering therapy ameliorates established atherosclerosis and reduces cardiovascular risk in patients. However, we have just begun to draw a small part of the whole picture, and a number of questions remain unanswered. For example, why do coronary events still occur in patients who undergo aggressive lipid-lowering therapy? Are MMPs really key players in plaque disruption? Why do some hypercholesterolemic patients have thrombotic complications, and others do not? Further studies employing new technologies may clarify more precisely the mechanisms of vascular inflammation and ultimately provide novel therapies.

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