Effect of Low Dose Atorvastatin Versus Diet-Induced Cholesterol Lowering on Atherosclerotic Lesion Progression and Inflammation in Apolipoprotein E*3–Leiden Transgenic Mice

Lars Verschuren, Robert Kleemann, Erik H. Offerman, Alexander J. Szalai, Sjef J. Emeis, Hans M.G. Princen, Teake Kooistra

Objective—To evaluate whether low-dose atorvastatin suppresses atherosclerotic lesion progression and inflammation in apolipoprotein E*3 (apoE*3)–Leiden mice beyond its cholesterol-lowering effect.

Methods and Results—ApoE*3–Leiden mice were fed a high-cholesterol (HC) diet until mild atherosclerotic lesions had formed. Subsequently, HC diet feeding was continued or mice received HC supplemented with 0.002% (w/w) atorvastatin (HC+A), resulting in 19% plasma cholesterol lowering, or mice received a low-cholesterol (LC) diet to establish a plasma cholesterol level similar to that achieved in the HC+A group. HC+A and LC diet reduced, significantly and to the same extent, lesion progression and complication in the aortic root, as assessed by measuring total atherosclerotic lesion area, lesion severity, and macrophage and smooth muscle cell area. In the aortic arch, HC+A but not LC blocked lesion progression. HC+A and LC reduced vascular inflammation (ie, expression of MIF, plasminogen activator inhibitor-1, matrix metalloproteinase-9), but HC+A additionally suppressed vascular cell adhesion molecule-1 expression and, in parallel, monocyte adhesion. In contrast, low-dose atorvastatin showed no anti-inflammatory action toward hepatic inflammation markers (serum amyloid A, C-reactive protein [CRP]) in apoE*3–Leiden mice and human CRP transgenic mice.

Conclusion—Low-dose atorvastatin cholesterol-dependently reduces lesion progression in the aortic root but shows anti-inflammatory vascular activity and tends to retard atherogenesis in the aortic arch beyond its cholesterol-lowering effect. (Arterioscler Thromb Vasc Biol. 2005;25:1-7.)

Key Words:

Despite remarkable advances in medical therapeutics and in understanding of its biology, atherosclerosis remains a principal cause of death in the Westernized world.1 Therefore, there is a clear need for more insight into the mechanisms underlying the atherosclerotic process and its (medical) treatment.

Atherosclerosis was previously thought to be a disease primarily involving lipid accumulation in the arterial wall. Current concepts of the disease include involvement of the immune system and chronic inflammation as crucial factors in all stages of the atherosclerotic process: the initiation of endothelial dysfunction, fatty streak formation, and lesion progression and complication.1 This central role of inflammation and immunity in atherogenesis suggests that anti-inflammatory therapies might have a beneficial role in management of the disease. In fact, it is now thought that the statin class of lipid-lowering drugs exerts part of the antiatherosclerotic effect via an anti-inflammatory property.2,3 Conceiving these so-called “pleiotropic” activities to statins is mainly based on in vitro studies.4 By interfering with intracellular signaling pathways, statins can suppress certain inflammatory responses in cultured cells.2–4 However, in most of these studies, statin concentrations are used that are not achieved under clinical circumstances. Support for the anti-inflammatory potential of statins also comes from human and animal studies that have shown decreases in plasma levels of inflammatory markers such as C-reactive protein (CRP) and serum amyloid A (SAA), parallel with lipid lowering by statin treatment.5 Although lipid lowering clearly causes inflammation associated with atherosclerosis to diminish, it is difficult to assess whether statins have direct anti-inflammatory effects (ie, independent of their cholesterol-
ol-lowering effects). Only recently, mouse studies have shown unequivocally that statins can reduce atherogenesis independent of and above and beyond their cholesterol-lowering effect, but the statin concentrations used in these animal studies were relatively high. The design of these and most other animal studies also deviates from the clinical norm because statin treatment was started simultaneously with onset of the experimentally controlled disease (ie, long before the first lesions were formed). Therefore, the question remains whether statins exert anti-inflammatory activity independent of their cholesterol-lowering effects under conditions mimicking current medical practice (viz, does a low-stat in dose show anti-inflammatory effects and retard the progression of existing atherosclerotic lesions beyond that attributable to its moderate lowering of plasma cholesterol?).

Herein, we addressed this question by evaluating the effect of a moderate dose of atorvastatin on progression of mild atherosclerotic lesions and on inflammation in apolipoprotein E*3 (apoE*3)–Leiden mice. ApoE*3–Leiden mice develop atherosclerotic lesions akin to their human counterparts with respect to morphological, histological, and immunohistochemical characteristics. Furthermore, apoE*3–Leiden mice display a lipoprotein profile that is very similar to the profile of patients experiencing familial dysbetalipoproteinemia, and cholesterol levels can easily be adjusted by modulating dietary cholesterol intake, thus allowing us to accurately assess the anti-inflammatory and antiatherosclerotic effects of atorvastatin versus dietary cholesterol lowering per se.

In this study, apoE*3–Leiden mice were fed a heterogenic high-cholesterol (HC) diet that induced mild atherosclerotic lesions as verified in reference mice. The effect of atorvastatin was then evaluated by comparing the progression of atherosclerosis and the inflammatory state in 3 groups of mice: (1) a group in which HC diet feeding was continued; (2) a group that received HC diet supplemented with 0.002% (w/w) atorvastatin; and (3) a group in which the dietary cholesterol intake was lowered to achieve the same plasma cholesterol level as that realized in the atorvastatin-treated group. Effects on plasma lipids were monitored during 16 weeks of experimental treatment. Then atherosclerotic lesions were analyzed, and lesion area, lesion severity, monocyte adhesion, macrophage content, smooth muscle cell (SMC) content, and the expression of inflammatory markers was determined to test whether atorvastatin reduces lesion progression and inflammatory state independent of cholesterol lowering. The anti-inflammatory potential of atorvastatin at the dosage used was further evaluated in human CRP transgenic (huCRPtg) mice, a mouse inflammation model that allows sensitive measuring of the anti-inflammatory potency of compounds in the absence of atherosclerosis.

Methods

Mice and Diets

Twelve-week-old female apoE*3–Leiden transgenic mice (n=48) received an atherogenic diet containing 0.35% (w/w) cholesterol (HC diet) for a run-in period of 9 weeks. Then 9 animals were euthanized (reference group). Remaining animals were divided into 3 groups (n=13 each) and fed an experimental diet for 16 weeks (ie, groups received HC [HC group] or HC supplemented with 0.002% [w/w] atorvastatin [Lipitor; Pfizer; HC+A group], or a low-cholesterol [LC] diet containing 0.15% [w/w] cholesterol for 4 weeks, followed by 0.20% [w/w] cholesterol for 12 weeks). The latter was done to achieve plasma cholesterol levels in the LC group equivalent to those in the HC+A group. The required atorvastatin dose to achieve a 20% reduction of plasma cholesterol has been defined as “low dose.” All animal experiments described were approved by the institutional animal care and use committee of TNO.

huCRPtg Mice

huCRPtg mice have been fully described previously. Three groups of male huCRPtg mice received chow containing 0.002% (w/w) atorvastatin or 0.1% (w/w) atorvastatin or vehicle for 3 weeks. Then murine recombinant interleukin-1β (IL-1β; Sanvertech) was injected intraperitoneally into each mouse (250 000 I.U.) to induce huCRP expression.

Lipid and Lipoprotein Analysis

Total plasma cholesterol and triglyceride levels were measured after 4-hour fasting using kits 1489437 (Roche Diagnostics) and 337-B (Sigma Aldrich Chemie BV), respectively. Lipoprotein profiles were obtained by SMART analysis using the AKTA-fast protein liquid chromatography system (Pharmacia) as described previously.

Monitoring Atherosclerosis

Five-micrometer serial cross-sections of the entire aortic root were prepared and stained with hematoxylin-phloxine-saffron. To determine the average cross-sectional lesion area, 4 cross-sections (interval 30 μm) of each specimen were analyzed blindly using QWin-software (Leica). Each cross-section consisted of 3 segments (viz, a segment represents the cross-sectional area between 2 heart valves). The number of lesions per segment was counted to determine the percentage of lesion-containing diseased segments, a measure of newly formed lesions during disease progression. Lesion severity was graded blindly according to the classification of the American Heart Association as reported. To determine the total plaque load in the aortic arch, perfusion-fixed aortas (from the aortic origin to the aortic bifurcation) were cleaned of extravascular fat, opened longitudinally, pinned en face, and stained for lipids with oil-red O (Aldrich Chemie BV) as described previously. Data were normalized for the analyzed surface area and expressed as percentage of the stained area. Monocyte adhesion, macrophage area, and SMC area were determined essentially as described previously.

Determination of Murine SAA and Human CRP

Plasma mouse SAA and human CRP were determined in tail blood samples by ELISA specific for human CRP or mouse SAA (kit KMA0012; BioSource). Of note, plasma SAA levels of healthy apoE*3–Leiden control mice are below the detection limit of the ELISA.

Aortic mRNA Expression

mRNA and cDNA (kit A3500; Promega) was prepared from n=5 aortas per experimental group. RT-PCR was performed using the RT-PCR–mastermix (Eurorgenetec) and the ABI7700 system (PE Biosystems) following the guidelines of the manufacturer and using established primer sets for vascular cell adhesion molecule-1 (VCAM-1), monocyte chemotactic protein-1 (MCP-1), MIF, matrix metalloproteinase-9 (MMP-9), plasminogen activator inhibitor-1 (PAI-1), and cyclophilin (PE Biosystems) as internal standard.

Statistical Methods

Significant differences were established by 1-way ANOVA test followed by a least significant difference post hoc analysis (SPSS). The level of statistical significance was set at P<0.05.
**Results**

**Dose Dependence of the Effect of Atorvastatin on Plasma Cholesterol**

In preliminary dose-finding experiments, we sought an atorvastatin dose that moderately reduced plasma cholesterol. ApoE*3–Leiden mice were fed a Western-type diet supplemented with increasing doses (0.0001% [w/w] to 0.01% [w/w]) of atorvastatin. Up to 0.001% (w/w) atorvastatin, no marked decrease in plasma cholesterol was observed, but plasma cholesterol decreased as atorvastatin dose increased to higher concentrations, achieving 53% (P<0.05) reduction at the highest dose tested (Figure 1, available online at http://atvb.ahajournals.org). On the basis of these results, we performed our atherosclerosis progression study using 0.002% (w/w) atorvastatin, which reduced plasma cholesterol by \( \approx 20\% \) (P<0.05).

**Atorvastatin Reduces Plasma Lipids and Atherogenic Lipoproteins During Atherosclerosis Progression in ApoE*3–Leiden Mice**

To induce atherosclerotic lesion development in apoE*3–Leiden mice, each animal received an HC diet containing 0.35% (w/w) cholesterol for 9 weeks. During this run-in, plasma cholesterol levels averaged 17.1 ± 2.6 mmol/L (Figure 1A), and mild atherosclerotic lesions developed (Figure 2; reference group). Subsequent progression of atherosclerosis was studied in 3 experimental groups (ie, a group in which the HC diet was continued [HC group], a group that received HC diet supplemented with 0.002% [w/w] atorvastatin [HC + A group], and a group in which the dietary cholesterol intake was lowered [LC group] to match the plasma cholesterol level of the HC + A group).

No differences in food intake and body weight gain were observed between the groups during the atherosclerosis progression study (data not shown). Plasma cholesterol levels in the HC group remained elevated throughout the treatment period and averaged 16.0 ± 1.9 mmol/L (Figure 1A). Atorvastatin moderately reduced plasma cholesterol levels by an average of 19% (P<0.05) between weeks 9 and 25, and a comparable reduction (25%; P<0.05) was achieved in the LC group fed the hypocholesterolemic diet. At the end of study, the total cholesterol exposure for the HC + A and LC groups was comparable and respectively 13% (P<0.05) and 19% (P<0.05) lower than for the HC group (Figure 1B). Very low-density lipoprotein (VLDL) and intermediate-density lipoprotein/LDL cholesterol were reduced similarly in the HC + A and LC groups (Figure 1C), and no significant differences in lipoprotein composition (ie, free cholesterol, cholesterol esters, triglycerides, and phospholipids) were observed between the groups (data not shown). Also, plasma triglyceride levels determined before and at the end of the experimental treatment period were not significantly different among the 3 groups (data not shown).

**Atorvastatin Reduces Progression and Severity of Atherosclerosis in ApoE*3–Leiden Mice**

After 16 weeks of treatment, animals were euthanized, and the extent of atherosclerosis in the aortic root area, the part of the aorta in which lesions develop most rapidly, was com-
The total plaque load in the HC and LC groups was increased 4.7-fold \( (P<0.05) \) and 3.2-fold \( (P<0.05) \), respectively, whereas the increase was not significant in the HC + A group (1.7-fold). Although lesion progression in the LC group tended to be faster than in the HC + A group, the difference between these groups was not statistically significant.

To assess differences in lesion severity in the aortic root area, the lesions were graded according to the American Heart Classification \(^{14} \) (Figure 2D). The HC group showed a significant skewing toward more severe lesions, with the skewing expressed as a ratio of “the number of type IV+V lesions/the number of type I+II+III lesions” \(^{18} \) (ratio of 2.8±2.1 in the HC group versus 0.6±0.2 in the reference group; \( P<0.05; \) Table I, available online at http://atvb.ahajournals.org). The bias toward more severe lesions was significantly reduced in the HC + A group (1.1±0.7; \( P<0.05 \)) and the LC (1.3±0.8; \( P<0.05 \)) groups, indicating that lesion development had been retarded. Figure 2D depicts the distribution of lesion types for each treatment group to illustrate this retarding effect. Approximately 75% of all pre-existing lesions in reference animals were mild type I–III lesions. During disease progression in the HC group, type V lesions increased strongly by 34% \( (P<0.05) \), thus being the most abundant lesion type. This increase was less pronounced in the LC group and was not observed in the HC + A group, which displayed 30% \( (P<0.05) \) fewer type V lesions than the HC group. This and the fact that type III lesions form the major lesion type in the HC + A and LC groups demonstrates that the development of atherosclerotic lesions is strongly delayed in these groups.

**Effect of Atorvastatin on Cellular Composition of the Lesions**

We sought a cellular correlate for the retarding effect of atorvastatin on lesion development. Figure 3A shows the number of monocytes adhering to the endothelium. Compared with the reference group, monocyte adhesion was 4.8-fold \( (P<0.05) \) and 4.0-fold \( (P<0.05) \) augmented in the HC and LC groups, respectively. In comparison, the number of adherent monocytes in the HC + A group was only 2.7-fold \( (P<0.05) \) increased compared with referents, being significantly lower compared with both control groups. This indicates an atorvastatin effect independent of and beyond its plasma cholesterol-lowering effect.

The HC group displayed a 5.5-fold \( (P<0.05) \) increased macrophage-containing area when compared with the reference group (Figure 3B). This increase was abated in the HC + A group (2.5-fold; \( P<0.05 \)) and the LC group (1.9-fold; \( P<0.05 \)); the difference observed between these groups was not significantly different. A similar effect was observed for the SMC-containing area. Compared with the reference group, the SMC area increased 4.9-fold \( (P<0.05) \) in the HC group.
group, which reflects the formation of severe lesions during disease progression (Figure 3C). This increase was significantly lower in the HC+A group (2.3-fold; \( P<0.05 \)) and the LC group (1.5-fold; \( P<0.05 \)).

Effects of Atorvastatin on Molecular Markers of Atherogenesis

The effect of atorvastatin on the aortic expression of markers specific for endothelial cell activation (VCAM-1), macrophage activation (MCP-1, MIF), collagen degradation (MMP-9), and atherothrombosis (PAI-1) was analyzed by RT-PCR (Table II, available online at http://atvb.ahajournals.org). With the exception of PAI-1, which was already elevated in reference animals, expression of all markers strongly increased during lesion development. Atorvastatin and the hypocholesterolemic diet prevented induction of VCAM-1, MIF, and MMP-9 expression and suppressed PAI-1 expression. The increase in MCP-1 mRNA expression was not affected by atorvastatin and hypocholesterolemic diet feeding. Western blot analysis of MCP-1 in aortic homogenates underlined this observation (data not shown). Remarkably, the expression of VCAM-1 was significantly lower in the HC+A group when compared with the HC and LC groups, pointing to an effect of atorvastatin independent of and beyond cholesterol lowering. The latter observation may constitute a possible molecular explanation for the cholesterol-independent reduction of monocyte adhesion by atorvastatin.

Systemic inflammation, as reflected by levels of the acute phase protein SAA, was already pronounced in the reference group and did not intensify during disease progression in the HC group (Figure 4A). However, in both cases, treatment with atorvastatin or a hypocholesterolemic diet dampened plasma levels of liver-derived SAA by 48% \( (P<0.05) \). The absence of an atorvastatin effect on SAA beyond that achieved by cholesterol lowering suggests that atorvastatin lacks anti-inflammatory activity in the liver at the dosage applied.

To further test this notion, atorvastatin was administered to huCRPtg mice. At the concentration used in the atherosclerosis progression study (0.002% [w/w]), atorvastatin treatment did not significantly change basal or IL-1β–induced plasma levels of the acute phase protein huCRP in huCRPtg mice (Figure 4B). In stark contrast, a higher dose of atorvastatin (0.1% [w/w]) did significantly reduce basal and IL-1β–induced expression of huCRP mice \( (P<0.005) \). Together, these data indicate that at a concentration sufficient to moderately lower plasma cholesterol levels, atorvastatin shows pleiotropic anti-inflammatory effects in the vessel wall but not in the liver.

Discussion

Therapeutic intervention for atherosclerosis is usually initiated at an advanced stage of disease and is directed at lowering plasma cholesterol; cholesterol-lowering statins are widely used to achieve this. Available evidence suggests that even moderate reduction of plasma cholesterol by statins significantly reduces human mortality.\(^{17} \) It is still unclear whether statins used at low doses reduce lesion progression in ways not merely attributable to their cholesterol-lowering effect. ApoE*3–Leiden mice with pre-existing lesions were used to address this question, with particular reference to the inflammatory component.

We demonstrated that an only 19% reduction of plasma cholesterol achieved by low-dose atorvastatin treatment is sufficient to markedly reduce progression of established atherosclerotic lesions in the aortic root (ie, the part of the aorta that represents the most advanced stage of the disease)\(^ {10} \) and to almost block the progression in the aortic arch, which represents a more initial stage of lesion development. This effect of atorvastatin in the aortic arch tends to be beyond its cholesterol-lowering effect because lesion progression not only continued in the HC group but also in the plasma cholesterol–matched LC group.

HC+A and LC reduced the expression of vascular inflammation markers, but atorvastatin additionally suppressed monocyte adhesion and VCAM-1 expression (ie, independent of cholesterol lowering). The cholesterol-independent effects of atorvastatin appear to be restricted to the vessel wall because atorvastatin lacks pleiotropic anti-inflammatory activity on hepatic SAA expression at the dose used.

Reduced progression of established atherosclerotic lesions has mainly been reported in cases in which very strong reductions of plasma cholesterol were achieved.\(^ {18} \) However, our study demonstrates that a 19% reduction of plasma cholesterol by a statin (ie, a cholesterol reduction achieved with most statins at their lowest pill dosage in humans)\(^ {19} \) is
already sufficient to reduce lesion progression in apoE*3–Leiden mice. This is in line with observations made in humans in which a comparable reduction (23%) by simvastatin reduced vessel wall thickness and vessel wall area.20 Furthermore, our study demonstrates that atorvastatin blocks de novo formation of lesions in the aortic root by its cholesterol-lowering effect and tends to retard the progression of pre-existing lesions in the aortic arch beyond cholesterol lowering. To our knowledge, this has not been documented before. Because the total cholesterol exposure of the HC+A group was slightly (6%) higher than in the LC group, we cannot exclude that we may even have underestimated the effects of atorvastatin.

In the present study, we showed that atorvastatin exerts anti-inflammatory activity in the liver of huCRPtg mice at high but not at low doses. Using the same mouse inflammation model, we showed recently that the statin concentration required for huCRP lowering is higher than the dose necessary for cholesterol lowering, indicating that both effects occur independently.16 The differential dose dependence of statin-mediated cholesterol and CRP lowering might explain why many of the pleiotropic anti-inflammatory effects of statins described in vitro and in animal models in vivo have not routinely been observed in patients; patients usually receive relatively low doses of statin to lower primarily their plasma cholesterol.2–4

The question whether statins have cholesterol-independent, in particular anti-inflammatory, therapeutic effects in humans is still open.23 We addressed this issue in the apoE*3–Leiden mouse model because cholesterol lowering by a statin in this model is observable at clinically relevant doses.6 As demonstrated here for the first time, low-dose atorvastatin, in this work defined as the dose required to reduce plasma cholesterol by 20% in apoE*3–Leiden mice, blocks progression of pre-existing lesions in the aortic arch, an effect that cannot be explained by the cholesterol-lowering effect of atorvastatin.

Our data clearly show that atorvastatin reduces monocyte adhesion and VCAM-1 expression independent of lowering cholesterol. In a recent study, we observed that rosuvastatin also reduced monocyte adhesion through its pleiotropic effects.6 In contrast to atorvastatin in this article, rosuvastatin exerted anti-inflammatory (pleiotropic) effects in the liver and reduced plasma SAA levels. This hepatic effect of rosuvastatin may be explained by the higher (2.5-fold) dosage used and its 2-fold longer elimination time compared with atorvastatin.21 Indeed, higher atorvastatin concentrations showed anti-inflammatory effects in the liver and reduced CRP expression as demonstrated in this study and previously.16

With exception of monocyte adhesion, we observed no cholesterol-independent effects of atorvastatin on cellular markers of the atherosclerotic process. A reduction of monocyte–endothelial cell interaction has also been seen for cerivastatin in vitro using human endothelial cells.23 The reduction of monocyte adhesion in the present study may be explained by a parallel anti-inflammatory cholesterol-independent effect on VCAM-1 expression. This explanation is supported by a recent human study that demonstrates that low-dose atorvastatin treatment reduces adhesion molecule expression.23 The recent in vitro observation that atorvastatin downregulates activation of the transcription factors nuclear factor κB and activator protein-1, both of which are required for VCAM-1 expression, provides a molecular rationale for this suppressive effect.24

A cholesterol-independent regulatory mechanism may also be applicable to PAI-1; the PAI-1 gene is regulated primarily by fatty acids under hypertriglyceridemic conditions,25 and the reduction of VLDL levels in the HC+A and LC groups may therefore, at least partly, explain the reduced PAI-1 expression levels in these groups.

In all, our data obtained from apoE*3–Leiden mice demonstrate that atorvastatin, applied at a dose that only moderately reduces plasma cholesterol, retards the progression of pre-existing atherosclerotic lesions in the aortic root through its cholesterol-lowering activity but cholesterol-independent reduces particular aspects of vascular inflammation (cf. monocyte adhesion) and also tends to suppress lesion development in the aortic arch beyond its cholesterol-lowering effect.

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References


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Figure I: The plasma cholesterol reducing effect of atorvastatin is dose-dependent. Groups of ApoE*3-Leiden mice (n≥6) were treated with increasing doses of atorvastatin mixed to a Western type diet. Plasma cholesterol was determined in tail blood samples taken after 4 weeks of treatment. Data represent the average reduction of plasma cholesterol compared to vehicle-treated control animals.
**Table I: Effect of atorvastatin on the shift towards more severe lesions.** The shift towards more severe lesions, as represented by the ratio (the number of type IV,V lesions/the number of type I-III lesions), was calculated per mouse. Data represent means±sd per group (n=13 per group). *P<0.05 compared to reference, #P<0.05 compared to HC.

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Table II: Effect of atorvastatin on aortic mRNA expression levels of genes involved in lesion progression. Effect of atorvastatin on VCAM-1, MIF, MCP-1, PAI-1 and MMP-9 gene expression as measured by RT-PCR in aortas of at least n=5 animals per group. Cyclophilin was used as an internal reference. The gene expression level in the HC group was set 100%. *P<0.05 HC compared to reference, #P<0.05 compared to HC, &P<0.05 HC+A compared to LC.

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