The interplay between cholesterol and inflammation in the evolution of atherosclerosis

Verschuren, L.

Chapter 5

Effect of low dose atorvastatin versus diet-induced cholesterol-lowering on atherosclerotic lesion progression and inflammation in ApoE*3Leiden transgenic mice

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Abstract

Background: To evaluate whether low dose atorvastatin suppresses atherosclerotic lesion progression and inflammation in ApoE*3Leiden mice beyond its cholesterol-lowering effect.

Results: ApoE*3Leiden mice were fed a high cholesterol (HC) diet until mild atherosclerotic lesions had formed. Subsequently, HC diet feeding was either 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.

Both 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. Both HC+A and LC reduced vascular inflammation, i.e. expression of MIF, PAI-1, MMP-9, but HC+A additionally suppressed VCAM-1 expression and, parallelly, monocyte adhesion. In contrast, low dose atorvastatin showed no anti-inflammatory action towards hepatic inflammation markers (SAA, CRP) in ApoE*3Leiden mice and huCRP transgenic mice.

Conclusions: 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.

Introduction

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.

Previously, atherosclerosis was 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 the management of the disease. In fact, it is now thought that the statin class of lipid-lowering drugs exerts part of the anti-atherosclerotic effect via an anti-inflammatory property.[2,3] Conferring 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 (summarized in [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] While lipid-lowering clearly causes inflammation associated with atherosclerosis to diminish, it is difficult to assess whether statins have direct anti-inflammatory effects, i.e. independent of their cholesterol-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.[6,7] 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, i.e. long before the first lesions were formed. The question remains therefore whether statins exert anti-inflammatory activity independent of their cholesterol-lowering effects under conditions mimicking current medical practice, *viz.* does a low statin 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 have addressed this question by evaluating the effect of a moderate dose of atorvastatin on progression of

mild atherosclerotic lesions and on inflammation in ApoE*3Leiden mice. ApoE*3Leiden mice develop atherosclerotic lesions akin to their human counterparts with respect to morphological, histological and immunohistochemical characteristics.[8-10] Furthermore, ApoE*3Leiden mice display a lipoprotein profile that is very similar to the profile of patients suffering from familial dysbetalipoproteinemia, and cholesterol levels can easily be adjusted by modulating dietary cholesterol intake, thus allowing us to accurately assess the anti-inflammatory and anti-atherosclerotic effects of atorvastatin versus dietary cholesterol-lowering *per se*.[6,9,11]

In this study, ApoE*3Leiden mice were fed an atherogenic 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 three groups of mice, viz. 1) a group in which HC diet feeding was continued; 2) a group which 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, 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 C-reactive protein transgenic mice (huCRPtg), a mouse inflammation model[12,13], that allows sensitive measuring of the anti-inflammatory potency of compounds in the absence of atherosclerosis.

Methods

Mice and diets

Twelve weeks-old female ApoE*3Leiden transgenic mice[9] (n=48) received an atherogenic diet containing 0.35%(w/w) cholesterol (high-cholesterol diet, HC) for a run-in period of 9 weeks. Then, nine animals were sacrificed (reference group). Remaining animals were divided into three groups (n=13 each) and fed an experimental diet for 16 weeks, i.e. groups received either 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.

Human CRP transgenic mice

CRP transgenic mice (huCRPtg) have been fully described.[12,13] Three groups of male huCRPtg 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, Heerhugowaard, The Netherlands) was injected intraperitoneally into each mouse (250,000 units) to induce huCRP expression.

Lipid and lipoprotein analysis

Total plasma cholesterol and triglyceride levels were measured after 4-hour fasting using kits No-1489437 (Roche Diagnostics, Almere, The Netherlands) and No.337-B (Sigma Aldrich Chemie BV, Zwijndrecht, The Netherlands), respectively. Lipoprotein profiles were obtained by SMART analysis using the AKTA-FPLC-system (Pharmacia, Roosendaal, The Netherlands) as described.[6]

Monitoring atherosclerosis

Five-µm serial cross-sections of the entire aortic root were prepared[9,11] and stained with hematoxylin-phloxine-saffron (HPS). To determine the average cross-sectional lesion area, four cross-sections (interval 30 µm) of each specimen were analyzed blindly using QWin-software (Leica).[6,11] Each cross-section consists of three segments, *viz.* a segment represents the cross-sectional area between two 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[14] as reported.[6,11] To determine the total plaque load in the aortic arch, perfusion-fixed aortas (from the aortic origin to the iliac bifurcation) were cleaned of extravascular fat, opened longitudinally, pinned en face and stained for lipids with oil-red O (Aldrich Chemie BV, Zwijndrecht, The Netherlands) as described previously.[8] Data were normalized for the analyzed surface area and expressed as percentage of the stained area. Monocyte adhesion, macrophage area, and smooth muscle cell area were determined essentially as described.[6,11]

Determination of murine SAA and human CRP

Plasma mouse SAA and human CRP was determined in tail blood samples by ELISA specific for human CRP[6,15] or mouse SAA (kit KMA0012; BioSource, Nivelles, Belgium).[11] Of note, plasma SAA levels of healthy ApoE*3Leiden control mice are below the detection limit of the ELISA.

Aortic mRNA expression

mRNA and cDNA (kit #A3500, Promega, Leiden, The Netherlands) was prepared from n=5 aortas per experimental group.[16] RT-PCR was performed using the RT-PCR-mastermix (Eurogentec, Seraing, Belgium) and the ABI7700 system (PE Biosystems, Nieuwekerk, The Netherlands) following the guidelines of the manufacturer and using established primer sets for VCAM-1, MCP-1, MIF, MMP-9, PAI-1, and cyclophilin (PE Biosystems) as internal standard.[16]

Statistical methods

Significant differences were established by one-way analysis of variance (ANOVA) test followed by a least significant difference (LSD) post hoc analysis (SPSS 11.5 for Windows; SPSS, Chicago, USA).[16] 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*3Leiden 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). Based on these results, we performed our atherosclerosis progression study using 0.002%(w/w) atorvastatin which reduced plasma cholesterol by about 20%($P<0.05$).

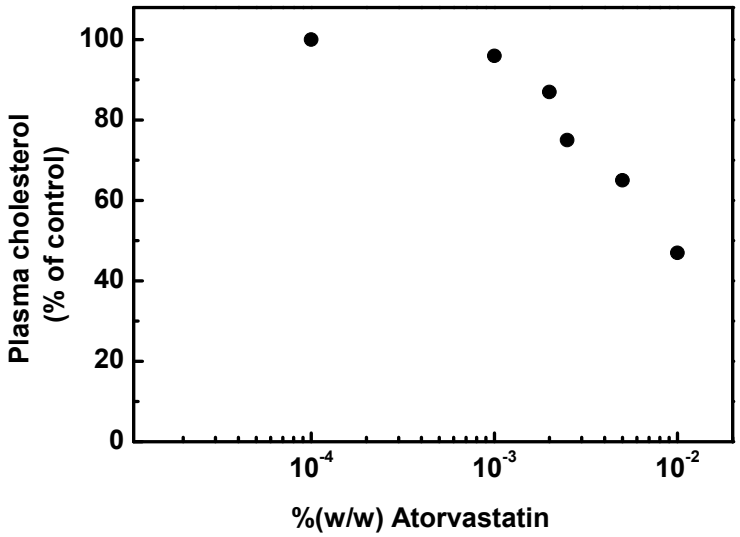

Figure 1: The plasma cholesterol reducing effect of atorvastatin is dose-dependent. Groups of ApoE*3Leiden mice (n≥6) were treated with increasing doses of atorvastatin mixed to a Western type diet. Plasma cholesterol was determined in tail blood samples taken after 4 weeks of treatment. Data represent the average reduction of plasma cholesterol compared to vehicle-treated control animals.

Atorvastatin reduces plasma lipids and atherogenic lipoproteins during progression of atherosclerosis in ApoE*3Leiden mice

To induce atherosclerotic lesion development in ApoE*3Leiden mice, each animal received a high cholesterol (HC) diet containing 0.35%(w/w) cholesterol for 9 weeks. During this run-in plasma cholesterol levels averaged 17.1±2.6 mM (Figure 2A), and mild atherosclerotic lesions developed (see Figure 3, reference group). Subsequent progression of atherosclerosis was studied in three experimental groups, i.e. 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 mM (Figure 2A). Atorvastatin moderately reduced plasma cholesterol levels by an average 19%($P<0.05$) between week 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 2B). VLDL and IDL/LDL cholesterol were reduced similarly in the HC+A and LC groups (Figure 2C) and no significant differences in lipoprotein composition, i.e. free cholesterol, cholesterol esters, triglycerides, and phospholipids were observed between the groups (not shown). Also, plasma triglyceride levels determined prior to and at the end of the experimental treatment period were not significantly different between the three groups (not shown).

Atorvastatin reduces progression and severity of lesions in ApoE*3Leiden mice

After 16 weeks of treatment animals were sacrificed and the extent of atherosclerosis in the aortic root area, the part of the aorta in which lesions develop most rapidly, was compared with that observed in the reference group at the end of the 9-wk run-in period. The total cross-sectional lesion area of the reference group was 25000±6500 μm^2 (Figure 3A). Compared to the reference group the total cross-sectional area was 4.5-fold increased in the HC group ($P<0.05$), and only insignificantly increased in the HC+A group (2.1-fold) and LC group (2.2- fold) groups. The total cross-sectional area of the HC+A group and the LC group were comparable and significantly lower than in the HC group. In reference animals, 55±5% of the segments (i.e. the cross-sectional area between two heart valves) contained lesions (Figure 3B). During subsequent disease progression, the lesion number increased in the HC group by 13%($P<0.05$), while it remained constant in the HC+A and the LC groups. At the end, the HC+A and LC groups displayed 18%($P<0.05$) and 16%($P<0.05$) fewer diseased segments than the HC group, indicating that atorvastatin and the hypocholesterolemic diet feeding impeded *de novo* lesion formation.

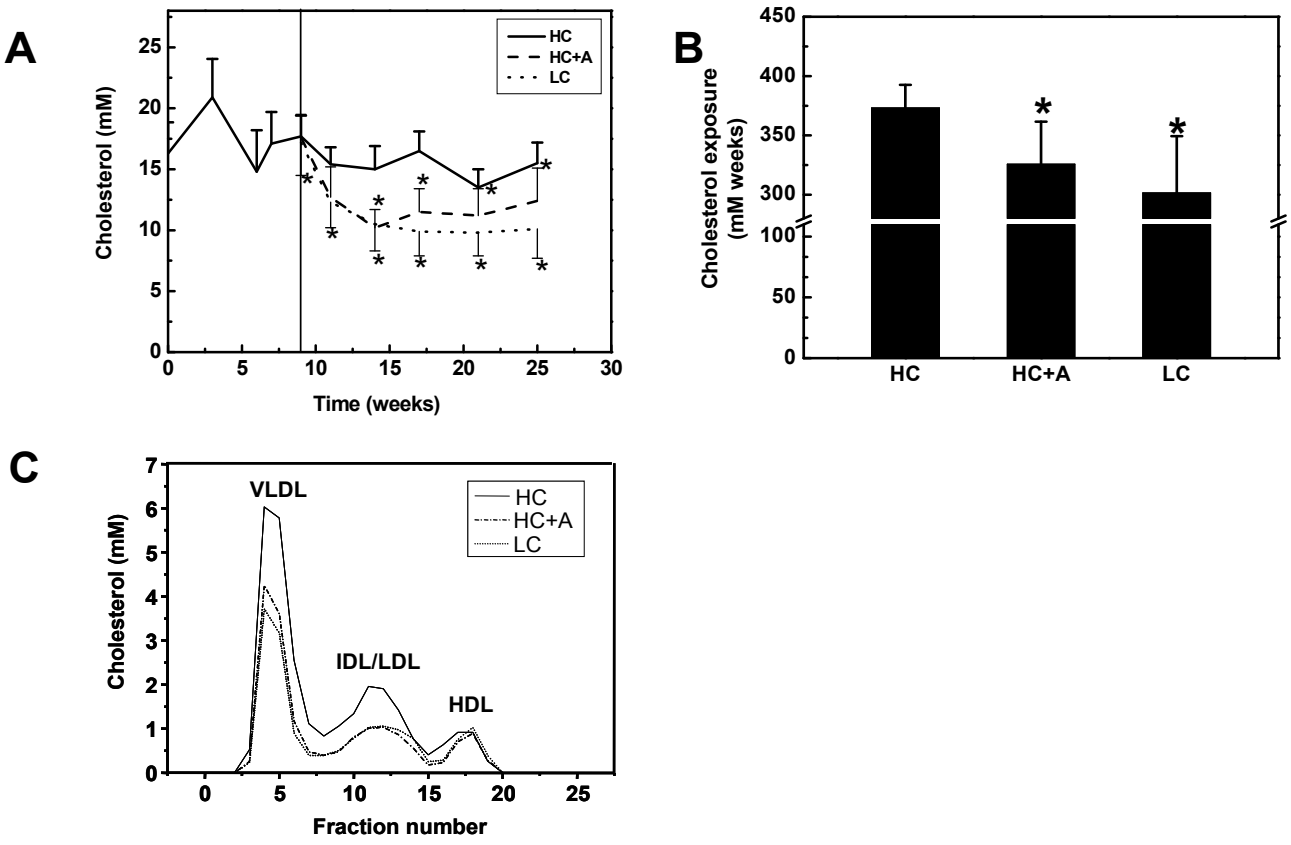

Figure 2: Effect of atorvastatin on plasma lipids. (A) Plasma cholesterol concentrations in ApoE*3Leiden mice over time (n=48 during run-in period and n=13 per group during experimental treatment period). **(B)** Total cholesterol exposures after 16 weeks of treatment. **(C)** Lipoprotein profiles of pooled plasma at 12 weeks. Data represent means±SD. *P<0.05 compared to HC.

For a global impression of atherosclerosis, the aortic arch, in which lesion development is delayed compared to the aortic root[10], was oil-red O-stained to determine the total atherosclerotic plaque area ('plaque load'). In reference animals, the total plaque load was 1.14 ± 0.47% of the stained area (Figure 3C). Compared to the reference group, 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, while 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 3D). The HC group showed a significant skewing towards 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' (ratio of 2.8±2.1 in the HC group versus 0.6±0.2 in the reference group; P<0.05; please see www.ahajournals.org, table I).

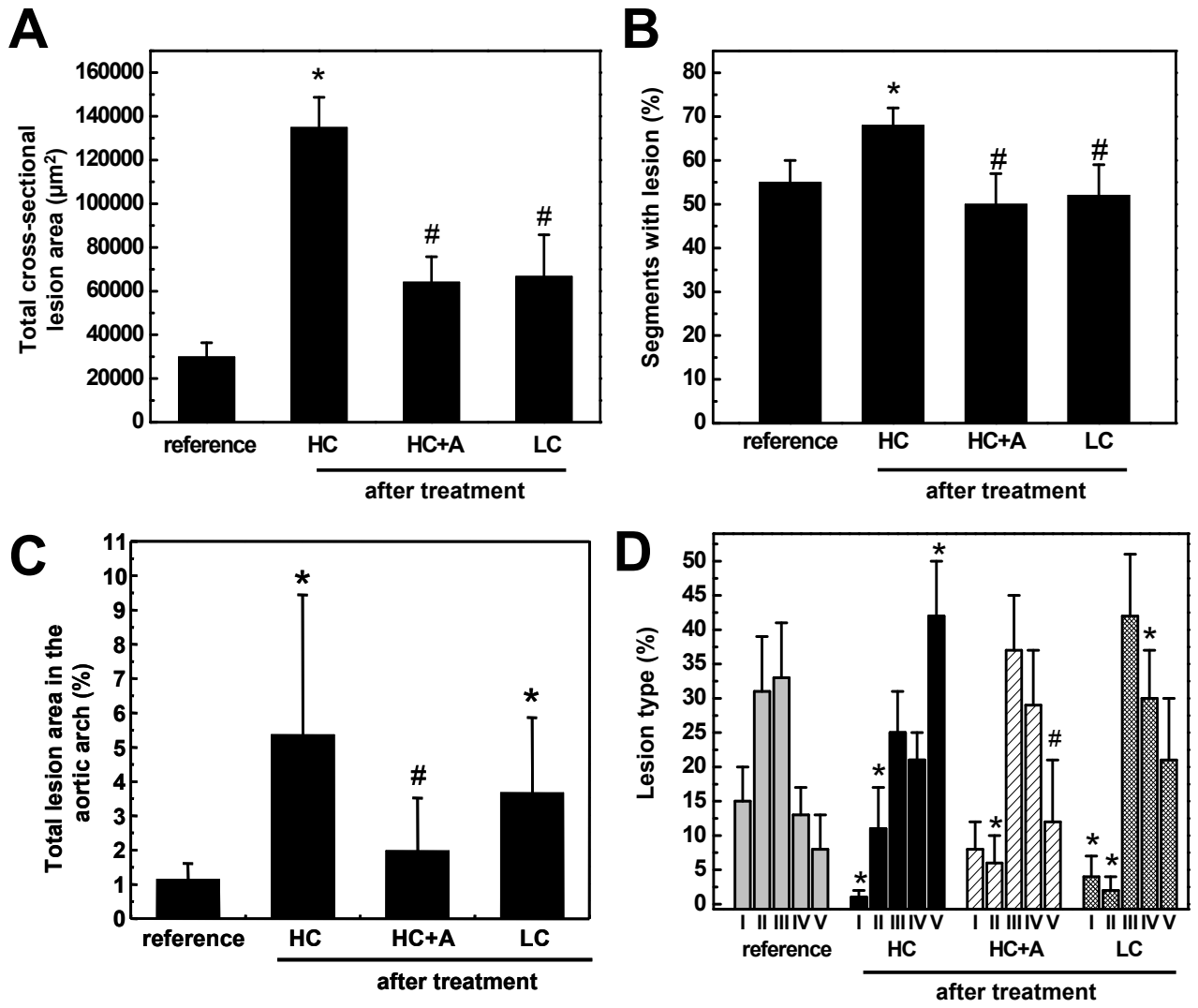

Figure 3: Effect of atorvastatin on the progression of mild lesions. (A) Total cross-sectional lesion area in the aortic root in reference animals (n=9) and treatment groups (n=13). **(B)** Effect of atorvastatin on the lesion number. Per mouse, 4 cross-sections with an interval of 30 µm were analyzed. **(C)** The total plaque load in the aortic arch was analyzed by oil-red O-staining (n≥6). Data are presented as percentage of the stained area. **(D)** Effect of atorvastatin on lesion severity in reference (n=9) and treatment groups (n=13). *P<0.05 compared to reference, and #P<0.05 HC+A or LC vs HC.

The bias towards more severe lesions was significantly reduced in the HC+A (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 strongly increased 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) less 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 lesion is strongly delayed in these groups.

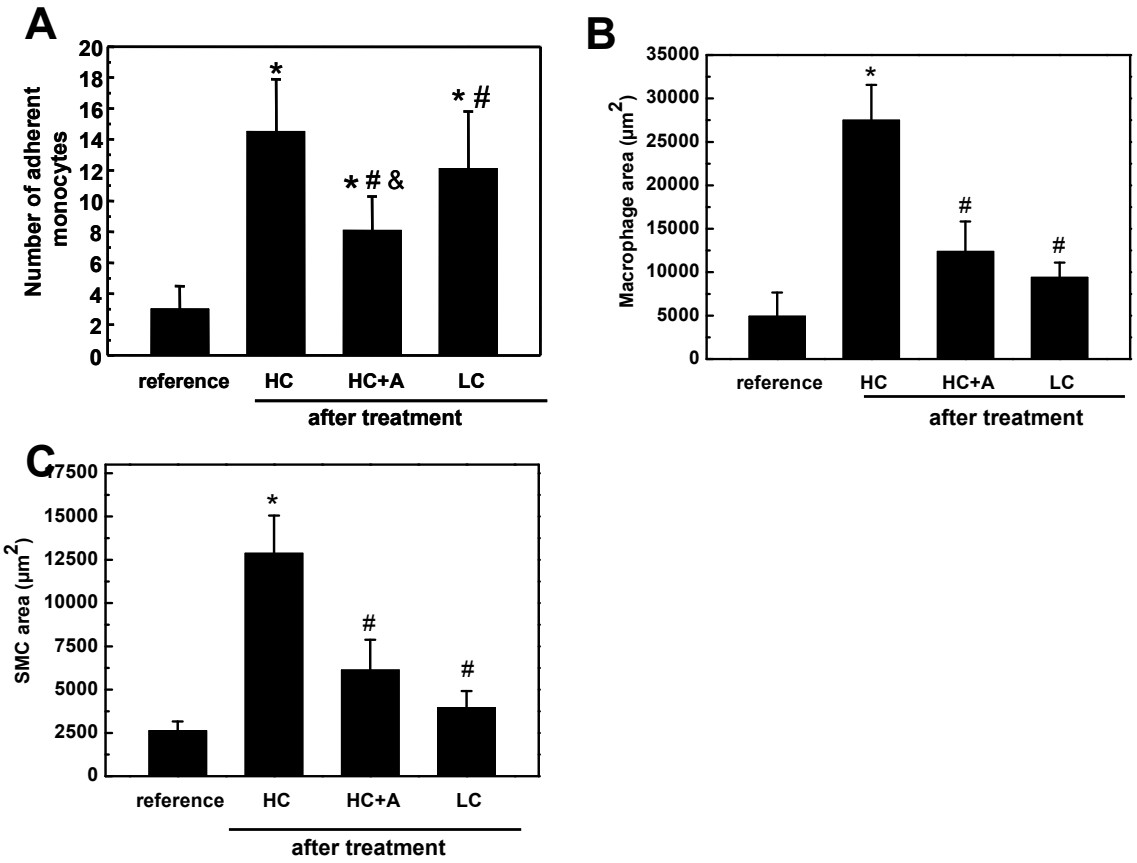

Figure 4: Effect of atorvastatin on aspects of atherogenesis in the aortic root. Effect of atorvastatin on **(A)** monocytes adhesion, **(B)** macrophage content, **(C)** smooth muscle cell content analyzed in reference (n=9) and treatment groups (n=11). *P<0.05 compared to reference, #P<0.05 HC+A or LC compared to HC, and &P<0.05 HC+A vs LC.

Effect of atorvastatin on the cellular composition of the lesions

We sought a cellular correlate for the retarding effect of atorvastatin on lesion development. Figure 4A shows the number of monocytes adhering to the endothelium. Compared to the reference group, monocyte adhesion was 4.8-fold (P<0.05) and 4.0-fold (P<0.05) augmented in the HC group and LC group, respectively. In comparison the number of adherent monocytes in the HC+A group was only 2.7-fold (P<0.05) increased compared to referents, being significantly lower compared to 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 to the reference group (Figure 4B). 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 smooth muscle cell (SMC)-containing area. As compared to the reference group the SMC area increased 4.9-fold (P<0.05) in the HC group which reflects the formation of severe lesions during disease progression (Figure 4C). This increase was significantly lower in the HC+A (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 (please see www.ahajournals.org, Table II). 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 protein in aortic homogenates underlined this observation (not shown). Remarkably, the expression of VCAM-1 was significantly lower in the HC+A group when compared to 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 serum amyloid A (SAA), was already pronounced in the reference group and did not intensify during disease progression in the HC group (Figure 5A). 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. 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 (Figure 5B). In stark contrast, a higher dose of atorvastatin (0.1% (w/w)) did significantly reduce both basal and IL-1β-induced expression of huCRP

(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*3Leiden 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 the progression of established atherosclerotic lesions in the aortic root, i.e. the part of the aorta which 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.

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

Reduced progression of established atherosclerotic lesions has mainly been reported in cases where very strong reductions of plasma cholesterol were achieved (reviewed in Stein *et al.*[18]) However, our study demonstrates that a 19% reduction of plasma cholesterol by a statin, i.e. a cholesterol-reduction which is achieved with most statins at their lowest pill dosage in humans[19], is already sufficient to reduce lesion progression in

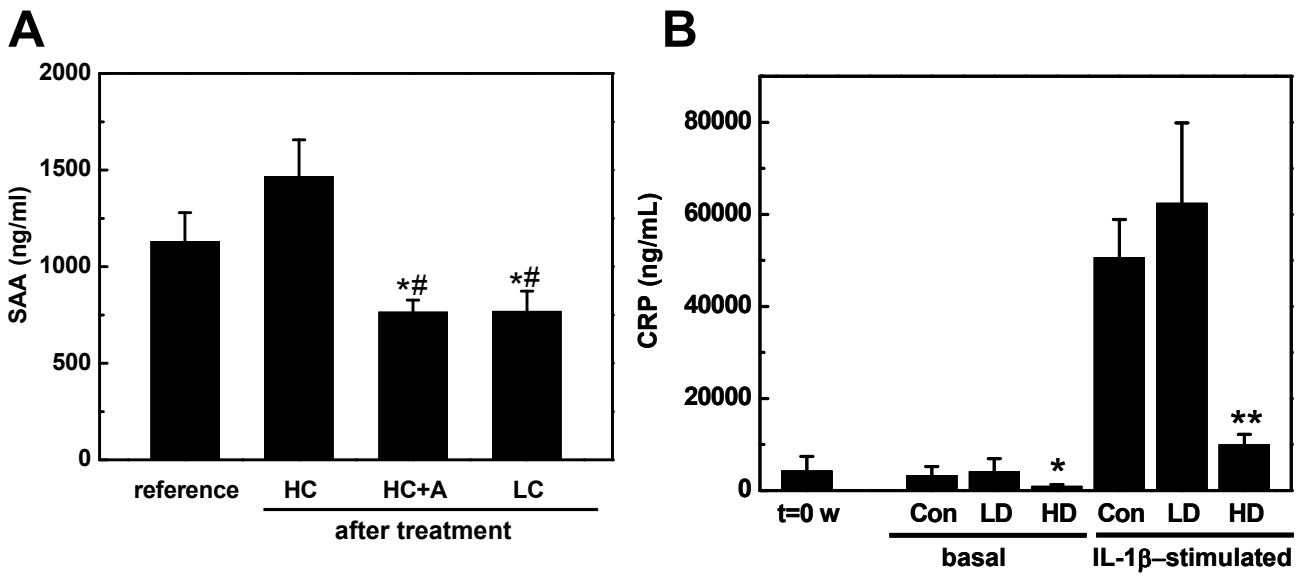

Figure 5: Effect of atorvastatin on plasma inflammation markers. (A) Effect of atorvastatin on plasma SAA (n≥11). *P<0.05 compared to reference, and #P<0.05 HC+A or LC compared to HC. **(B)** HuCRPtg mice received chow containing a low dose (LD; 0.002%(w/w)) or a high dose (HD; 0.1%(w/w)) atorvastatin, or vehicle control (Con). huCRP levels were determined before treatment (t=0 weeks), after 3 weeks of atorvastatin-treatment (basal), and 10 hrs after an intraperitoneal injection of IL-1□ (250,000 U/mouse). Data represent mean±SEM. *P<0.05 HD vs Con, **P<0.005 HD vs Con.

ApoE*3Leiden 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. Since 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 recently showed that the statin concentration which is required for huCRP-lowering is higher than the dose which is 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.[2,3] We have addressed this issue in the ApoE*3Leiden 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*3Leiden mice, blocks the progression of pre-existing lesions in the aortic arch, an effect which cannot be explained by atorvastastin's cholesterol-lowering effect.

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 paper, 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 as compared to atorvastatin.[21] Indeed, higher atorvastatin concentrations showed anti-inflammatory effects in the liver and reduce CRP expression as demonstrated in this study and elsewhere.[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 shown for cerivastatin *in vitro* using human endothelial cells.[22] 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 which demonstrates that low dose atorvastatin treatment reduces adhesion molecule expression.[23] The recent *in vitro* observation that atorvastatin downregulates the activation of the transcription factors NF-κB and AP-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 in ApoE*3Leiden mice demonstrate that atorvastatin, applied at a dose which only moderately reduces plasma cholesterol, retards the progression of pre-existing atherosclerotic lesions in the aortic root through its cholesterol-lowering activity, but cholesterol-independently 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.

Acknowledgements

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Reference List

(1) Ross R. Atherosclerosis--an inflammatory disease. *N Engl J Med.* 1999;340:115-126.

(2) Comparato C, Altana C, Bellosta S, Baetta R, Paoletti R, Corsini A. Clinically relevant pleiotropic effects of statins: drug properties or effects of profound cholesterol reduction? *Nutr Metab Cardiovasc Dis.*2001;11:328-343.

(3) Davignon J, Laaksonen R. Low-density lipoprotein-independent effects of statins. *Curr Opin Lipidol.* 1999;10:543-559.

(4) Takemoto M, Liao JK. Pleiotropic effects of 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitors. *Arterioscler Thromb Vasc Biol.* 2001;21:1712-1719.

(5) Kinlay S, Schwartz GG, Olsson AG, Rifai N, Leslie SJ, Sasiela WJ, Szarek M, Libby P, Ganz P. High-dose atorvastatin enhances the decline in inflammatory markers in patients with acute coronary syndromes in the MIRACL study. *Circulation.* 2003;108:1560-1566.

(6) Kleemann R, Princen HM, Emeis JJ, Jukema JW, Fontijn RD, Horrevoets AJ, Kooistra T, Havekes LM. Rosuvastatin reduces atherosclerosis development beyond and independent of its plasma cholesterol-lowering effect in ApoE*3Leiden transgenic mice: evidence for antiinflammatory effects of rosuvastatin. *Circulation.* 2003;108:1368-74.

(7) Sparrow CP, Burton CA, Hernandez M, Mundt S, Hassing H, Patel S, Rosa R, Hermanowski-Vosatka A, Wang PR, Zhang D, Peterson L, Detmers PA, Chao YS, Wright SD. Simvastatin has anti-inflammatory and antiatherosclerotic activities independent of plasma cholesterol lowering. *Arterioscler Thromb Vasc Biol.* 2001;21:115-121.

(8) Groot PH, van Vlijmen BJ, Benson GM, Hofker MH, Schiffelers R, Vidgeon-Hart M, Havekes LM. Quantitative assessment of aortic atherosclerosis in APOE*3 Leiden transgenic mice and its relationship to serum cholesterol exposure. *Arterioscler Thromb Vasc Biol.* 1996;16:926-933.

(9) van Vlijmen BJ, van den Maagdenberg AM, Gijbels MJ, Van Der BH, HogenEsch H, Frants RR, Hofker MH, Havekes LM. Diet-induced hyperlipoproteinemia and atherosclerosis in apolipoprotein E3-Leiden transgenic mice. *J Clin Invest.* 1994;93:1403-10.

(10) Lutgens E, Daemen M, Kockx M, Doevendans P, Hofker M, Havekes L, Wellens H, de Muinck ED. Atherosclerosis in ApoE*3Leiden transgenic mice: from proliferative to atheromatous stage. *Circulation.* 1999;99:276-283.

(11) Delsing DJ, Offerman EH, van Duyvenvoorde W, Van Der BH, de Wit EC, Gijbels MJ, van Der LA, Jukema JW, Havekes LM, Princen HM. Acyl-CoA:cholesterol acyltransferase inhibitor avasimibe reduces atherosclerosis in addition to its cholesterol-lowering effect in ApoE*3Leiden mice. *Circulation.* 2001;103:1778-1786.

(12) Ciliberto G, Arcone R, Wagner EF, Ruther U. Inducible and tissue-specific expression of human C-reactive protein in transgenic mice. *EMBO J.* 1987;6:4017-4022.

(13) Szalai AJ, McCrory MA. Varied biologic functions of C-reactive protein: lessons learned from transgenic mice. *Immunol Res.* 2002;26:279-287.

(14) Stary HC. Natural history and histological classification of atherosclerotic lesions: an update. *Arterioscler Thromb Vasc Biol.* 2000;20:1177-1178.

(15) Kleemann R, Gervois PP, Verschuren L, Staels B, Princen HM, Kooistra T. Fibrates down-regulate IL-1-stimulated C-reactive protein gene expression in hepatocytes by reducing nuclear p50-NFkappa B-C/EBP-beta complex formation. *Blood.* 2003;101:545-551.

(16) Kleemann R, Verschuren L, De Rooij BJ, Lindeman J, De Maat MM, Szalai AJ, Princen HM, Kooistra T. Evidence for anti-inflammatory activity of statins and PPAR{alpha}-activators in human C-reactive protein transgenic mice in vivo and in cultured human hepatocytes in vitro. *Blood.* 2004;103:4188-4194.

(17) Influence of pravastatin and plasma lipids on clinical events in the West of Scotland Coronary Prevention Study (WOSCOPS). *Circulation.* 1998;97:1440-1445.

(18) Stein Y, Stein O. Does therapeutic intervention achieve slowing of progression or bona fide regression of atherosclerotic lesions? *Arterioscler Thromb Vasc Biol.* 2001;21:183-188.

(19) Edwards JE, Moore RA. Statins in hypercholesterolaemia: a dose-specific meta-analysis of lipid changes in randomised, double blind trials. *BMC Fam Pract.* 2003;4:1-19.

(20) Corti R, Fuster V, Fayad ZA, Worthley SG, Helft G, Smith D, Weinberger J, Wentzel J, Mizsei G, Mercuri M, Badimon JJ. Lipid lowering by simvastatin induces regression of human atherosclerotic lesions: two years' follow-up by high-resolution noninvasive magnetic resonance imaging. *Circulation.* 2002;106:2884-2887.

(21) White CM. A review of the pharmacologic and pharmacokinetic aspects of rosuvastatin. *J Clin Pharmacol.* 2002;42:963-970.

(22) Yoshida M, Sawada T, Ishii H, Gerszten RE, Rosenzweig A, Gimbrone MAJ, Yasukochi Y, Numano F. Hmg-CoA reductase inhibitor modulates monocyte-endothelial cell interaction under physiological flow conditions in vitro: involvement of Rho GTPase-dependent mechanism. *Arterioscler Thromb Vasc Biol.* 2001;21:1165-1171.

(23) Nawawi H, Osman NS, Yusoff K, Khalid BA. Reduction in serum levels of adhesion molecules, interleukin-6 and C-reactive protein following short-term low-dose atorvastatin treatment in patients with non-familial hypercholesterolemia. *Horm Metab Res.* 2003;35:479-485.

(24) Dichtl W, Dulak J, Frick M, Alber HF, Schwarzacher SP, Ares MP, Nilsson J, Pachinger O, Weidinger F. HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol.* 2003;23:58-63.

(25) Chen Y, Schneider DJ. The independence of signaling pathways mediating increased expression of plasminogen activator inhibitor type 1 in HepG2 cells exposed to free fatty acids or triglycerides. *Int J Exp Diabetes Res.* 2002;3:109-118.