Atorvastatin inhibits plaque development and adventitial neovascularization in ApoE deficient mice independent of plasma cholesterol levels

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1. Introduction

Acute cardiovascular syndromes (ACS) such as myocardial infarction and stroke are commonly caused by erosion or rupture of an unstable atherosclerotic plaque. Both lipid accumulation and inflammation have been regarded to contribute substantially to the initiation and progression of atherosclerosis, the underlying cause of ACS [1,2]. Inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A HMG-CoA reductase (statins) have evolved into the drugs of choice to lower elevated lipids levels [3]. While the therapeutic efficacy of statins in cardiovascular disorders has been established in numerous studies, evidence is mounting that statins protect against cardiovascular disorders not only by virtue of their hypolipidemic activity but also by quenching NFκB [4] and by increasing PPARγ [5,6] as well as PPARγ activation [7]. Both processes are deemed to affect not only vascular inflammation but also angiogenesis, a process widely regarded as a risk factor in atherosclerosis [8]. In effect, Panigrahy et al. have demonstrated that PPARγ activation inhibits angiogenesis by reducing VEGF synthesis at a transcriptional level [9] and enhances endothelial cell apoptosis [10]. In addition, Oitzinger et al. have shown that the ability of endothelial cells to form capillary tubes in a matrigel assay was impaired after inhibition of NFκB activation [11], while endothelial cell survival was governed in part by NFκB [12]. Current literature on statin treatment and neovascularization is rather contradictory. For example, Kureishi et al. demonstrated that simvastatin promotes angiogenesis in normocholesterolemic animals [13], while more recently it was shown that atorvastatin strongly induced angiogenesis by enhancing the expression of angiogenic cytokines such as VEGF, Ang-1, Ang-2 and eNOS, and by increasing HO-1 and EPC numbers in a hindlimb ischemia model [14]. In contrast, atorvastatin was demonstrated to impair myocardial angiogenic response to chronic ischemia [15]. In human endothelial cells, statin treatment was shown to induce thrombomodulin expression which may be favorable in disorders associated with endothelial dysfunction [16]. Wilson et al. showed that simvastatin treatment inhibited the expansion of the vasa vasorum in pigs [17]. Likewise, cerivastatin appeared to have anti-angiogenic effects in a panel of in vitro and in vivo models [18,19]. These discrepancies may arise from differences in experimental setup, such as animal model, diet and statin...
dosage [20]. Indeed, atorvastatin affected endothelial function in a dose-dependent manner, being pro-angiogenic at 10 nM, and anti-angiogenic in the μM range [21].

Microvessel formation in the intima and adventitia of advanced atherosclerotic plaques is increasingly considered as an important factor in plaque destabilization [8,22]. The PPAR and NfκB modulatory activity of statins led us to propose that statins may lower the risk of acute cardiovascular syndromes at least partly by inhibiting plaque neoangiogenesis. In this study we aimed to establish the effect of atorvastatin treatment of apoE−/− mice on perivascular microvessel formation in advanced atherosclerotic plaques. We demonstrate that atorvastatin treatment inhibits atherosclerotic plaque development independent of cholesterol levels and in addition reduces the number of perivascular CD31+ microvessels. Our study thus identifies an alternative mechanism by which atorvastatin treatment may prevent atherogenesis.

2. Materials and methods

2.1. Animals

All animal works were performed in compliance with the Dutch government guidelines. Male apoE−/− mice, obtained from the local animal breeding facility, were fed a Western type diet, containing 0.25% cholesterol and 15% cacao butter (SDS, Sussex, UK). After 2 weeks of diet feeding, carotid artery plaque formation was induced by perivascular collar placement in male apoE−/− mice as described previously [23], after which the mice were fed either Western-type diet (control) or Western-type diet supplemented with a low dose of atorvastatin (0.003%, w/w) [24]. After 8 weeks of atorvastatin treatment, the mice were anaesthetized and in situ perfusion and carotid artery embedding occurred as described previously [23]. Transverse 5 μm cryosections were prepared on a Leica CM 3050S Cryostat (Leica Instruments) in a proximal direction for histology and immunohistochemical analysis.

2.2. Histology and morphometry

Cryosections were stained with hematoxylin (Sigma–Aldrich, Zwijndrecht, The Netherlands) and eosin (Merck Diagnostica). Macrophage content of the lesions was assessed using a rat monoclonal MOMA-2 antibody (Serotec, Kidlington, Oxford, UK). Collagen was visualized using a Masson’s Trichrome staining kit (Sigma–Aldrich). Mast cells and neutrophils were visualized by staining of cryosections with naphthol AS-D chloroacetate esterase (Sigma–Aldrich). Endothelial cells were visualized by incubation with a CD31 (PECAM-1) antibody (1:50, Thermo Fisher Scientific) overnight at 4°, after which the slides were incubated with Powervision Ready to use Poly-HRP anti Rabbit IgG (Immunologic, Duiven, The Netherlands) for 1 h at room temperature and subsequently stained with Nova Red. Nuclei were visualized with hematoxylin.

Morphometric analysis (Leica Qwin image analysis software) was performed on hematoxylin-eosin stained sections of the carotid arteries at the site of maximal stenosis (controls: n = 12, atorvastatin: n = 11). The number of perivascular CD31+ microvessels, mast cells and neutrophils were assessed manually. MOMA-2 and collagen positive areas were quantified by Leica Qwin image analysis software. All morphometric analyses were performed by blinded independent operators (I.M.L./I.B.).

2.3. Cell culture

The mouse endothelial cell line H5 V was cultured in a humidified atmosphere (5% CO2) at 37 °C in Dulbecco’s Modified Eagle Medium (DMEM) containing 10% Foetal Bovine Serum (FBS), 2 mmol/L l-glutamine, 100 U/mL penicillin and 100 μg/mL streptomycin (all from PAA, Colbe, Germany). To measure cell proliferation, H5 V cells were detached by short incubation with trypsin-EDTA (PAA) and seeded at a density of 4.0 × 104 cells per well. The cells were allowed to attach, serum starved in DMEM containing 1% FBS for 8 h to synchronize cell cycle and incubated overnight with medium containing atorvastatin or control medium. After incubation 0.5 μCi [3H]thymidine (GE healthcare) per well was added and the cells were incubated at 37 °C for 5 h. [3H]Thymidine incorporation was quantified in a liquid scintillation analyzer (Packard 1500 Tricarb, USA), n = 4 per concentration used.

To measure the effects of atorvastatin on endothelial tubule formation, H5 V cells (4 × 104 cells/well) were plated on Matrigel (BD Biosciences) coated plates in the presence or absence of atorvastatin. After 16 h, representative pictures were taken from each well and the number of sprouts (measured by the number of cellular extensions) relative to cellular surface area was determined using Leica Qwin image analysis. The experiment was performed in triplicate and of each sample three microscopic fields were analyzed.

2.4. Statistical analysis

Data are expressed as mean ± SEM. A 2-tailed Student’s t-test was used to compare individual groups. Non-parametric data were analyzed using a Mann–Whitney U test. A level of P ≤ 0.05 was considered significant.

3. Results

We delineated the effect of atorvastatin treatment on atherosclerotic lesion progression. Atherosclerotic plaques were induced in apoE−/− mice by non-constrictive perivascular collar placement at the carotid arteries. During 8 weeks of lesion development, the mice were fed plain Western-type diet (control) or Western-type diet supplemented with 0.003% (w/w) atorvastatin. During the study, atorvastatin treatment did not affect body weight gain (data not shown) and did not lower average plasma total cholesterol levels (control: 1357 ± 45 versus atorvastatin: 1354 ± 46 mg/dL, P = NS, Fig. 1). Despite the unaltered plasma lipid profile, carotid artery plaque size at 8 weeks after collar
Fig. 2. (A) Treatment of apoE−/− mice with atorvastatin (0.003%, w/w, in Western-type diet for 8 weeks) resulted in a significant reduction in atherosclerotic plaque development (*P < 0.05, controls: n = 12, atorvastatin: n = 11), while media size remained unaffected (B). (C) Relative necrotic core size was also significantly reduced by atorvastatin treatment (*P < 0.05). Lower panels: representative micrographs of carotid artery lesions from a control (left) and an atorvastatin treated ApoE−/− (right, 100× magnification).

placement was reduced by almost 40% from 85 ± 11 × 10³ μm² in control mice to 52 ± 9 × 10³ μm² in the atorvastatin treated mice (P < 0.05, Fig. 2A). Media size did not differ between the groups (47 ± 3 × 10³ μm² in control mice to 41 ± 2 × 10³ μm² in the atorvastatin treated mice, Fig. 2B), while also intima/media ratio and intima/lumen ratio were not significantly affected by atorvastatin treatment. Furthermore, the relative necrotic area was reduced by 35% from 42 ± 4% in control mice to 28 ± 5% in atorvastatin treated mice (P < 0.05, Fig. 2C).

As to plaque morphology, atorvastatin treatment significantly reduced the number of perivascular CD31+ neovessels in the adventitia by over 40% (6.6 ± 1.1 versus 11.1 ± 1.6 CD31+ neovessels/mm² perivascular tissue for controls; P < 0.05, Fig. 3) pointing to an inhibitory effect of atorvastatin on inflammatory angiogenesis. MOMA-2 macrophage positive area and collagen content, as measured with a Masson’s Trichrome staining did not differ between the groups (Fig. 4A and B). As we and others have previously shown that activation of adventitial mast cells leads to an acute deterioration of plaque stability with intraplaque hemorrhages and de novo cell influx [25], and mast cells were seen to harbor potent proangiogenic activity, we studied whether mast cell numbers and activation status were affected by atorvastatin treatment. However, atorvastatin did not alter the number of perivascular mast cells (controls: 3.3 ± 0.6 mast cells/mm² perivascular tissue versus atorvastatin: 2.7 ± 0.8 mast cells/mm² perivascular tissue, Fig. 4C), while the percentage of activated mast cells tended to be reduced from 57 ± 11% in controls to 38 ± 12% activated mast cells in the atorvastatin treated mice. Also the number of perivascular neutrophils remained unaffected by atorvastatin treatment (controls: 67 ± 16 neutrophils/mm² perivascular tissue versus atorvastatin: 72 ± 17 neutrophils/mm² perivascular tissue, Fig. 4D). These data suggest that atorvastatin at the dose used affects plaque size in particular by affecting perivascular neovascularization.

To assess whether atorvastatin affected endothelial proliferation, we stimulated mouse endothelial cells (HSV cells) with increasing concentrations of atorvastatin, after which cell proliferation was measured by [3H]thymidine incorporation. Atorvastatin inhibited proliferation in a dose-dependent fashion from 484 ± 4 × 10³ dpm in untreated cells up to 262 ± 9 × 10³ dpm at the highest concentration of 25 μM of atorvastatin (equals 0.003%, P < 0.001, Fig. 5A), suggesting that atorvastatin indeed inhibits endothelial growth as seen in vivo. Interestingly, atorvastatin treatment did not affect the formation of endothelial sprouts in a Matrigel assay, as measured by the number of cellular extensions per mm² cellular surface area (P = NS, Fig. 5B).

4. Discussion

In this study, we demonstrate that atorvastatin inhibits perivascular neovascularization and lesion development in apoE−/− mice independently of plasma cholesterol levels. HMG-CoA reductase inhibitors or statins are widely used in lipid-lowering therapy, but evidence is accumulating that statins may not only act atheroprotective by lowering cholesterol levels but also by modulation of NKx8, PPARα, and PPARγ dependent responses [4–7]. These transcription factors have been demonstrated to affect inflammation but have recently also been implicated in the process of angiogenesis [8–12]. As this is an independent risk factor for atherosclerosis it is conceivable that the beneficial effects of statin treatment may at least partly proceed by inhibition of neovascularization. Literature on statin treatment and angiogenesis is rather conflicting, as a number of studies demonstrate pro-angiogenic effects of statins.
For example, Kureishi et al. revealed that simvastatin promoted angiogenesis in a rabbit model of hindlimb ischemia [13]. Similarly, atorvastatin treatment was seen to induce angiogenesis in ischemic limbs of rats [14]. Others showed an inhibitory capacity of statin treatment on endothelial proliferation in different models of ischemia [15–19]. As mentioned earlier, these contradictory findings may arise from differences in experimental setup, such as animal model, diet, statin type and dosage [20].

Here, we hypothesized that statins may have a beneficial effect on perivascular angiogenesis in a model of collar-induced atherosclerosis in mice because of its PPAR and NFκB modulatory activity. Western-type diet fed apoE−/− mice, treated with atorvastatin (0.003% in western-type diet) showed reduced atherosclerotic lesion progression independently of plasma cholesterol levels. Necrotic core size was reduced as well in the atorvastatin treated plaques, which may either be due to reduced plaque progression.

Fig. 3. Perivascular CD31+ microvessel density was significantly reduced by atorvastatin treatment (*P < 0.05, left panel, controls: n = 12, atorvastatin: n = 11). Right panel: representative micrographs (400× magnification) of perivascular tissue of control mice (upper) and atorvastatin treated mice (lower) stained with a CD31 antibody.

Fig. 4. (A) Macrophage content of collar-induced carotid artery plaques was not affected by atorvastatin treatment as demonstrated by a MOMA-2 staining. (B) Lesional collagen content also did not differ between control and atorvastatin treated mice as shown by Masson’s Trichrome staining. Furthermore, perivascular mast cell (C) and neutrophil (D) numbers remained unaffected by atorvastatin treatment (controls: n = 12, atorvastatin: n = 11).
in general or possibly by reduced apoptosis of plaque leukocytes [26]. Strikingly, we observed a significant reduction of the CD31+ microvessel content in adventitial tissue of atorvastatin treated plaques. These findings concur with our in vitro data that show that atorvastatin inhibits endothelial cell proliferation at all tested concentrations, while not affecting endothelial sprouting. Our data confirm previous studies by Wilson et al. [17] reporting reduced vasa vasorum formation in pigs after simvastatin treatment. Also, cerivastatin studies showed similar effects on endothelial cell proliferation and in vivo angiogenesis in a matrigel setup [18,19]. Our study demonstrates that atorvastatin inhibits perivascular neovascularization in an in vivo model of atherosclerosis. These data furthermore suggest that perivascular angiogenesis may contribute to plaque progression and indeed can be regarded a risk factor in atherosclerosis as described earlier [8]. Finally, they demonstrate an additional atheroprotective feature of statin treatment.

In conclusion, atorvastatin treatment was seen to inhibit plaque development in ApoE deficient mice independent of plasma total cholesterol levels. The concomitant inhibitory effect of atorvastatin on adventitial neovascularization fuels the intriguing notion that part of its beneficial effects occurs via this pathway. Thus, the present data suggest that inhibition of neovascularization is an additional mechanism by which atorvastatin treatment may prevent the development and progression of atherosclerosis.

Conflict of interest

The authors have no conflicts of interest to disclose.

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