Pravastatin Reduces Marfan Aortic Dilation

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Background—The sequelae of aortic root dilation are the lethal consequences of Marfan syndrome. The root dilation is attributable to an imbalance between deposition of matrix elements and metalloproteinases in the aortic medial layer as a result of excessive transforming growth factor-beta signaling. This study examined the efficacy and mechanism of statins in attenuating aortic root dilation in Marfan syndrome and compared effects to the other main proposed preventative agent, losartan.

Methods and Results—Marfan mice heterozygous for a mutant allele encoding a cysteine substitution in fibrillin-1 (C1039G) were treated daily from 6 weeks old with pravastatin 0.5g/L or losartan 0.6 g/L. The end points of aortic root diameter (n=25), aortic thickness, and architecture (n=10), elastin volume (n=5), dp/dtmax (maximal rate of change of pressure) (cardiac catheter; n=20), and ultrastructural analysis with stereology (electron microscopy; n=5) were examined. The aortic root diameters of untreated Marfan mice were significantly increased in comparison to normal mice (0.161±0.001 cm vs 0.252±0.004 cm; P<0.01). Pravastatin (0.22±0.003 cm; P<0.01) and losartan (0.221±0.004 cm; P<0.01) produced a significant reduction in aortic root dilation. Both drugs also preserved elastin volume within the medial layer (pravastatin 0.23±0.02 and losartan 0.29±0.03 vs untreated Marfan 0.19±0.02; P=0.01; normal mice 0.27±0.02). Ultrastructural analysis showed a reduction of rough endoplasmic reticulum in smooth muscle cells with pravastatin (0.022±0.004) and losartan (0.013±0.001) compared to untreated Marfan mice (0.035±0.004; P<0.01).

Conclusions—Statins are similar to losartan in attenuating aortic root dilation in a mouse model of Marfan syndrome. They appear to act through reducing the excessive protein manufacture by vascular smooth muscle cells, which occurs in the Marfan aorta. As a drug that is relatively well-tolerated for long-term use, it may be useful clinically. (Circulation. 2011;124[suppl 1]:S168–S173.)

Key Words: aneurysm  aorta  pathology  remodeling  vessels

Marfan syndrome is a multisystem connective tissue disorder characterized by cardiovascular, musculoskeletal, and ocular abnormalities, with the most life-threatening complications being cardiovascular and particularly aortic regurgitation, thoracic aortic aneurysm, and dissection. Without intervention, most patients have a life expectancy of only 45 years. It is attributable to a defect in fibrillin-1, which is a component of microfibrils, which provide the connectivity between the connective tissue matrix of the aortic medial layer and the surrounding vascular smooth muscle cells, allowing for appropriate responses and remodeling to wall strain. A relative lack or abnormal fibrillin-1 means that the vascular smooth muscle cells lose their ability to sense aortic wall strain and excessive transforming growth factor-beta (TGF-β) is released from the connective tissue matrix, causing excessive activation of the smooth muscle cells and a haphazard and inappropriate remodeling response. This is characterized by excess deposition of matrix elements such as collagen and proteoglycans, excess metalloproteinases, and infiltration of macrophages. Weakening and progressive dilatation of the aortic root ensues. It is this pathological remodeling changes initiated by vascular smooth muscle cells that may be a target for therapeutic intervention. Dysregulation of TGF-β attributable to a deficiency of fibrillin-1 rather than a purely structurally deficient aorta is thought to predispose to this aneurysmal dilatation.

Pravastatin is a HMG-CoA reductase inhibitor or “statin.” It is primarily used as a cholesterol-lowering agent and is effective in reducing deaths from cardiovascular disease in patients with hypercholesterolemia. Pravastatin also has a
number of beneficial pleiotropic antiinflammatory effects. Pravastatin reduces the excess risk of coronary events attributable to inflammation after myocardial infarction. In carotid plaques removed at endarterectomy, previous treatment with pravastatin was associated with reduced metalloproteinases and macrophage infiltration. It also has been shown to reduce cardiac expression of TGF-β in an in vivo model of diabetic glucose intolerance. Thus, we became interested in pravastatin as a potential therapeutic agent to prevent the cardiovascular complications of Marfan syndrome. We investigated its efficacy in reducing aortic root dilation and inappropriate remodeling of the medial layer and compared it to the main medication that is currently undergoing clinical trials for aortic root dilation prevention in Marfan syndrome, the angiotensin-2 antagonist, losartan. We used ultrastructural analysis of the smooth muscle cells of the aortic root as a preliminary tool for analysis of the mechanism of any potential beneficial effect.

Methods

A male pair of C1039G Marfan mice (courtesy of Dr Hal Dietz, Johns Hopkins Hospital, Baltimore, MD) were cross-bred with C57Bl6 females. Genetic analysis of the offspring was performed using polymerase chain reaction to amplify a 600-bp fragment of the fibrillin-1 gene containing the missense mutation. The primers used for polymerase chain reaction were intron 24s (TTGCTCATGTGCTTTAAGTAGC) and intron 25s (ACAGAGGTCAAGGATATGC; Genosys; Sigma). C1039G Marfan mice were identified by the presence of a KpnI restriction site introduced by the mutation into the fibrillin-1 gene. This was achieved using the restriction enzyme Asp 718 (Roche), which split the polymerase chain reaction product containing the missense mutation into a 350-bp and 250-bp fragment. The substrate was then subject to agarose gel electrophoresis. Male mice identified to be heterozygous for the C1039G mutation were entered into the study in different treatment groups. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Royal College of Surgeons in Ireland.

The first group was administered pravastatin (Bristol Myers Squibb) from 6 weeks of age. The dose was 0.5 g/L, equivalent to 100 mg/kg per day. This dose was chosen after a dose–response study was performed in C57Bl6 mice in which increasing doses of pravastatin were administered, and 2 weeks later serum cholesterol levels were measured. A dose of 50 mg/kg per day led to a significant reduction in mean cholesterol levels from 1.71 to 1.29 mmol/L (P<0.05). The second group was administered losartan (Sigma) from 6 weeks of age. The dose was 0.6 g/L, which was the dose used in previous studies. A third group included untreated Marfan mice and a fourth group included normal offspring that did not have the C1039G mutation. Mice were housed in a temperature-controlled environment with 12-hour light/dark cycles and they had access to food and water ad libitum.

Primary End Points

Aortic root diameter (n=10 in each group) was assessed using stereology. Semithin sections (0.75-μm-thick) were cut using a glass knife and a Reichert-Jung Ultracut ultramicrotome. The sections were then placed on glass slides and stained for 8 seconds with toluidine blue (Sigma). Sections were examined on a Leica bright field microscope connected to a computer with Leica application suite microscope software. Two images from each mouse were taken at ×10 and ×40 objective magnification to perform stereological analysis. For calculating the volume fraction of the elastic lamellae in the aortic wall (Vv), ImageJ software was used. The images were thresholded at a final magnification of ×310 to estimate the area of aortic wall and area of elastic lamellae. The area fraction and corresponding volume fraction were estimated using the formulæ:

$$\frac{A_{\text{medial lamellae}}}{A_{\text{aortic wall}}} \cdot \frac{V_{\text{elastic lamellae}}}{V_{\text{aortic wall}}} \cdot V_v$$

Verhoeff-van Gieson stains were used for assessing the elastin architecture of the aortic root. The degree of fragmentation of the elastic fibers was examined by 2 independent observers blinded to the genotype and treatment group who scored each slide using an arbitrary scoring system that counted the number of “islands of damage” within an aortic cross-section from each mouse (n=10 in each group). An island of damage was defined as an isolated area of aortic wall where 2 adjacent elastic fibers were fragmented with interposed excessive connective tissue matrix (Figure 1).

Cardiac function was assessed using hemodynamic variables from cardiac catheter recordings, particularly dp/dt (maximal rate of change of pressure) maximum as a measure of left ventricular systolic function at 8 months. After anesthesia with intraperitoneal xylazine 10 mg/kg (Xylapan; Vetoquinol) and ketamine 100 mg/kg (Ketalar; Pfizer), a midline vertical neck incision was made and the right carotid artery was exposed. A 1.2-French Millar cardiac catheter was introduced into the carotid artery, and aortic and left ventricular pressure tracings were recorded. Powerquest software was used to analyze the pressure tracings to calculate dp/dt maximum and pulse pressure. Samples of aortic root were harvested at 8 months for ultrastructural examination with electron microscopy of aortic vascular smooth muscle cell activity. Ultrathin sections (0.5 μm) were cut using a diamond knife and a Reichert-Jung UltracutE ultramicrotome. The sections were placed on 200-μm mesh copper grids and stained with uranyl acetate and lead citrate using a Leica EM AC20 Autocontrastor. Sections were examined on a Hitachi H7000 transmission electron microscope at an accelerating voltage of 75 kV. Six images were taken of each tissue sample at ×5000 and ×20 000 magnification to perform stereological analysis. Volume of smooth muscle cells was estimated using a variation of the nucleator principle. Twenty smooth muscle cells were chosen from images of each animal at final magnification of ×310. The profile of each smooth muscle cell was traced and a 4-way nucleator was applied. The distance from the nucleus of the cell to

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**Figure 1.** Verhoeff-van Gieson–stained axial section of the aortic root showing a normal aortic wall (A) and a higher-power image of an island of damage (red arrow) within the aortic wall (B).
the cell membrane was measured. This was cubed and the mean multiplied by \(\pi/3\) to give an estimate of the individual cell volumes. This gave an estimate of the number-weighted mean smooth muscle cell volume for each animal in all groups.\(^1\)

The volume ratio of rough endoplasmic reticulum-to-cytoplasm in vascular smooth muscle cells in aortic axial sections was estimated on images at a final magnification of \(\times 34,560\) by randomly applying a stereological grid onto the images using a simple point counting method and the formula: \(Vv (volume\ fraction) = \frac{P(ROUGH\ ENDOPRASMIC\ RETICULUM)}{P(CYTOPLASM)}\), where \(P(ROUGH\ ENDOPRASMIC\ RETICULUM)\) represents the points that hit the cisternae of rough endoplasmic reticulum and \(P(CYTOPLASM)\) represents the points that hit the cytoplasm of smooth muscle cell (Mayhew 1991\(^1\)).

By similar means, the volume ratios of the nucleus, smooth endoplasmic reticulum, golgi apparatus, and mitochondria to the smooth muscle cell cytoplasm were calculated. Also, the volume ratios of euchromatin and heterochromatin to the nucleus were examined.

Statistical analysis was performed using the Kruskal-Wallis 1-way analysis of variance for nonparametric data, with \(P<0.05\) considered significant. There was no adjustment for multiple comparisons. All results reported in the text are means \(\pm\ SEM\).

## Results

The aortic root diameter enlarged from 0.161\(\pm\)0.001 cm in the normal group to 0.252\(\pm\)0.004 cm in the Marfan untreated group (\(P<0.01\)). The aortic root diameter enlargement was attenuated significantly by pravastatin treatment (0.221\(\pm\)0.004 cm; \(P<0.01\)), and to the same degree by losartan treatment (0.221\(\pm\)0.004 cm; \(P<0.01\); Figure 2). We then examined whether this reduction in root dilation was associated with preservation of more normal aortic wall architecture by assessing medial layer thickness, mural architecture disturbance, and elastin content of the aortic wall. The architecture by assessing medial layer thickness, mural architecture disturbance, and elastin content of the aortic wall. The degree of elastin loss from the medial layer was compared between treatment groups by looking at the volume fraction of elastin in the medial layer. Untreated Marfan mice showed a predictable reduction in volume fraction of elastin (0.19\(\pm\)0.02 vs normal 0.27\(\pm\)0.02; \(P<0.01\)). Pravastatin preserved some of the elastin within the medial layer (0.23\(\pm\)0.02; \(P=0.01\)), and losartan had an even greater beneficial effect (0.29\(\pm\)0.03; \(P<0.01\); Figure 3).

Using a stereological method that directly assessed whether there were any differences in the hemodynamic forces being exerted on the aorta between the losartan-treated and statin-treated mice. The dp/dt maximum was reduced in the Marfan mice (4793\(\pm\)257 mm Hg/sec) compared to normal wild-type mice (6574\(\pm\)173 mm Hg/sec; \(P<0.01\)). Losartan pretreatment was associated with a further reduction in dp/dt maximum compared to the untreated Marfan mice (4068\(\pm\)209 mm Hg/sec; \(P=0.02\)). The pulse pressure (systolic–diastolic pressure) was similar in the normal untreated Marfan and pravastatin-treated Marfan group. However, the losartan-treated Marfan group had a significantly lower pulse pressure compared to the other groups (Figure 4), demonstrating a hypotensive effect with losartan treatment.

Because the vascular smooth muscle cells in the aortic wall are affected by all the pathological factors that lead to medial layer damage in Marfan syndrome, we examined for any differences in the ultrastructure of these cells between the groups to screen for any possible protective mechanism of pravastatin. Stereology was used to assess the volume ratio/fraction of each organelle compared to the whole smooth muscle cell cytoplasm volume, and the volume of heterochromatin and euchromatin in the nucleus. The only organelle that showed a predictable reduction in volume fraction of elastin (0.19\(\pm\)0.02 vs normal 0.27\(\pm\)0.02; \(P<0.01\)). Pravastatin preserved some of the elastin within the medial layer (0.23\(\pm\)0.02; \(P=0.01\)), and losartan had an even greater beneficial effect (0.29\(\pm\)0.03; \(P<0.01\); Figure 3).

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<th>Table. Aortic Wall Architectural Score Based on Number of Islands of Damage</th>
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\(N=10\) in each group.
showed any significant changes was the rough endoplasmic reticulum (Figure 5), which was significantly increased in the untreated Marfan group compared to controls (0.035 ± 0.004 vs 0.009 ± 0.002; \( P < 0.01 \)).

Both pravastatin treatment (0.022 ± 0.004; \( P = 0.02 \)) and losartan treatment (0.013 ± 0.001; \( P < 0.01 \)) reduced the excessive rough endoplasmic reticulum activity, but losartan reduced it to a significantly greater degree than pravastatin, such that there was no significant difference in rough endoplasmic reticulum activity between losartan-treated Marfan mice and normal mice that did not have Marfan syndrome at all (\( P = 0.08 \); Figure 6).

**Discussion**

This study demonstrates that pravastatin treatment has a beneficial effect in attenuating aortic root dilation and preserving elastin within the aortic wall; however, significant pathological changes still exist within the aortic wall. Losartan, in comparison, reduces aortic root dilation to the same degree but has a more significant effect on preserving elastin within the aortic wall and in reducing aortic wall thickening. Our findings with losartan are of a similar magnitude to those reported previously.\(^{10}\) The beneficial effect of pravastatin in Marfan syndrome is not surprising when you look at the pathological mechanism of Marfan syndrome. Contrary to the previous understanding of aortic disease in Marfan syndrome, which assumed a mechanically deficient vessel attributable to reduced levels of structurally important fibrillin-1 microfibrils, a more complex homeostatic role for fibrillin-1 leading to tissue remodeling has evolved in recent years. Fibrillin-1 is a component of microfibrils that provide a connection between elastin fibers and vascular smooth muscle cells to provide signaling for homeostasis within the aortic wall.\(^{13}\) Structural homology between the fibrillins and latent TGF-β-binding proteins suggests an additional role for fibrillin-1 in targeting TGF-β to the extracellular matrix and keeping it in an inactive state. A deficiency of fibrillin-1 therefore would lead to excessive TGF-β signaling.\(^{4,14}\) Dysregulation of TGF-β signaling has been observed to contribute to pathology in the lung,\(^{4}\) aorta,\(^{10}\) and mitral valve\(^{15}\) in mouse models of Marfan syndrome. This also is supported by the observation of a Marfan phenotype in humans with mutations in the TGF-β receptor.\(^{14}\) Fibrillin-1 deficiency leading to dysregu-
lation of TGF-β signaling prompts vascular smooth muscle cells to attempt to remodel the extracellular matrix via the SMAD transcription factor-mediated pathway. This is characterized by excess deposition of matrix elements such as proteoglycans and collagen in a haphazard fashion and excess metalloproteinase deposition, which is associated with fragmentation of elastic fibers. This excessive metalloproteinase activity in the aortic wall is similar to that seen in atheromatous plaques. Statins, which are HMG-CoA reductase inhibitors, are used as cholesterol-lowering agents with proven efficacy in reducing cardiovascular morbidity, even in those patients with normal or only moderately elevated cholesterol levels. The cholesterol-independent pleiotropic antiinflammatory effects of pravastatin also contribute to this reduction in cardiovascular morbidity. This has been demonstrated by the abrogation of the excess risk of recurrent coronary events associated with evidence of inflammation after myocardial infarction. Elucidation of the mechanism of these antiinflammatory effects, which are thought to be attributable to plaque stabilization, revealed a beneficial effect of pravastatin at a routine dose of 40 mg/d to reduce inflammation, particularly metalloproteinases and macrophage infiltrates in human carotid plaques. This effect is not just confined to atherosclerosis, however, because a reduction in matrix metalloproteinsase-9 in serum samples also has been observed in humans treated with pravastatin. Therefore, statins were a logical choice for a potential therapy for Marfan syndrome.

To investigate for the potential mechanisms involved, we chose to look at the ultrastructure of the vascular smooth muscle cell in the aortic wall, and we and other investigators have previously found it difficult to reliably perform immunohistochemistry on C1039G murine aortic cross-sections. As stated already, we noted that losartan and pravastatin reduced aortic rough endoplasmic reticulum activity. The rough endoplasmic reticulum is the site of protein manufacture within the cell. It is called rough because of the ribosomes, which are stuck to its folds, giving it a studded appearance. When the ribosomes are adhered to the rough endoplasmic reticulum, they are producing proteins that are destined for the secretory pathway. Therefore, in Marfan syndrome, we would expect to see more rough endoplasmic reticulum activity attributable to chronic TGF-β activation leading to excess protein production, as we found. In the case of losartan, there was no increase in TGF-β activity compared to normal mice, which is explained by it blocking the Marfan pathogenic sequence at the TGF-β receptor level. Statins, however, produced some reduction in rough endoplasmic reticulum activity but not as marked as losartan, suggesting that their beneficial effect is likely a posttranslational effect. Statins inhibit HMG-CoA reductase, which is an enzyme involved in the synthesis of cholesterol and the prenylation of proteins. Prenylation is a process whereby an additional factor is added to a protein to enable its secretion from a cell to be functional in the extracellular matrix. Statins have been shown to reduce metalloproteinase secretion from vascular smooth muscle cells, likely through this mechanism, which would explain the reduced but not completely normal rough endoplasmic reticulum activity in statin-treated Marfan mice.

One surprising finding was that although the volume of elastin in the aortic wall was improved in the losartan-treated and pravastatin-treated groups compared to the Marfan group, this did not result in any improvement in the architecture scores. This is at odds with previous studies that have compared losartan treatment to the β-blocker propranolol and to doxycycline. In both these studies, losartan was shown to have a marked effect.
in preserving aortic wall architecture similar to that of wild-type mice that did not have Marfan syndrome. This may be explained by the fact that the scoring system that we designed for the architecture scores is based on counting the number of foci of damage (island of damage) in a given axial section. The score does not provide any information on the size of each island of damage, ie, whether an island crosses 2 or 4 elastin fibers, it is still counted as 1 island of damage. This may suggest that the initiating stimulus for the island of damage is still present, but that losartan and pravastatin attenuate the degree of elastin damage at each island of damage, because the volume of elastin in each axial section was greater in these groups compared to the untreated Marfan group. This will require further study.

The additional beneficial effects of losartan over statins also could be contributed from losartan’s antihypertensive effect, as seen by a reduced pulse pressure in our study, leading to less hemodynamic strain on the aortic root. Future studies of potential pharmacological therapies for the prevention of aortic pathology in Marfan syndrome should probably include another antihypertensive drug to delineate exactly the contribution of the antihypertensive effect. However, previously, losartan was compared to the β-blocker propranolol in a study in which doses were adjusted so that they both produced an equivalent decrease in blood pressure of 10% to 20%. This study found that although propranolol treatment produced the same antihypertensive effect, it failed to reduce elastin damage or to reduce histological damage in the aortic wall.  

This study demonstrates a potential therapeutic effect for statins in the slowing of aortic root dilation and elastin loss in Marfan syndrome. It is a proof of principle study, and further work now needs to be performed to examine further the mechanism involved. However, as a drug treatment that would be potentially required lifelong, it has a proven relative safety record with use over long periods as a cholesterol-lowering treatment. This study also emphasizes the beneficial effects of losartan and demonstrates that at the ultrastructural level, Marfan smooth muscle cells can appear like normal smooth muscle cells with losartan treatment.

Sources of Funding
This research was conducted in The Royal College of Surgeons in Ireland with funding from the Marfans Research Foundation Ireland and the Marfan Syndrome Support Group Ireland Limited. Additional funding was provided by the Howard Hughes Medical Institute, the National Institutes of Health, the National Marfan Foundation (USA), and the Smilow Center for Marfan Syndrome Research.

Disclosures
H.C.D. has previously received research grants from the National Institutes of Health, the Howard Hughes Medical Institute, the National Marfan Foundation, and the Smilow Centre for Marfan Syndrome Research. H.C.D. also previously received TGF-beta activation contributes to pathogenesis in Marfan syndrome. Nat Genet. 2003;33:407–411.


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Circulation. 2011;124:S168-S173
doi: 10.1161/CIRCULATIONAHA.110.012187

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