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Circ Res. 2008;102:1529-1538; originally published online May 22, 2008;
doi: 10.1161/CIRCRESAHA.108.175976

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Chronic Apoptosis of Vascular Smooth Muscle Cells Accelerates Atherosclerosis and Promotes Calcification and Medial Degeneration

Murray C.H. Clarke, Trevor D. Littlewood, Nichola Figg, Janet J. Maguire, Anthony P. Davenport, Martin Goddard, Martin R. Bennett

Abstract—Vascular smooth muscle cell (VSMC) accumulation is implicated in plaque development. In contrast, VSMC apoptosis is implicated in plaque rupture, coagulation, vessel remodeling, medial atrophy, aneurysm formation, and calcification. Although VSMC apoptosis accompanies multiple pathologies, there is little proof of direct causality, particularly with the low levels of VSMC apoptosis seen in vivo. Using a mouse model of inducible VSMC-specific apoptosis, we demonstrate that low-level VSMC apoptosis during either atherogenesis or within established plaques of apolipoprotein (Apo)E−/− mice accelerates plaque growth by two-fold, associated with features of plaque vulnerability including a thin fibrous cap and expanded necrotic core. Chronic VSMC apoptosis induced development of calcified plaques in younger animals and promoted calcification within established plaques. In addition, VSMC apoptosis induced medial expansion, associated with increased elastic lamina breaks, and abnormal matrix deposition reminiscent of cystic medial necrosis in humans. VSMC apoptosis prevented outward remodeling associated with atherosclerosis resulting in marked vessel stenosis. We conclude that VSMC apoptosis is sufficient to accelerate atherosclerosis, promote plaque calcification and medial degeneration, prevent expansive remodeling, and promote stenosis in atherosclerosis. (Circ Res. 2008;102:1529-1538.)

Key Words: VSMC ■ apoptosis ■ atherosclerosis ■ mouse models ■ calcification

Human atherosclerotic plaques consist of an accumulation of vascular smooth muscle cells (VSMCs), inflammatory cells (predominantly macrophages, T lymphocytes, and mast cells), and both intracellular and extracellular lipid. Although inflammatory cells promote atherosclerosis at all stages of plaque development, the role of VSMCs is less clear. In the original “response to injury” hypothesis of atherogenesis, accumulation of both inflammatory cells and VSMCs were viewed as responsible for lesion formation.1,2 Thus, prevention of VSMC accumulation, either by blocking cell division or inducing apoptosis, might be predicted to be beneficial. Indeed, antiproliferative therapy reduces neointima formation after stenting in humans, and induction of VSMC apoptosis reduces neointima formation in animal models.3 In contrast, in established plaques, VSMC accumulation is viewed as beneficial, stabilizing the plaque by maintaining a thick fibrous cap and secreting collagen and extracellular matrix (ECM).4 In atherosclerosis, VSMC apoptosis has been associated with plaque rupture, promotion of outward remodeling as plaques grow (the Glagov phenomenon5), and aneurysm formation.6 These effects have been viewed as a passive consequence of VSMC loss from the cap (plaque rupture) or media (aneurysm/aneurysm formation), in part, because of reduced tensile strength from loss of ECM and collagen secreted by VSMCs. Furthermore, VSMC apoptosis is implicated in medial degeneration seen in a variety of human genetic diseases including Marfan’s syndrome, “cystic” medial necrosis (CMN), Hutchison–Gilford progeria (HGP), and cerebral autosomal dominant arteriopathy with subcortical infarcts (CADASIL). These conditions are characterized, to a variable extent, by medial VSMC loss and abnormal ECM deposition (reviewed elsewhere7). Finally, in vitro, we have shown that VSMC apoptosis accelerates calcification of VSMCs8 and promotes coagulation,9 although neither of these consequences has been proven in vivo.

Although VSMC apoptosis accompanies much vascular pathology, it has been difficult to ascribe a role for it per se, predominantly because of a paucity of animal models of selective cell death in the absence of changes in blood flow, surgical manipulation, or genetic manipulation of cell death in multiple cell types. Furthermore, existing models rely on acute high-level induction of apoptosis, rather than reproducing the chronic low (almost undetectable) levels seen in...
atherogenesis in vivo in animals and humans.10 We have recently described SM22α-hDTR mice, where transgenic expression of the human diphtheria toxin receptor (DTR) from the minimal SM22α promoter conditionally induces VSMC-specific apoptosis in both normal arteries and atherosclerotic plaques.11 Acute high-level induction of VSMC apoptosis in SM22α-hDTR/ApoE−/− mice within established plaques induced multiple features of plaque vulnerability.11 However, apoptotic frequencies obtained were more than 10-fold higher than those seen in atherogenesis or established plaques in humans or animal models of atherosclerosis. In addition, only the short-term effects (3 weeks) of apoptosis were studied. Although this study demonstrates that acute high-level VSMC apoptosis promotes features of plaque vulnerability, it does not establish a role for the low levels of VSMC apoptosis seen in atherosclerosis, or identify whether VSMC apoptosis promotes other vascular pathologies. The present study analyzed the effects of chronic low-level VSMC apoptosis in both atherogenesis and progression of established plaques, focusing on plaque development and putative sequelae such as calcification, inflammation, fragmentation of the elastic lamina, remodeling, contractile responses, and features of vulnerability. We demonstrate that inducing chronic VSMC apoptosis at levels comparable to those in human plaques is sufficient to chronically deplete VSMCs from plaques, to promote both atherogenesis and progression of established plaques, and induce profound pathological changes in plaque and vessel morphology.

**Materials and Methods**

A detailed description of methods is presented in the online data supplement, available at http://circres.ahajournals.org. All materials were purchased from Sigma-Aldrich unless otherwise stated.

**Animal Protocols**

SM22α-hDTR/ApoE−/− mice were generated and characterized as described previously.11 Animal experiments were performed under UK Home Office licensing. Control animals were littermates negative for the SM22α-hDTR transgene. Recombinant diphtheria toxin (DT) (Quattertech Diagnostics) was injected intraperitoneally 3 times weekly for long-term treatment. The high-fat diet was comprised of 21% fat, 0% cholate, and 0.21% cholesterol (D12079B; Research Diets). Serum lipids were determined using a Dade-Behring Dimension autoanalyzer. Serum cytokine levels were quantified using the Mouse Inflammation Cytometric Bead Array (Becton Dickinson).

**Histology and Immunohistochemistry**

Experimental animals were euthanized, tissues were perfused in situ with 10% neutral-buffered formalin, and sections were prepared as described previously.11 Primary antibodies were as follows: α-smooth muscle actin (1A4, Dako); Mac 3 (M3/84; Pharmingen); and monocyte chemoattractant protein-1 (MCP-1/JE) antigen (AF-479-NA; R&D). Elastic laminae were stained with Verhoeff–van Giesen (Bio-Optica; no. 053812). Masson’s trichrome staining was performed with Accustain Kit (HT-15). TUNEL was performed as described previously.11 Images were captured and quantified as described previously.11

**In Vitro Pharmacology**

Assessment of aortic contractile responses was performed as described previously.11

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**Figure 1. VSMC apoptosis and serum lipids.**

A, TUNEL staining for apoptotic cells (arrows) in the media of SM22α-hDTR/ApoE−/− and control ApoE−/− mice treated with 2 doses of 1 ng/g DT for 72 hours. Scale bars represent 100 μm and 50 μm in higher-power sections. Blood was collected midway through the DT treatment period and analyzed for serum lipid composition in both the atherogenesis (B) and atheroprogression (C) studies for control ApoE−/− (gray bars) and SM22α-hDTR/ApoE−/− (white bars) mice. Data represent means±SEM. *P<0.03.
The minimal SM22α promoter (−445 to +88) directs expression to VSMCs of large- and medium-sized arteries in adult mice. We have previously shown that administration of DT to SM22α-hDTR mice results in apoptosis of VSMCs only, in both the media and intima. We, therefore, used SM22α-hDTR/ApoE−/− mice to examine the direct consequences of chronic VSMC apoptosis on both the initial development and progression of atherosclerotic plaques.

Chronic Apoptosis of VSMCs Accelerates Plaque Growth

Although previous work has demonstrated that acute high-level VSMC apoptosis in vivo induces inflammation and fibrous cap thinning, no animal model has recapitulated the low-level “chronic” VSMC apoptosis seen in human plaques throughout the time required to generate atherosclerosis in mice. We, therefore, examined the effect of low-dose DT administration (1 ng/g) on VSMC apoptosis. Short-term treatment (72 hours) with 1 ng/g DT induced a comparable low level of medial VSMC apoptosis in both fat-fed and non–fat-fed SM22α-hDTR/ApoE−/− mice (0.79±0.15% versus 0.86±0.21% TUNEL-positive cells, respectively). This apoptotic index is approximately 16-fold lower than previous short-term studies with 5 ng/g DT and similar to the observed rates of apoptosis seen in human plaques of 0.8 to 1.1%. In control apolipoprotein (Apo)E−/− mice, apoptosis was not observed in non–fat-fed mice and was only very rarely seen in fat-fed animals (0.07±0.07%) (Figure 1A).

To study the effects of VSMC death during atherogenesis, SM22α-hDTR/ApoE−/− and littermate control ApoE−/− mice were fed a high fat “western” diet for 2 weeks, followed by simultaneous administration of 1 ng/g DT and continued fat feeding for 10 weeks. To examine the effects of chronic VSMC apoptosis on established plaques (atheropropygression), mice were initially fat-fed for 12 weeks, followed by concurrent high-fat feeding and DT administration for 10 weeks. Blood was taken at midpoints for lipid profile and at early, middle, and euthanasia time points for cytokine analysis. Atherosclerotic plaque burden and composition were assessed in aortic roots and brachiocephalic arteries. Aortic rings were used to analyze the effects of VSMC loss on passive and active contractile responses, and descending aorta was used to examine medial structure.

The lipid profile of SM22α-hDTR/ApoE−/− or control ApoE−/− mice was similar in atherogenesis studies (Figure 1B) and also similar to that seen in previous studies. In
Chronic VSMC Apoptosis Induces Features of Plaque Vulnerability

Previous studies have demonstrated that acute high-level VSMC apoptosis within established plaques induces features of plaque vulnerability, including thin fibrous caps, enlarged necrotic cores, and foci of inflammation around apoptotic bodies. This level of VSMC apoptosis also induced increased levels of local MCP-1 and systemic interleukin (IL)-6. A 1 ng/g DT treatment led to a near doubling of the percentage of apoptotic cells detected at the end of the atherogenesis study compared with control ApoE−/− mice (Table). This apparent increase in the percentage of TUNEL-positive cells at the end of the study compared with the beginning, in spite of the ongoing loss of VSMCs, is possibly attributable to the presence and apoptosis of other cell types, in particular, macrophages. An accumulation of apoptotic debris in the fibrous cap was not seen in either group (Figure 3A), and the fibrous caps of brachiocephalic lesions in SM22α-hDTR/ApoE−/− mice were significantly smaller compared with control ApoE−/− mice (cap area/plaque area of 0.12±0.03 versus 0.31±0.09, respectively, P=0.03). SM22α-hDTR/ApoE−/− mice also showed an increased percentage necrotic core, a significantly reduced VSMC content, and a general reduction in plaque cellularity in both aortic root and brachiocephalic plaques in both atherogenesis and atheroprogression studies, compared with control ApoE−/− mice (Table and Figure 3A). These data confirm depletion of VSMCs via apoptosis.

Multiplex cytokine analysis showed significant increases in serum levels of tumor necrosis factor-α and IL-6 after 7 days, and IL-12, interferon-γ, and MCP-1 after 5 weeks of DT treatment in SM22α-hDTR/ApoE−/− mice, compared with ApoE−/− mice (Figure 3B). However, after 10 weeks of DT treatment, no difference in inflammatory cytokines was evident (Figure 3B), suggesting any inflammatory response to apoptotic VSMCs had resolved. In keeping with this, no significant difference in macrophage content and no increase in local MCP-1 was evident (Figure 3A and Table), and in contrast to previous studies, no foci of inflammation were seen at euthanasia (Figures 2 and 3A). Despite the increase in proinflammatory cytokines after 7 days of DT treatment, no inflammatory infiltrate into the vessels was seen (data not shown). We did not see evidence of plaque rupture (defects in fibrous caps, intraplaque, or lumen thrombus) in either group of mice in either study (data not shown).

Chronic Apoptosis of VSMCs Promotes Calcification

We have previously found that apoptotic bodies can initiate calcification in vitro, and phagocytosis of apoptotic bodies has been suggested to be reduced within plaques. We, therefore, determined whether induction of VSMC apoptosis could accelerate plaque calcification. Although plaque calcification has previously been demonstrated within ApoE−/− mice, it generally develops in much older animals (>40 weeks). Strikingly, calcification was evident in 19% of plaques in SM22α-hDTR/ApoE−/− animals and not seen in control ApoE−/− during atherogenesis (Figure 4A). Similarly, although 13% of control plaques showed calcification in...
atheroprogession studies, 31% of plaques demonstrated calcification in SM22α-hDTR/ApoE<sup>−/−</sup> mice (n=54 ApoE<sup>−/−</sup> and n=43 SM22α-hDTR/ApoE<sup>−/−</sup> plaques). Confluent areas of calcification were confined to the intima and localized around the necrotic core in SM22α-hDTR/ApoE<sup>−/−</sup> mice (Figure 4A). To confirm that calcification was a local phenomenon and not attributable to change in serum biochemistry or renal failure, we examined serum biochemistry of these mice. Serum calcium, phosphate, urea, and creatinine showed no significant difference between groups and were within normal ranges (data not shown).

Low-Level VSMC Apoptosis Induces Medial Expansion and Degeneration

Loss of medial VSMCs accompanies remodeling of vessels, particularly under the atherosclerotic plaque. Indeed, medial atrophy is associated with positive remodeling seen in human arteries in response to atherosclerotic plaques. Both cystic medial degeneration (as seen in Marfan’s syndrome) and aneurysm formation also show medial VSMC apoptosis, associated with breakdown of collagen, ECM, and elastin. Finally, medial VSMC loss is a characteristic feature of genetic diseases such as HGP and CADASIL. We, therefore, examined the effects of DT on both medial area and structure below atherosclerotic plaques, as well as in remote vessels not affected by plaques.

Large areas of medial expansion in successive layers of the elastic lamina were observed under plaques in SM22α-hDTR/ApoE<sup>−/−</sup> mice (Figure 4B). These areas appeared collagen-rich, as evidenced by Masson’s trichrome staining, and demonstrated extensive degradation of the elastic lamina (Figure 4B). Cellular content of the media was α-smooth muscle actin–negative, although a few cells were Mac-3–positive, suggesting the potential migration of monocyte/macrophages. The extent of the “expanded” medial areas was significantly increased in SM22α-hDTR/ApoE<sup>−/−</sup> mice compared with control ApoE<sup>−/−</sup> mice, within both aortic roots and brachiocephalic arteries and during both studies (Table).

We have previously shown that inducing VSMC apoptosis either acutely or chronically in the absence of high-fat feeding did not alter the medial structure. In contrast, fat-fed...
SM22α-hDTR/ApoE−/− mice demonstrated areas of extensive elastic lamina degradation, often associated with medial expansion (Figure 4B), and areas containing multiple localized lamina breaks (Figure 5). Quantification revealed a significantly increased frequency of lamina breaks in SM22α-hDTR/ApoE−/− mice in atherogenesis studies (Figure 5E). Although lamina degradation and frank breaks were mostly present underlying and proximal to plaques, plaque-independent breaks were also found (Figure 5C).

Atherosclerotic plaque development is usually accompanied by a compensatory increase in vessel diameter, such that the lumen is not compromised until late in the disease. Failure of positive remodeling is a major cause of stenosis in native vessels and restenosis after angioplasty. To examine arterial remodeling in atherosclerosis, we quantified areas circumscribed by the internal elastic lamina (IEL) in brachiocephalic arteries, a vessel segment that has been shown to undergo compensatory outward remodeling in response to plaque formation. We also compared IEL areas in a group of ApoE−/− mice of similar age that had not been fat-fed. As expected, the brachiocephalic IEL area in plaque-bearing ApoE−/− mice was significantly increased compared with non–fat-fed ApoE−/− mice (Figure 5F). Remarkably, DT-induced VSMC loss in SM22α-hDTR/ApoE−/− mice completely inhibited IEL expansion in response to atherosclerosis, despite overall increased plaque size (Figure 5F). Thus, the degree of stenosis within brachiocephalic arteries was significantly higher in SM22α-hDTR/ApoE−/− mice than control ApoE−/− mice in both studies (Table), indicating a loss of compensatory enlargement. Vessels were examined throughout the arterial tree for aneurysm formation and, in particular, the suprarenal aorta, the most frequent site of aneurysm formation in ApoE−/− mice. No gross aneurysmal-like dilatations were seen in either group or study (data not shown), suggesting that although medial disruption may precede or promote aneurysms, final dilatation, and
rupture may require hemodynamic alterations such as increased blood pressure.

Elastic lamina breaks are also seen in human pathologies such as CMN, Marfan’s syndrome, and aneurysms. In CMN and Marfan’s syndrome, there is degeneration of elastin fibers accompanied by loss of medial VSMCs (producing holes or “cysts”) and changes in ECM composition, particularly accumulation of glycosaminoglycans (Figure IA and IB in the online data supplement).24 This accumulation is characterized by increased Alcian blue staining of glycosaminoglycans around the cysts and is often associated with speckled calcification of the cyst. SM22α-hDTR/ApoE−/− mice showed reduced numbers of medial VSMCs compared with control ApoE−/− mice (3406 ± 236 versus 5101 ± 420 cells/mm², respectively), accompanied by cystic degeneration of the media demonstrated by marginal Alcian blue staining (Figure 6A) and increased medial calcification evidenced by punctate Von Kossa staining (Figure 6B).

**Chronic VSMC Apoptosis Inhibits Vasoconstriction Responses**

We have previously found that loss of up to 50% of medial VSMCs in normal vessels of SM22α-hDTR mice does not affect vasoconstrictor responses to a variety of agents,11 suggesting that compensatory processes can occur in normal vessel walls. We therefore examined the responses to vasoconstrictor agents in aortas of SM22α-hDTR/ApoE−/− and control ApoE−/− mice following the atherogenesis protocol. ApoE−/− mice showed a hypersensitivity to vasoconstrictors compared with control SM22α-hDTR mice (Table I in the online data supplement). In contrast, responses in DT-treated SM22α-hDTR/ApoE−/− vessels displayed a normalization of response to agonists, similar to the contractile responses seen...
in SM22α-hDTR controls (Figure 7 and supplemental Table I), suggesting that loss of VSMCs diminishes the ability of the vessel to undergo vasoconstriction to a variety of agonists.

Discussion

VSMC apoptosis occurs in a wide range of cardiovascular diseases, such as atherosclerosis, CMN, and aneurysm formation, that develop over months (genetically modified mice) or decades (humans). The low frequency of VSMC apoptosis in plaques and the media (<1%) and the extended time periods for disease to develop have meant that the direct consequences of VSMC apoptosis in these pathologies are unclear. Plaque rupture, remodeling, aneurysm formation, coagulation, and calcification have all been suggested as consequences of chronic low-level or “pathophysiological” levels of VSMC apoptosis. In particular, observations that VSMC accumulation occurs in atherogenesis and induction of VSMC apoptosis can reduce neointima formation suggest that VSMC apoptosis might limit atherogenesis and retard progression of plaques. In contrast, using a mouse model of DT-induced VSMC-specific apoptosis, we demonstrate that chronic low-level VSMC death over extended time periods accelerates atherosclerosis, promotes intimal calcification, medial expansion, and medial degeneration, but impairs vessel remodeling in response to atherosclerosis.

The most striking finding of this study is that VSMC apoptosis accelerates plaque growth at both stages of the disease studied. Although it is reasonable to assume that plaque rupture and repair might accelerate atherosclerosis in established lesions, we did not see evidence of rupture, and this is an unlikely explanation during atherogenesis. In contrast, our findings argue that VSMCs slow plaque growth even in the absence of plaque rupture. For example, the presence of a thick VSMC-rich fibrous cap might slow monocyte/macrophage migration and accumulation. An alternative explanation is that VSMC apoptosis itself is detrimental, for example, by inducing inflammation. In our previous study, extensive VSMC apoptosis resulted in foci of macrophages associated with increased expression of inflammatory cytokines such as MCP-1 and IL-6 that remained at high levels in the circulation for 4 weeks after cessation of DT treatment (M.C.H.C. and M.R.B., unpublished observations, 2006). The present findings suggest that although chronic low-level VSMC apoptosis initially induces systemic proinflammatory cytokines, no increased monocyte accumulation occurred. In addition, as the plaque matures, the inflammatory response resolves, despite ongoing VSMC apoptosis. The “biphasic” inflammatory cytokine profile may be related to clearance of apoptotic VSMCs, a process that has significant effects on the regulation of inflammation and its resolution.26 In particular, ingestion of apoptotic cells can both switch off production of proinflammatory cytokines and induce antiinflammatory cytokines, thus helping resolution of inflammation.27,28

We also demonstrate that chronic low-level VSMC apoptosis is sufficient to cause marked fibrous cap thinning with expansion of the necrotic core. Fibrous cap thinning is a characteristic feature of progressive and advanced human
atherosclerosis, with caps <65 μm being particularly associated with plaque rupture. Apoptosis of VSMCs in the fibrous cap has long been considered an important mechanism of cap thinning; however, VSMC apoptosis has been difficult to demonstrate in the cap. We demonstrate that even small increases in VSMC apoptosis can induce marked cap thinning. Necrotic core formation is also crucial to the formation of thin-capped fibroatheromata, the plaque morphology most frequently correlated with plaque rupture. Necrotic core formation is considered to be attributable to death of inflammatory cells and accumulation of lipid (reviewed elsewhere). We demonstrate that VSMC death alone can accelerate enlargement of the necrotic cores of both developing and established plaques. Indeed, in previous studies, we have found that the effects of monocyte/macrophage apoptosis depend on the stage of atherosclerosis.

Intimal calcification is a characteristic feature of advanced plaques in humans and is also seen in mice with increasing age. Calcification is regulated in vivo by a variety of factors (reviewed elsewhere) and is often seen adjacent to areas of apoptosis or the necrotic core. In culture, we have previously shown that apoptotic VSMCs promote calcification, and others have found that inhibition of phagocytosis with acetylated LDL or scavenger receptor ligands accelerates calcification. However, it was not known whether VSMC apoptosis alone could induce calcification in atherosclerosis. We find that low-level VSMC apoptosis accelerates calcification during atherogenesis and in established plaques. Interestingly, the local plaque environment appears crucial for this effect because extensive confluent medial calcification did not occur, despite DT also inducing VSMC apoptosis in the media. This also suggests that the mechanisms underlying intimal calcification in atherogenesis may be different from those underlying medial calcification in diabetes or chronic renal failure.

Chronic VSMC apoptosis had profound effects on the media in atherosclerosis. Unlike previous studies in which medial VSMC apoptosis in normal vessels was effectively silent, medial VSMC apoptosis in atherosclerosis induced medial expansion, elastin breaks, and prevented positive vessel remodeling, resulting in greater loss of lumen. The latter observations are surprising. Outward remodeling has previously been shown to be associated with medial atrophy, and it was widely assumed that increased “stretch” of the vessel to accommodate the plaque was essentially a passive process (reviewed elsewhere). Our studies demonstrate that outward remodeling might be an active process that requires VSMCs, perhaps via VSMC-mediated degradation and/or subsequent deposition of matrix and collagen, and reformation of elastin laminae as the vessel expands. Our observations support the idea that vessel stenosis in advanced atherosclerotic vessels may result in part from death of medial VSMCs.

In contrast, VSMC apoptosis in otherwise atherosclerosis-free areas was sufficient to induce features of CMN, a pathology that is both idiopathic and associated with disorders of collagen and fibrillin, such as Marfan’s syndrome. CMN predisposes to dissection of the artery and may also prelude aneurysm formation. We find that VSMC apoptosis induces elastin breaks and cyst-like structures within the media, associated with changes in ECM composition and “speckled” calcification. Even considering the structural differences between human and mouse arteries, the histological similarities between DT-treated SM22α-hDTR/ApoE−/− mice and human Marfan’s vessels are striking. Given that inducing VSMC apoptosis recreates such similar pathologies to Marfan’s syndrome without the fibrillin mutation, these data suggest that fibrillin mutations in Marfan’s might have a direct effect on VSMC survival. If confirmed, the SM22α-hDTR/ApoE−/− mouse may be a valuable model of this unusual pathology. Similarly, HGP is associated with profound medial VSMC loss, with abnormal deposition of collagen and matrix, and the appearance of cell debris, matrix vesicles, and focal calcification. Many of these features have been reproduced in a mouse model of HGP vascular disease that showed progressive loss of VSMCs, breakage of elastic fibers, accumulation of collagen and proteoglycans, and calcification, without evidence of inflammation and with an intact endothelium. Although apoptosis of VSMCs was not demonstrated in HGP mice, we show that chronic VSMC apoptosis is sufficient alone to reproduce many of these features.

The effects of chronic VSMC apoptosis on vascular function in response to vasoconstrictors are also surprising. We have previously shown that the contractile response of the normal artery is unaffected by loss of 50% of VSMCs, suggesting either a large redundancy in contractile responses or an ability of remaining VSMCs to compensate. ApoE−/− mice on a high-fat diet demonstrate a hypersensitivity to vasoconstrictors compared with SM22α-hDTR controls. However, VSMC loss in the SM22α-DTR/ApoE−/− animals normalizes this hyperreactivity, suggesting that in this model, a beneficial consequence of apoptosis might be to limit detrimental vasoconstriction.

In conclusion, we have demonstrated that chronic apoptosis of VSMCs accelerates atherogenesis and progression of atherosclerosis in established plaques. Fibrous cap thinning, enlargement of the necrotic core, plaque calcification, medial expansion and degeneration, elastin breaks, and failure of outward remodeling are all direct consequences of VSMC apoptosis. In addition, chronic VSMC apoptosis may mimic multiple features of the medial degeneration seen in a variety of human pathologies.

Sources of Funding
This study was supported by British Heart Foundation grants PG/06/024/20354 and RG 04/001 (to M.R.B.) and PG/05/127/19872 and PS/02/001 (to A.P.D.), the European Vascular Genomics Network of Excellence (FP6), and a National Institute for Health Research Biomedical Research Centre grant to Cambridge University Hospitals NHS Foundation Trust/Cambridge University.

Disclosures
None.

References


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Online Data Supplement

Methods

All materials were purchased from Sigma-Aldrich unless otherwise stated.

Animal protocols

SM22α-hDTR/ApoE−/− mice were generated and characterised as previously described⁴. All animal experiments were performed under UK Home Office licensing. Control animals were littermates negative for the SM22α-hDTR transgene. Purified recombinant DT (Quadratech Diagnostics) was prepared in 0.9% saline/0.2% BSA (low endotoxin), sterile-filtered and stored at -80°C until used. Experimental animals (Control ApoE−/− and SM22α-hDTR/ApoE−/−) were weighed and injected intraperitoneally with DT (doses as indicated) thrice weekly for long-term treatment. Mice were given a high fat diet comprising 21% fat, 0% cholate, 0.21% cholesterol (D12079B; Research Diets) where indicated. Blood was collected via tail veins and serum lipids determined using a Dade-Behring Dimension auto analyzer, with LDL calculated using the Friedwald formula. Serum cytokine levels were quantified using the Mouse Inflammation Cytometric Bead Array (Becton Dickinson).

Histology and Immunohistochemistry

Experimental animals were euthanized under CO₂ and tissues perfused in situ with 10% neutral-buffered formalin at physiological pressures, followed by removal and overnight fixation. Washed tissues were processed then embedded in paraffin blocks, 5μm serial
sections cut, deparaffinated, and stained with Haematoxylin and Eosin. Antigen retrieval was achieved by boiling in citrate buffer (pH 6.0). Blocking and primary antibody conditions were as follows: αSMA (1A4, Dako; 1:500); Mac 3 (M3/84; Pharmingen, 1:200); MCP-1/JE antigen (AF-479-NA; R&D Systems, 1:100). All sections were stained with biotinylated secondary antibodies and detected with ABC reagents (Vector Laboratories). Elastic lamina were stained using a modified Verhoeff’s van Giesen stain (Bio-Optica; #053812). Masson’s Trichrome staining was achieved with Accustain Kit (HT-15). Calcification was detected with standard Von Kossa staining. TUNEL was performed as previously described with the exception of dUTP-DIG incorporation (Roche), detection with anti-DIG-alkaline phosphatase (Roche), and development with BCIP/NBT (Vector). TUNEL positive cells are expressed as a % of the total cells (counted by nuclei using H&E staining) in the plaque. Images were captured using a BX51 microscope (Olympus), cooled CCD camera and imaging software (Soft Imaging Systems). Final images were prepared using Photoshop 6.0 (Adobe). Plaque constituent areas were quantified as number of DAB-positive (Mac-3 and αSMA) pixels as a percentage of total plaque pixels. Fibrous caps were defined as the VSMC- and proteoglycan-rich area overlying the cholesterol-rich, matrix-poor, acellular regions of the necrotic cores. Non-specific staining of lipid within the core was excluded from analysis.

**In Vitro pharmacology**

Control ApoE−/− and SM22α-hDTR/ApoE−/− mice were killed by a rising CO₂ concentration and thoracic aortas removed and cleaned of fat and connective tissue. For
each animal four consecutive rings (1-2 mm long) were mounted in wire myographs (Linton Instrumentation) and maintained at 37°C in oxygenated Krebs’ solution. Aortic rings were automatically normalised and set to 90% of the internal circumference they would adopt if fully relaxed under a transmural pressure of 100 mmHg. Vessels were then challenged three times with high K⁺ Krebs’ solution (95 mM K⁺) at 15 min intervals and allowed to re-equilibrate for 60 min. Cumulative concentration-response curves were constructed to the vasoconstrictors phenylephrine (10⁻⁹-10⁻⁵ M) and U-46619 (10⁻¹⁰-10⁻⁶ M). Agonist responses were expressed as wall tension in mN/mm of vessel wall.

References for Online Supplement

Supplementary Figure 1A: (A) Human aorta section from a patient with Marfan’s syndrome demonstrating hypocellularity with H&E (I), extensive elastin breaks with Verhoeff’s van Giesen (II), Alcian blue (III) staining of cysts (high power to the right), and localised punctate Von Kossa (IV) staining (high power to the right).
Supplementary Figure 1B: (A) Normal human aorta section demonstrating cellularity with H&E (I), elastin with Verhoeff's van Giesen (II), Alcian blue (III) staining (high power to the right), and Von Kossa (IV) staining (high power to the right).
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<td>SM22α-hDTR Control</td>
<td>6.08 ± 0.20</td>
<td>5</td>
</tr>
</tbody>
</table>

**Online Table 1:** Values of potency (pD₂, mean±SEM) are shown for each agonist, for the two groups compared to data previously obtained for non-DT treated SM22α-hDTR animals. n = number of mice from which aortas were obtained. * p<0.05, ** p<0.01.