Smooth Muscle Cell Apoptosis Promotes Vessel Remodeling and Repair via Activation of Cell Migration, Proliferation, and Collagen Synthesis

Haixiang Yu, Murray C.H. Clarke, Nichola Figg, Trevor D. Littlewood, Martin R. Bennett

Objective—Although vascular smooth muscle cell (VSMC) apoptosis occurs after vessel injury and during remodeling, the direct role of VSMC death in determining final vessel structure is unclear. We sought to determine the role of VSMC apoptosis in vessel remodeling, medial repair, and neointima formation and to identify the mediators involved.

Methods and Results—The left common carotid artery was ligated in SM22α-human diphtheria toxin receptor mice, in which diphtheria toxin treatment selectively induces VSMC apoptosis. Apoptosis induced from day 7 to day 14 after ligation significantly increased neointimal and medial areas, cell proliferation, migration, and vessel size. Neointima formation depended on VSMCs, as VSMC depletion before ligation significantly reduced neointimal area and cellularity. In culture, conditioned media from apoptotic VSMCs promoted VSMC migration, proliferation, and collagen synthesis. Interleukin-6 (IL-6) secretion increased 5-fold and IL-1α 1.5-fold after apoptosis, whereas IL-6 inhibition negated the effect of apoptotic VSMC supernatants on VSMC migration, proliferation, and matrix synthesis.

Conclusion—Signaling from apoptotic VSMCs directly promotes vessel remodeling, medial repair, and neointima formation after flow reduction. Although lumen size appears to depend on flow, VSMC apoptosis is an important determinant of vessel, medial, and neointimal size after flow reduction. (Arterioscler Thromb Vasc Biol. 2011;31:2402-2409.)

Key Words: apoptosis ■ remodeling ■ smooth muscle

Remodeling of a vessel describes an increase or decrease in its lumen size accompanied by changes in vessel wall area and components. Remodeling can be inward (negative), for example following physiological or surgical flow reduction or angioplasty restenosis, or outward (positive), for example after increased flow following changing tissue perfusion requirements, or in aneurysm formation. Both positive and negative remodeling occurs in atherosclerosis, even at the same site at different times during atherogenesis.1,2 For the vessel to be fully functional at its new dimensions, remodeling requires orderly destruction and synthesis of vessel wall tissue, involving cell death (usually by apoptosis), proliferation, and migration and matrix destruction, synthesis, and reorganization. Although cell proliferation has been studied extensively in remodeling, apoptosis, matrix changes, and cell migration have not. In particular, as many processes occur simultaneously after injury or surgery, it has been difficult to define the direct role of a single process in remodeling and how it affects other processes.

We have previously described the SM22α-human diphtheria toxin receptor (hDTR) mouse, in which the vascular smooth muscle cell (VSMC)–specific minimal SM22α promoter drives expression of the hDTR.3 Wild-type mice are resistant to diphtheria toxin (DT); however, transgenic hDTR expression allows selective induction of apoptosis in medial and intimal VSMCs in the aorta and the brachiocephalic and carotid arteries.3 The model of flow reduction following common carotid artery ligation is also well established,4–6 in which there is first positive and then negative remodeling over 28 days. The contralateral carotid artery receives increased flow,7 which can result in positive remodeling. Medial VSMC proliferation in mice begins at day 2, peaks at ~day 8, and then subsequently declines.5,6 Remodeling-induced VSMC apoptosis begins at day 7, peaks at ~day 14, and then declines until day 28 (5 and our unpublished observations). A neointima is evident beginning at day 3 and subsequently increases; both neointima formation and negative vessel remodeling contribute to lumen shrinkage.5,6 Cell migration has been demonstrated indirectly via the presence of a calculated excess of neointimal cells over the predicted numbers based on cell proliferation indices.6 Although these studies did not include a possible contribution from circulating or local adventitial VSMC progenitors, the model recapitulates many processes that contribute to both physiological and pathological arterial responses to changes in blood flow and injury in humans.

In this study, we found that VSMC apoptosis promoted neointimal formation and medial repopulation in negatively
remodeled vessels, in part by increasing cell proliferation, migration, and cell matrix formation. Apoptotic and adjacent live VSMCs released both mitogens and chemotactic factors to mediate this response, including interleukin-6 (IL-6), indicating that VSMC apoptosis is a major regulator of vessel remodeling after flow reduction, for example in physiological conditions, surgery, or disease.

Methods
Full methods are provided in the supplemental materials, available online at http://atvb.ahajournals.org. All reagents were from Sigma-Aldrich unless otherwise stated.

SM22α-DTR Mice
The generation and characterization of SM22α-hDTR mice has been described previously.3

Vessel Remodeling
All animal experimentation was performed under United Kingdom Home Office licensing following ethical approval. Sex-matched littermate SM22α-hDTR heterozygous C57BL/6 mice (Mus musculus, ~4 months old, 20–28 g) were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg). The left common carotid artery (LCCA) was exposed and ligated near the bifurcation. The wound was closed, and animals were allowed to recover for 3 hours. To detect cell proliferation, 0.8 mg/mL bromodeoxyuridine (BrDU) plus 1% sucrose was added to drinking water immediately after carotid surgery and throughout the experiment. Mice were euthanized at 0, 14, and 28 days after surgery by perfusion-fixation with formalin at physiological pressure. Common carotid arteries were harvested, the proximal and distal 2 mm were discarded, and the remaining portion (~5–6 mm) was cut into 3 pieces of identical lengths. Five-micrometer sections were cut from each portion for immunostaining and morphometric analysis as described previously.4 Morphometric data represent the mean of the 3 sections per artery.

Histology and Immunohistochemistry
Immunohistochemistry of mouse vessels and antibodies used were as previously described.

In Vitro Assays of Cell Proliferation, Migration, and Matrix Synthesis
These assays have been described previously; full details are given in the supplemental materials.

Antibody Cytokine Array and Enzyme-Linked Immunosorbent Assay
Custom-designed murine cytokine antibody arrays were performed according to the manufacturer’s instructions (RayBiotech).

Statistical Analysis
Values were expressed as mean±SD. For control and treated groups showing similar variances, group means were examined using unpaired Student t test. Multiple groups were compared using ANOVA followed by the Tukey test using GraphPad Prism software. The significance level was set at *P<0.05.*

Results

VSMC Apoptosis Increases Tissue Mass in Ligated Vessels
SM22α-hDTR mice underwent LCCA ligation. DT was administered by intraperitoneal injection between day 7 and day 14, with vessels perfusion fixed and examined histologically at day 14 to confirm increased VSMC apoptosis and at day 28 to examine remodeling (Supplemental Figure 1, Protocol A). Mice received BrDU throughout the experimental period to determine cumulative cell proliferation. At day 14, ligated (LCCA) and unligated (right common carotid artery) vessels treated with saline showed 0.3%±0.1% and 0% TUNEL-positive cells, respectively (mean±SEM); DT treatment increased apoptosis to 1.6%±0.4% and 0.8%±0.2%, respectively (Figure 1A and Supplemental Figure II), confirming both that VSMC apoptosis occurred during remodeling after ligation and that DT administration increased remodeling-associated apoptosis. At days 0 and 28, no apoptotic VSMCs were identified. At day 28, there was a marked neointima composed of α-smooth muscle actin–positive cells (Figure 1B). Although small amounts of thrombus were seen at the ligation site, similar to previous studies,5 no CD45-positive cells (Supplemental Figure II) and few Mac-3 positive cells (Figure 1B) were present rostral to the ligation site by day 28.

As previously described,7 LCCA ligation reduced lumen area (Figure 2A). LCCA and the contralateral (right) carotid artery lumen areas were not further altered by concomitant DT administration. Ligation induced neointima formation, which was further increased significantly after DT administration (Figure 2B). Medial area was also increased after DT administration 2-fold compared with ligated, control mice (Figure 2C). Intimal+medial area, a composite of vessel wall area, increased after ligation and was further significantly increased by DT (Figure 2D). Lumen perimeter was decreased after ligation, similar to results seen in previous studies,5 with no further reduction following DT administration. In contrast, DT administration increased total vessel
perimeter (Supplemental Figure III). The increased intimal + medial area after DT treatment therefore produces a larger vessel with a thicker wall, but the same-sized lumen. DT administration did not alter lumen or vessel perimeter in the contralateral (right) common carotid artery (Supplemental Figure III). To confirm that these effects on medial and neointimal areas were not a nonspecific action of DT after vessel ligation, we administered DT or saline to wild-type mice after ligation; DT had no effect on lumen, neointimal, or medial areas at 28 days in wild-type mice (Supplemental Figure IV).

VSMCs normally comprise ≈30% of the medial area, the remainder composed of extracellular matrix, including proteoglycans and fibrillar proteins. The mouse has few if any intimal VSMCs in normal vessels; we therefore did not compare intimal counts or cellularity against unligated vessels. However, despite augmentation of medial cell apoptosis (Figure 1A), both neointimal and medial cell counts in DT-treated mice were not significantly different from control mice at day 28 (Figure 3A and 3B), suggesting that transient VSMC apoptosis might stimulate proliferation of adjacent VSMCs to maintain normal medial cellularity. DT treatment did not reduce neointimal cellularity versus control ligated mice (Figure 3C). In contrast, despite similar medial cell counts (Figure 3B), DT-treated mice showed 40% lower medial cellularity (Figure 3D), suggesting that medial VSMC apoptosis promotes matrix production. We have previously shown that chronic VSMC apoptosis over many months induces features consistent with cystic medial necrosis, including reduced collagen formation, microcalcification, and deposition of Alcian Blue proteoglycans around gaps left by apoptotic cells that had been removed.10 None of these features were found in the media of DT-treated or control arteries.

VSMC Apoptosis Promotes VSMC Proliferation and Migration

Despite induction of VSMC apoptosis, the maintenance of medial cell counts after DT administration suggests that apoptotic VSMCs may signal to adjacent cells to increase cell proliferation, migration, or both. To measure proliferation, mice were administered BrdU throughout the 28-day experimental period (Supplemental Figure V). Migration is difficult to measure directly in this model; however, previous studies following vessel ligation in mice and balloon injury in rats assessed migration by calculating predicted intimal VSMC numbers (based on proliferation rates)6 or by counting intima cells that had not undergone division (BrdU-negative cells).11 Although intimal VSMCs and invading progenitor cells can also divide, we did not find CD45-positive cells in the intima to indicate transdifferentiation from invading
inflammatory cells. It is therefore most likely that neointimal BrdU-negative VSMCs have arisen by migration from the media.

Ligation significantly increased cumulative VSMC proliferation in both neointima and media; DT administration further increased proliferation in both compartments (Figure 3E and 3F), and also medial proliferation in the contralateral right carotid artery (Figure 3F), whose medial VSMCs also undergo DT-induced apoptosis. We have previously shown that proliferation markers such as Ki67 are not increased when VSMCs undergo apoptosis, suggesting that these BrdU percentages truly represent cell division. After ligation, BrdU-negative cells were present in the neointima, suggesting that some of the neointima is derived from cells that undergo migration only. DT administration significantly increased neointimal BrdU-negative cells by 41% (Figure 3G), suggesting that VSMC apoptosis also promotes migration of medial VSMCs.

VSMC Apoptosis Actively Promotes Remodeling

These studies suggest that augmentation of VSMC apoptosis during remodeling increases vessel (intimal + medial) mass via increased cell proliferation, migration, and matrix synthesis. However, it is not clear whether these effects are due to the remaining VSMCs simply reacting to loss of cells, for example via loss of cell-cell contact inhibition, or whether apoptosis actively stimulates remaining cells to repair the vessel, for example via release of factors from dying cells. Similarly, it is not clear whether VSMC apoptosis before the remodeling stimulus (ligation) also promotes cell proliferation/migration. To determine whether loss of cells alone could promote increased medial or neointimal areas, we examined the effects of VSMC apoptosis before ligation (Supplemental Figure I, Protocol B). SM22α-hDTR mice were administered DT for 28 days before ligation, and remodeling was examined 28 days later. Again, ligation reduced lumen area, and prior VSMC apoptosis did not affect this (Figure 4A). However, DT administration before ligation completely inhibited neointima formation (Figure 4B). Ligation increased medial area in control mice, which was not affected by prior DT administration (Figure 4C). Both neointimal and medial cell counts were markedly reduced by prior VSMC apoptosis (Figure 4D and 4E). Thus, VSMC apoptosis increases both medial and neointimal areas when apoptosis is augmented after ligation and during remodeling but inhibits neointima formation if medial apoptosis is induced before remodeling.

Apoptotic VSMCs Promote VSMC Proliferation, Migration, and Collagen Synthesis

To examine whether VSMC apoptosis augments proliferation, migration, or matrix synthesis by adjacent viable cells, we studied VSMCs exposed to conditioned media from apoptotic VSMCs in culture. As proapoptotic stimuli such as DT would be carried over in conditioned medium, potentially affecting cell proliferation and migration, we also induced VSMC apoptosis with UV irradiation. We found that 20 mJ/cm² UV irradiation induced reproducible apoptosis in mouse VSMCs, albeit with some secondary necrosis (Supplemental Figure VI). Conditioned media from apoptotic VSMCs increased VSMC proliferation and collagen synthesis by 24% and 21%, respectively (Figure 5A and 5B). However, apoptotic VSMC-conditioned media increased migration by ~3.5 fold (Figure 5C), which could be inhibited by treatment of the apoptotic cells with the broad-spectrum caspase inhibitor zVAD-fmk or small interfering RNA to caspase 3 before collection of conditioned media (Figure 5C and 5D), the latter also reducing caspase 3 protein (Supplemental Figure VII). This suggests that the effect of conditioned media is due to the VSMCs undergoing apoptosis rather than primary necrosis. Matrix metalloproteinase-2 and -9 activities were previously shown to increase after carotid ligation, promoting VSMC migration. However, apoptotic cell-conditioned media did not change matrix metalloproteinase-2 or matrix metalloproteinase-9 activity on zymography (Figure 5E).

VSMCs Undergoing Apoptosis Cause Release of IL-6

To identify cytokines released either directly or by viable cells adjacent to apoptotic VSMCs that promote cell proliferation, migration, or collagen synthesis, we performed anti-
body arrays on conditioned media from apoptotic mouse aortic VSMCs (Supplemental Figure VIII). There was a marked increase in IL-6 in the conditioned media after VSMC apoptosis (Figure 6A), confirmed by ELISA after both DT and UV treatment (Supplemental Figure IX). IL-6 expression was also increased by DT treatment in ligated vessels (Figure 6B and Supplemental Figure X), but no increase in serum IL-6 was found (Supplemental Figure X).

To examine whether IL-6 was responsible for the proliferative and migratory activity of conditioned media from apoptotic VSMCs, we repeated migration and cell proliferation assays in the presence of neutralizing antibodies to IL-6. Nevertheless, there were potent enough that, despite induction of VSMC apoptosis, no change in lumen size, and no change in active or passive mechanical properties.

Here, VSMCs released increased amounts of IL-1α with increasing doses of UV irradiation as more cells undergo necrosis (Supplemental Figure XI). However, neutralizing antibodies to IL-1α did not significantly reduce migration, collagen synthesis, or cell proliferation induced by conditioned media from apoptotic VSMCs (Figure 6C–6E).

Discussion

VSMC apoptosis occurs in both positive and negative vessel remodeling. Medial VSMC apoptosis is rapid and profound after injury, with loss of ~70% of VSMCs within 90 minutes in some models. The media is subsequently repopulated over several days or weeks by proliferation of a relatively small number of adjacent viable VSMCs. Both dividing and nondividing medial VSMCs can also migrate and subsequently proliferate to form a neointima, with a proportion potentially derived from smooth muscle cell progenitors located within and remote from the vessel wall. VSMC apoptosis also occurs in atherosclerosis (reviewed in) and in both aneurysm formation and flow-induced neointimal regression, where it contributes to intimal regression or medial atrophy, respectively, and has been implicated in positive remodeling in both conditions. Although VSMC apoptosis accompanies these pathologies, our recent studies have shown that VSMC apoptosis in a normal vessel has few sequelae, with no repopulation, no inflammation, no change in lumen size, and no change in active or passive mechanical properties. In contrast, VSMC apoptosis in atherosclerosis has profound consequences, promoting multiple features of vulnerable plaques, and accelerating both atherogenesis and progression of established lesions. VSMC apoptosis also appears to prevent rather than promote the normal outward remodeling seen in atherosclerosis.

To better understand the role of VSMC apoptosis in remodeling, we induced VSMC apoptosis in a mouse model of flow reduction-induced inward remodeling. Ligation caused reduction in lumen size, as previously reported. Augmentation of VSMC apoptosis from day 7 to day 14 increased neointimal and medial areas, in part by effects on medial and neointimal VSMC proliferation, migration, and matrix components. Medical proliferation increased, allowing medial repopulation despite an initial reduction in medial cell number due to transient VSMC apoptosis. Calculated VSMC migration from media to intima increased, and there was also an increase in matrix components after VSMC apoptosis, as medial cellularity (cells/mm²) did not return to normal, despite there being normal medial cell counts. These effects were potent enough that, despite induction of VSMC apoptosis, there was an increase in total vessel area and perimeter in DT-treated mice. These effects were not a response to reduced vessel cellularity per se, as apoptosis induced before ligation was associated with reduced neointimal formation. In contrast, this suggests that VSMC apoptosis drives adjacent cells to proliferate, migrate, and synthesize matrix, thus repairing the artery following injury.

To better examine the mechanisms underlying these effects of VSMC apoptosis on bystander VSMCs, we studied proliferation, migration, and matrix synthesis in culture after induction of VSMC apoptosis. IL-6 secretion was increased...
approximately 5-fold in cultures containing apoptotic and live VSMCs. IL-6 is the eponym of a cytokine family, all of which require gp130 as a coreceptor to exert their biological activity. Although the specific IL-6 receptor is only expressed on defined cell types, including monocytes, lymphocytes, and hepatocytes, IL-6 can bind to soluble IL-6 receptor to form a complex that binds cell surface gp130 and activates intracellular signaling, including cells lacking the membrane IL-6 receptor. As a consequence, IL-6 acts on a huge variety of cell types, including endothelial cells and VSMCs. IL-6 has been shown to promote VSMC migration\textsuperscript{21–23} and proliferation,\textsuperscript{24–26} and indeed hepatic gp130 promotes neointima formation after ligation in mice.\textsuperscript{27} In our study, IL-6 neutralization completely blocked the increased VSMC prolifera-
tion, migration, and matrix synthesis induced by conditioned media from apoptotic + live VSMC cultures. Vessel wall (but not serum) IL-6 was also increased by DT in vivo, indicating that IL-6 release and activity is a local vessel wall effect rather than a systemic action. Thus, we consider that IL-6 is the predominant effector cytokine for VSMC responses to VSMC apoptosis in this model.

We also examined the upstream signals that cause IL-6 release. The antibody arrays measure cytokines released from both dying cells when they undergo apoptosis and from adjacent viable cells responding to cytokines released from dying cells. We have previously found that IL-1α is released from VSMCs undergoing necrosis and that IL-1α and IL-1β are released from cells undergoing secondary necrosis. Although there was a small increase in IL-1α in the conditioned media, the effects of conditioned media from apoptotic cells did not depend on IL-1α. Interestingly, cell death induced by Fas/Fas-associated death domain activation in VSMCs also induces neo-intima formation and release of multiple cytokines, including monocyte chemoattractant protein-1, IL-6, and IL-8,28,29 although the predominant cytokines were different in the studies by Schaub et al28,29 and ourselves, most likely because of the differing contributions of apoptosis and secondary necrosis in the 2 models.9

Our study raises a number of unanswered questions. In particular, what are the signals that cause VSMC apoptosis after flow remodeling, and why does VSMC apoptosis during remodeling have such profound effects increasing neo-intimal and medial areas when similar levels of apoptosis in normal vessels are effectively silent, and apoptosis induced before remodeling has the opposite effect? Carotid artery ligation rostral to the origin of the thyroid artery reduces flow in the carotid upstream of this branch and causes a significant (<70%) reduction in shear stress.4 Physical forces, growth factors, and extracellular matrix drive vascular cell survival pathways, and considerable evidence points to reduced endothelial cell function with subsequent reduced local nitric oxide production as an important but complex regulator of vascular cell death after flow reduction.30 For example, flow reduction causes endothelial cell loss, and monocyte migration across the endothelium. Both monocyte macrophages31,32 and reactive oxygen species14 induced by cytokines produced by these cells can induce VSMC apoptosis. It is likely that VSMC apoptosis cannot instigate migration and proliferation of adjacent live VSMCs alone but can augment these processes when accompanied by the second hit of injury or changes in flow. For example, VSMCs in the normal vessel wall are kept quiescent by matrix restraint or lack of exposure to growth factors. Matrix metalloproteinase activation, which occurs as early as day 1 after ligation,6 may release VSMCs, allowing them to proliferate/migrate when they receive the appropriate stimulus, which is augmented by signals released by VSMCs apoptosis. Indeed, DT-induced VSMC apoptosis promoted medial VSMC proliferation, including in the contralateral artery after ligation that also undergoes remodeling, whereas apoptosis before remodeling did not. Intriguingly, lumen size was unaffected by manipulation of VSMC apoptosis at any stage, suggesting that independent mechanisms control vessel caliber.

Our studies suggest the following scenario following arterial ligation or flow reduction: ligation is associated with a rapid activation of matrix metalloproteinases,6 releasing VSMCs from matrix restraint, allowing them to undergo proliferation and migration when they receive the appropriate stimulus. VSMC apoptosis from day 7 to day 14 in part provides that stimulus, inducing release of IL-6. IL-6 promotes proliferation, migration, and matrix synthesis from adjacent VSMCs, and these processes promote neo-intimal formation and recovery of medial cell number. Matrix synthesis allows further increases in both medial and neo-intimal tissue mass and enlargement of the vessel. Recovery of medial cellularity and matrix synthesis, together with loss of exposure to cytokines/growth factors, allows VSMCs to return to quiescence at the new vessel size. Formal proof of this mechanism requires in vivo inhibition of IL-6 only in VSMCs, most likely via genetic means, as previous studies have shown that neutralization of IL-6 signaling in the liver also reduces neo-intima formation after carotid artery ligation.27 Specifically, the gp130 (IL-6)-dependent systemic acute phase response was important for vascular inflammation and VSMC migration, as well as proliferation, and, subsequently, for vascular remodeling.27 Systemic administration of an IL-6 neutralizing antibody would also reduce this systemic response and therefore could not determine whether any reduction in neointima and remodeling was due to a reduced systemic response or local IL-6.

In conclusion, we show that apoptotic VSMCs cause release of factors that promote VSMC proliferation, migration, and matrix synthesis. VSMC apoptosis occurring after flow reduction actively promotes neo-intimal formation and artery repair. These signaling events may represent built-in feedback pathways to repair the vessel after acute or chronic injury and to remodel the vessel wall to a new size after changes in blood flow.

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Disclosures
None.

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Supplement material

Methods

Cell proliferation
Aortic VSMCs from SM22α-hDTR mice were seeded in 96-well plates at 10,000 cells/well and growth arrested by culture in 0% FCS for 48 hours. Cells were incubated with conditioned media from control or apoptotic VSMCs for 24 h; media were also preincubated with control goat IgG (sc-2028, Santa Cruz), neutralizing antibodies to IL-1α (AF400, R&D) or IL-6 (AF-407, R&D) at a concentration of 1 µg/mL for 1 h. Cell proliferation was measured using a CyQUANT NF cell proliferation kit according to the manufacturer's instructions (Invitrogen Ltd, Paisley, UK).

Cell migration
Cell migration was performed using a microchemotaxis chamber containing a polycarbonate filter (Transwell) with 8 µm pores. 10,000 cells were added to the upper chamber and the lower chamber was filled with 600 µL of conditioned media from 100,000 apoptotic or control VSMCs. After 5 h, cells on the upper side of membrane were removed and inserts fixed in ice-cold methanol for 15 min. Cells were stained with Hoechst 33342 (1µg/ml) and viewed on an Olympus epi-fluorescent microscope. The number of VSMCs that migrated to the lower surface of the filter was counted in 10 fields from triplicate experiments.

Collagen synthesis
Collagen synthesis was assayed as described previously, after exposure of VSMCs to conditioned media pre-treated with neutralizing antibodies at 1 µg/mL for 24 h.
**Supplemental Figures**

Supplemental Figure I. Mouse protocols

(A) High dose DT administration between weeks 1-2 after ligation. (B) Low dose DT administration 4w prior to ligation.

Supplemental Figure II.

(A) % TUNEL-positive cells at Day 0 (LCCA), and Day 14 (LCCA and RCCA) after ligation and treated with DT or Control from 7-14 Days. Data are means, errors bars represent SEMs. (B) CD45 immunohistochemistry in LCCA at 28 day (upper panel) vs. spleen (positive control – lower panel)
Supplemental Figure III. VSMC apoptosis increases total vessel size
(A) Vessel lumen perimeter and (B) total vessel external elastic lamina (EEL) perimeter for mice undergoing carotid ligation. Analyses were performed on both ligated left (LCCA) and contralateral right common carotid arteries (RCCA). n=8 mice / group, *p<0.05, *** p<0.001.

Supplemental Figure IV. DT does not affect vessel areas or neointimal formation in wild-type mice
Wild-type mice (not expressing DTR) underwent LCCA ligation with DT administered between 7-14 days or control, and vessel areas examined at 28 Days. n=10 mice/group.
Supplemental Figure V.
Immunohistochemistry for BrdU in common carotid arteries at Day 28 after DT administration between Days 7-14. Scale bars represent 200 \( \mu \)m (LCCA) and 100 \( \mu \)m (RCCA).

Supplemental Figure VI
Flow cytometry of VSMCs treated with increasing doses of UV irradiation, stained with annexin V-FITC and propidium iodide. Representative data are shown and a total of at least 10,000 cells were analyzed (n=3).
Supplemental Figure VII

Western blot for caspase-3 in cell extracts 24 h after mock transfection (Control), transfection with 50 nmol/L control (scrambled) siRNA, or 50 nmol/L Caspase-3 siRNA.
### Mouse cytokine antibody array

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**Supplemental Figure VIII**

Antibody array and legend for conditioned media from control and apoptotic VSMCs.
Supplemental Figure IX
(A-B) IL-6 concentration in conditioned media after DT treatment of DTR+/− or control (wild-type) VSMCs (A), or after UV irradiation of wild-type VSMCs (B). **p<0.01, Values represent means ±SD of three individual experiments.

Supplemental Figure X.
(A) Area of LCCA at 28 D stained positively for IL-6 or (B) Serum cytokines at 28 D in mice undergoing LCCA ligation and treatment with DT or control from 7-14 Days. n=6. *** p<0.001
Supplemental Figure XI

(A-B) Scratch assay for VSMC migration in the presence of an antibody to IL-6 (α-IL-6) or isotype control (IgG).

(C) IL-1α concentration in conditioned media after increasing doses of UV irradiation. **p<0.01, *** p<0.001.