Vascular Smooth Muscle Cell Apoptosis Induces Interleukin-1–Directed Inflammation
Effects of Hyperlipidemia-Mediated Inhibition of Phagocytosis
Murray C.H. Clarke, Sara Talib, Nichola L. Figg, Martin R. Bennett

Rationale: Atherosclerosis is characterized by lipid accumulation in the vessel wall, inflammation, and both macrophage and vascular smooth muscle cell (VSMC) apoptosis. However, whereas VSMC apoptosis in mice with established atherosclerotic plaques or hyperlipidemia increases serum levels of the proatherogenic cytokines monocyte chemotactic protein (MCP)-1, tumor necrosis factor α, and interleukin (IL)-6, the link between hyperlipidemia, apoptosis and inflammation, and the mechanisms by which apoptotic cells promote inflammation in atherosclerosis are unknown.

Objective: To determine whether hyperlipidemia affects apoptotic cell clearance, and identify the molecular pathways downstream of VSMC apoptosis that may promote inflammation.

Methods and Results: We find that human VSMCs are potent and efficient phagocytes of apoptotic human VSMCs, but phagocytosis is significantly reduced by oxidized low-density lipoprotein in vitro or hyperlipidemia in vivo. Necrotic human aortic VSMCs release IL-1α, which induces IL-6 and MCP-1 production from viable human VSMCs in vitro. In contrast, secondary necrotic VSMCs release both IL-1α and caspase-activated IL-1β, augmenting IL-6 and MCP-1 production. Conditionally inducing VSMC apoptosis in situ in hyperlipidemic SM22α-hDTR/ApoE−/− mice to levels seen in human plaques increases serum MCP-1, tumor necrosis factor α, and IL-6, which is prevented by blocking IL-1.

Conclusions: We conclude that VSMC necrosis releases IL-1α, whereas secondary necrosis of apoptotic VSMCs releases both IL-1α and IL-1β from necrotic VSMCs induces the surrounding viable VSMCs to produce proinflammatory cytokines. Thus, failed clearance of apoptotic VSMCs caused by hyperlipidemia in vivo may promote the increased serum cytokines and chronic inflammation associated with atherosclerosis. (Circ Res. 2010;106:363-372.)

Key Words: apoptosis ▪ phagocytosis ▪ atherosclerosis ▪ inflammation ▪ hyperlipidemia

The phagocytosis of senescent or dead cells plays a pivotal role in development, normal tissue turnover, regulation of the immune system, and the resolution of inflammation. Phagocytosis of apoptotic cells generally leads to reduced expression of inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-12, and increased production of antiinflammatory cytokines such as IL-10 and transforming growth factor β. However, if dying or apoptotic cells are not swiftly phagocytosed they may undergo necrosis, with lysis releasing endogenous “danger signals” able to induce inflammation. Indeed, a growing body of evidence suggests that defective phagocytosis may be involved, or even causative, in many inflammatory diseases including rheumatoid arthritis, lung injury, autoimmune diseases, and, more recently, atherosclerosis. Many advanced atherosclerotic plaques consist of a fibrous cap of vascular smooth muscle cells (VSMCs), collagen, and extracellular matrix, with macrophages and T lymphocytes present in superficial regions of the plaque. Myocardial infarction occurs when the fibrous cap ruptures to expose the lipid-rich core, with subsequent thrombosis occluding the vessel. Plaque stability is determined by its mechanical properties, in particular the number of VSMCs and their ability to synthesize extracellular matrix. Both apoptosis and necrosis occur in advanced plaques at higher levels than those seen in normal vessels, and levels are increased in patients with unstable versus stable angina along with serum levels of inflammatory factors such as IL-6 and C-reactive protein. It has been suggested that reduced phagocytosis within atherosclerotic plaques may promote both chronic inflammation and plaque progression. However, the identity of...
phagocytes in atherosclerosis, the mechanisms linking failed phagocytosis and inflammation, and the cytokines promoting inflammation are all unknown. The phagocyte surface expresses a number of scavenger receptors responsible for uptake of apoptotic cells that are also involved in atherosclerosis. For example, scavenger receptor-A, CD36, and LOX-1 (lectin-like oxidized low-density lipoprotein receptor) mediate uptake of modified lipoproteins by macrophages and foam cells and thus oxidized low-density lipoprotein (oxLDL) can delay engulfment by competing for scavenger receptors. Furthermore, a hallmark of conditions that lead to an accumulation of apoptotic cells is loss of immune tolerance, whereby apoptotic antigens may be cross-presented by dendritic cells, which can lead to autoimmunity. Many autoantibodies develop as atherosclerosis progresses, including anti-oxLDL, anti-nuclear, anti–heat shock proteins, and anti-cardiolipin.

We have recently described SM22α-hDTR/ApoE−/− mice, in which transgenic expression of the human diphtheria toxin receptor (hDTR) from the minimal SM22α promoter enables conditional induction of VSMC-specific apoptosis. VSMC apoptosis in normal arteries is silent, with no inflammation or pathology. In contrast, VSMC apoptosis in hyperlipidemic SM22α-hDTR/ApoE−/− mice resulted in both local and systemic inflammation and pathological changes in vessel morphology. Although these findings suggest that phagocytosis may be reduced enough during hyperlipidemia to allow necrosis, with leakage of intracellular contents able to induce inflammation, this has not previously been proven. Therefore, we have used SM22α-hDTR/ApoE−/− mice to determine the mechanisms underlying apoptosis-induced inflammation during atherosclerosis. We show that VSMCs are efficient phagocytes of apoptotic VSMCs in vitro and in vivo. Hyperlipidemia alone is sufficient to reduce phagocytosis in the vessel wall, resulting in necrosis of apoptotic VSMCs and leakage of intracellular IL-1α. IL-1α subsequently acts on the surrounding viable VSMCs inducing them to release IL-6 and monocyte chemotactic protein (MCP)-1, thus perpetuating a chronic inflammatory state.

**Methods**

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

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### Animal Protocols

Animal experiments were performed under United Kingdom Home Office licensing. Recombinant DT (Quadtech Diagnostics) was prepared in 0.9% saline/0.2% BSA and stored at −80°C until use. To assess phagocytosis in vivo, mice were either left on normal chow (SDS), or placed on high fat diet (21% fat, 0% cholate, 0.2% cholesterol [SDS]) for 2 weeks, followed by 3 doses of DT (5 ng/g) intraperitoneally, each a day apart. Mice were euthanized under CO2, blood was collected, and vessels were removed and fixed on days −1, 1, 3, and 6 after DT treatment. IL-1 receptor antagonist (IL-1RA) or carrier control (Amgen) was administered intraperitoneally (25 mg/kg per day) from the first DT injection until euthanasia. Serum cytokine levels were quantified using the mouse inflammation array (Becton Dickinson), and lipids were determined using a Dade-Behring Dimension analyzer.

### Histology and Immunohistochemistry

Tissue was processed as previously described. Primary antibodies used were Mac 3 (Pharmingen) and cleaved caspase-3 (CC3) (Cell Signaling). All sections were stained with biotinylated secondary antibodies and detected with ABC reagents (Vector Laboratories). TUNEL was performed as previously described. Images were captured as previously described. Total number of apoptotic VSMCs were counted per whole aortic section and expressed per millimeter squared. Counts within mice were made on a minimum of 3 independent planes at least 50 μm apart.

### Cell Culture

Human VSMCs were isolated from aortas of cardiac transplant patients with informed consent and local ethics approval. Cells were cultured in DMEM supplemented with 10% FCS. Subconfluent VSMCs were trypsinized and resuspended in serum-free DMEM at 7×10⁵ cells/mL. Cell membranes were disrupted by 2 rounds of freeze/thaw in liquid nitrogen, then stored at −20°C or used as indicated. Apoptotic VSMCs were prepared by incubating with the Fas antibody CH.11 (100 ng/mL; Upstate) and cycloheximide (20 μg/mL) for 10 hours and made necrotic as above. Necrotic lysates were also prepared by incubating VSMCs with digitonin at RT for 10 minutes.

### In Vitro Phagocytosis Assay

VSMCs were either labeled with cell tracker orange and cultured overnight, or with cell tracker green (Molecular Probes) and apoptosis induced. Green apoptotic bodies were incubated with orange VSMCs for 2 hours, uneaten bodies were washed off, cells were trypsinized and analyzed by flow cytometry. Where indicated, phagocytic VSMCs were pretreated with fucoidan (100 μg/mL), poly-I (100 μg/mL), glucosamine (20 mM), or oxLDL (100 μg/mL; Biogenesis) for 15 minutes at 37°C.

### Cytokine Release Assay

VSMCs were plated at 15×10⁵ cells per well of a 24-well plate, allowed to adhere, and incubated in serum-free DMEM for 24 hour. Fresh serum-free DMEM was added to a final volume of 500 μL including treatments, incubated for 6 hours and then assayed by ELISA (PeproTech) for cytokine level. Where indicated necrotic lysates were treated with protease K (0.2 mg/mL), DNase (20 U/mL), or lipase (3000 U/mL) at 37°C for 30 minutes. Where indicated, VSMCs were preincubated with Box A (10 μg/mL) or IL-1RA (100 ng/mL), and necrotic lysates were preincubated with Hirudin (10 U/mL) or neutralizing antibodies (2 μg/mL) for 15 minutes. Immunodepletion was achieved by crosslinking IL-1α to protein G, followed by 2 rounds of depletion at 4°C for 1 hour. “Sham IL-1α depleted” controlled for carryover of neutralizing antibody.
Glycerol Gradients
Two continuous 5% to 25% glycerol gradients were poured in parallel, overlayed with either necrotic VSMC lysate or native molecular weight markers (Amersham) and ultracentrifuged at 280,000 g for 27 hours at 4°C. Fractions were collected from bottom to top from both separations. Necrotic VSMC fractions were assayed for cytokine-inducing activity, whereas marker fractions were subjected to SDS-PAGE and silver staining to allow estimation of peak molecular weight content within each fraction.

Western Blotting
Western blotting was performed according to standard laboratory techniques. Control and apoptotic samples were whole cell lysates of control and apoptotic VSMCs, whereas necrotic and secondary necrotic

Figure 1. VSMCs phagocytose apoptotic VSMCs. Flow cytometric assay for VSMCs phagocytosis. VSMCs were labeled “orange” and cultured overnight or “green” and cultured overnight to undergo Fas-induced apoptosis. Green apoptotic bodies were incubated with orange VSMCs for 2 hours, uneaten bodies were washed off, and cells were analyzed by flow cytometry. Orange VSMCs are easily distinguished (gate R1) (A) from green apoptotic bodies (gate R3) (B). Thirty-five percent of VSMCs ingest apoptotic VSMCs, as evidenced by increased green fluorescence of the previously orange-only VSMCs (gate R2) (C). Preincubation of the phagocyte VSMCs with fucoidan (gate R2) (D) inhibits phagocytosis. E, Confirmation of internalization was verified with epi-fluorescence and confocal microscopy. Scale bars = 50 μm. F, Histogram displaying levels of phagocytosis with various inhibitors. Data represent means ± SD (n = 3). *P < 0.007, **P = 0.015, compared to control.
samples were control and apoptotic VSMCs that had been freeze/thawed before lysis, respectively. Samples were loaded on the basis of equal numbers of extracted cells.

Statistics
Nonparametric tests were used for immunohistochemical and morphological analysis, as normality of data distribution could not be guaranteed. Comparison between 3 or more unpaired groups was made with the Kruskal–Wallis test, whereas comparisons between 2 unpaired groups were made with the Mann–Whitney *U* test (SPSS). Continuous data were analyzed by ANOVA (Excel).

Results
VSMC Phagocytosis of Apoptotic VSMCs Is Inhibited by Hyperlipidemia
We have previously shown that primary rat VSMCs can phagocytose apoptotic VSMCs in vitro; however, these findings were entirely descriptive, any consequences of phagocytosis (such as inflammation) or their mechanism were not examined, and we did not study how perturbation of phagocytosis might alter vessel structure or regulate inflammation in vivo. Using a quantitative FACS-based phagocytosis assay we confirmed that VSMCs are efficient phagocytes of apoptotic VSMCs, via a mechanism that was attenuated with the pan–scavenger receptor inhibitors poly-I and fucoidan (Figure 1). Importantly, phagocytosis was also significantly reduced by oxLDL (Figure 1).

We have previously demonstrated that apoptotic VSMCs are cleared from the vessel wall of mice without the presence of mononuclear phagocytes, suggesting that VSMCs also readily phagocytose apoptotic VSMCs in vivo. We therefore examined whether hyperlipidemia alone could reduce phagocytosis within the vessel wall before plaque development and before the appearance of monocyte/macrophages. The SM22α-hDTR/ApoE−/− mice allow conditional and dose-dependent induction of VSMC apoptosis. By inducing VSMC apoptosis with a “bolus” of DT and subsequently counting apoptotic cells over time, we can calculate a rate of clearance for VSMCs in vivo. This unique in vivo model measures phagocytosis kinetics, rather than apoptotic frequency at a single time point, as is more usual. This removes the uncertainty as to whether manipulations also alter the rate of apoptosis, and avoids trying to determine histologically whether apoptotic bodies are free, bound, or ingested. Two weeks of fat-feeding resulted in a significant 2.3-

![Figure 2](https://example.com/figure2.png)

Hyperlipidemia inhibits phagocytosis in SM22α-hDTR/ApoE−/− mice. SM22α-hDTR/ApoE−/− mice were either fat-fed for 2 weeks or left on normal chow. All mice were administered DT at 5 ng/g and then euthanized, and vessels were collected at the times indicated. A and B, Representative aortic sections following cessation of DT showing progressive removal of apoptotic bodies (arrows) by TUNEL staining (A) and absence of mononuclear phagocytes with MAC-3 (B). Scale bars=50 μm. C, Time course displaying number of cells actively undergoing apoptosis labeled by CC3 (triangles) or number of apoptotic cells labeled by TUNEL (squares) from aorta of normal chow (dashed line) and fat-fed (solid line) mice. Data represent means of n=4 (day −1), n=7 (day +1, +6), n=11 (day +3) mice at each time point and condition, *P*<0.03, **P*<0.002.
and 2.7-fold increase in serum cholesterol and LDL, respectively, compared to normal chow fed SM22α-hDTR/ApoE−/−mice (Online Figure I, available in the Online Data Supplement at http://circres.ahajournals.org). Following cessation of DT administration, apoptotic VSMCs marked by TUNEL were progressively removed from the vessel wall, which occurred in the absence of macrophages (Figure 2A and 2B). The rate of VSMC phagocytosis in fat-fed mice was markedly slower than chow-fed mice, such that almost double the number of apoptotic cells remained in fat-fed mice at day 6, despite an equivalent initial level of apoptotic VSMCs at day +1 (Figure 2C). To further confirm that fat feeding did not alter induction of apoptosis, we also examined the level of CC3-positive cells (Online Figure II). DT administration (Day 1) induced significant numbers of CC3- and TUNEL-positive cells; there was no significant difference in CC3 or TUNEL staining found between chow and fat-fed mice at this time point (Figure 2C), suggesting no effect of diet on induction of apoptosis. After cessation of DT the level of CC3 positive cells rapidly fell, with no positive cells detectable at days 3 and 6, despite the presence of TUNEL-positive cells. Given that CC3 only marks cells actively undergoing apoptosis, whereas TUNEL labels apoptotic cells until they are phagocytosed, this confirms that the differences in TUNEL-positive cells between chow and fat-fed mice at days 3 and 6 are governed by phagocytosis and not apoptosis induction.

Direct Release of IL-1β From Necrotic VSMCs Induces IL-6 and MCP-1 Production From VSMCs

We have previously found that VSMC apoptosis in hyperlipidemic SM22α-hDTR/ApoE−/−mice increases serum levels of the proinflammatory cytokines IL-6, MCP-1, TNF-α, and IL-12.24,25 Given the decrease in phagocytosis observed during hyperlipidemia (Figure 2C) we examined whether secondary necrosis of VSMCs (for example, attributable to failed phagocytosis) could release a factor that increases serum cytokines. Necrotic lysate supernatants made by freeze/thawing human aortic VSMCs induced release of IL-6 and MCP-1 from monolayers of human VSMCs (Figure 3A), via de novo production (Online Figure III). Necrotic VSMC lysates made by gentle permeabilization with the detergent digitonin gave equivalent results (Online Figure IV), although necrotic lysates themselves contained negligible IL-6 and MCP-1 (data not shown). The factor(s) responsible was proteinaceous, heat-inactivated, but remained stable at 22°C over 24 hour (Figure 3B). The previously described candidates HMGB-127 and thrombin28 were eliminated following a lack of inhibition with their antagonists Box A and hirudin, respectively (Online Figure V).

Previous studies have identified more than 15 proteins able to mediate release of IL-6 and MCP-1 from VSMCs. We therefore first used glycerol density centrifugation...
gation to determine the molecular weight of the factor(s), which revealed a single peak of activity for both cytokines within the same fraction of 17 kDa (Figure 3C). We examined the previous candidates identified for a 17-kDa protein exported via nonclassical pathways that may be released by cell necrosis. Both IL-1α and IL-1β are potent proinflammatory cytokines synthesized by VSMCs that do not possess signal peptides, and are released by nonclassical export (reviewed elsewhere).

Indeed, IL-1RA, which blocks all ligands binding to the IL-1 receptor 1, completely abolished all response to necrotic VSMC lysates (Figure 4A). Individual neutralizing antibodies for IL-1α and IL-1β subsequently identified IL-1α to be the single protein from necrotic VSMCs able to mediate IL-6 and MCP-1 release from VSMCs (Figure 4B). Multiplex cytokine analysis of supernatants collected from viable VSMCs incubated with necrotic VSMC lysates confirmed that only IL-6 and MCP-1 are released, with no significant production of IL-1β, IL-2, IL-10, IL-12, IL-18, interferon-γ, or TNF-α (Online Figure VI).

The finding that IL-1α can mediate inflammation in response to necrotic cells has recently been reported by others, however controversy exists as to whether IL-1α acts as a secondary signal released by macrophages in response to unknown necrotic factors, or whether IL-1α is directly released on cell lysis. By immunodepleting IL-1α from necrotic VSMC lysates (Figure 4C), we found a complete loss of IL-6 and MCP-1-inducing activity (Figure 4D), confirming that IL-1α alone is indeed a direct molecular danger signal released from necrotic VSMCs.

**Secondary Necrotic VSMCs Are More Inflammatory Because of the Combined Effect of IL-1α and IL-1β**

Although we have shown that primary necrosis releases IL-1α, our original hypothesis requires IL-1α to be functional when released on necrotic lysis after apoptosis. We therefore examined the efficacy of necrotic lysates made from equal numbers of both viable and apoptotic (secondary necrotic) VSMCs to induce VSMC cytokine release. Surprisingly, secondary necrotic VSMC lysates were more effective at activating live VSMCs to produce cytokines (Figure 5A). Examination of necrotic lysates by Western blotting revealed equal amounts of IL-1α present within control and apoptotic lysates, and equal amounts of calpain-cleaved IL-1α within necrotic and secondary necrotic lysates (Figure 5B). How-

![Graph A](image1.png)

![Graph B](image2.png)

![Graph C](image3.png)

![Graph D](image4.png)

**Figure 4.** Direct release of IL-1α is required to induce cytokine production in response to necrotic VSMC lysates. IL-6 (gray) or MCP-1 (black) concentrations within supernatants collected from viable VSMCs incubated with necrotic VSMC lysates with or without IL-1RA (A) or neutralizing antibodies (pAb) to IL-1α or β (B). Antibody specificity was determined by incubation with human recombinant IL-1α or -β (hrIL-1α/hrIL-1β). Immunodepleting IL-1α from necrotic VSMC lysates (C) results in loss of all stimulatory activity (D). Data represent means ± SD (of n=5). *P<0.001, **P<0.01.
ever, activated IL-1β was increased in apoptotic and secondary necrotic VSMC lysates (Figure 5C), suggesting the increased activity of secondary necrotic lysates is attributable to the combined action of IL-1α and caspase-activated IL-1β.

The Increase in Serum Proinflammatory Cytokines After VSMC Apoptosis In Vivo Depends on IL-1
We have previously reported that acute induction of VSMC apoptosis in mice with established plaques or chronic VSMC apoptosis during atherogenesis increases serum inflammatory cytokines. However, in both cases the vessel wall contained substantial numbers of inflammatory cells, which could have been a potential source of cytokines. We therefore examined the serum cytokine response to VSMC apoptosis in SM22α-hDTR/ApoE–/– mice after only 2 weeks of fat-feeding, when plaques are not present. Acute medial VSMC apoptosis in fat-fed mice induced significant increases in the serum level of IL-6, MCP-1 and TNF-α, which rapidly returned to baseline (Figure 6A) with a time course which paralleled the removal of most of the remaining apoptotic cells (Figure 2C). However, normal chow-fed SM22α-hDTR/ApoE–/– mice showed no increase in serum cytokines (Figure 6A), despite the same initial level of apoptotic VSMCs (Figure 2C). Importantly, fat feeding alone, without induction of VSMC apoptosis, resulted in no increase in serum cytokines compared to control chow-fed animals (Online Figure VII). We have previously found that VSMC apoptosis also increases local expression of MCP-1 within established plaques. However, despite an increase in serum MCP-1 (Figure 6A), no inflammatory cells were detected within the vessel wall (Figure 2B). To determine whether IL-1 was responsible for inducing serum inflammatory cytokines, we administered recombinant IL-1RA or carrier control concurrent with DT treatment throughout the experimental time course. IL-1RA administration resulted in a statistically significant decrease in IL-6, and a borderline reduction in TNF-α (Figure 6B). IL-1RA did not reduce the number of apoptotic bodies (67.0±7.6 versus 65.6±5.6 cells/mm², IL-1RA versus carrier, P=0.89) present at death or reduce serum lipids (data not shown), indicating that its effect on IL-6 and TNF-α levels were attributable to neutralization of IL-1, most likely released from necrotic VSMCs.

Discussion
Atherosclerosis is a disease characterized by lipid accumulation in the vessel wall, cell death and inflammation. Apoptosis in VSMCs and macrophages may both promote inflammation and alter plaque composition. However, the mechanisms by which apoptotic cells promote inflammation in atherosclerosis are unclear. One possibility is that failure to clear apoptotic vascular cells results in secondary necrosis, with either subsequent release of inflammatory cytokines or signaling to local healthy cells to stimulate cytokine release. Although much research has endeavored to assess the impact of failed phagocytosis in vivo, many manipulations intended to alter phagocytosis (e.g., knock out of phagocytic receptors) are marred by redundancy in the clearance system and result in little pathology. In contrast, we report that conditional induction of...
VSMC apoptosis during hyperlipidemia results in accumulation of apoptotic bodies, followed by a robust IL-1-dependent increase in serum inflammatory cytokines, which rapidly resolves after removal of the stimulus (summarized in Online Figure VIII). To our knowledge, this is the first direct demonstration of the inflammatory consequences of failed VSMC phagocytosis in situ, in vivo.

Although it has been suggested that failed phagocytosis within atherosclerotic plaques may be responsible for the inflammation associated with the disease,6,7 the complex microenvironment makes it difficult to quantify kinetics of phagocytosis, the cells involved, and the number of uncleared apoptotic cells that would elicit an inflammatory response. We have previously shown that systemic inflammation results from apoptosis in ~15% of VSMCs within established plaques,24 or in ~1% of VSMCs in normal vessels after fat-feeding.25 In the present study, we demonstrate that in hyperlipidemic mice, induction of apoptosis in ~1% of VSMCs (≈66 cells/mm²) is sufficient to induce high levels of circulating IL-6, TNF-α, and MCP-1, which return to baseline by day 6 when <0.5% of apoptotic VSMCs (≈27 cells/mm²) remain. These changes in inflammatory cytokines were not seen in normolipidemic mice despite similar initial levels of apoptosis, supporting the concept that inflammation requires both VSMC apoptosis and hyperlipidemia acting in concert, most likely via reduced phagocytosis resulting in secondary necrosis. Apoptotic frequencies of 1% are commonly found in advanced plaques in humans and mice, suggesting that uncleared apoptotic cells may be potent stimuli sustaining the inflammation associated with atherosclerosis.

Our study supports recent observations that inhibition of local phagocytosis in the vessel wall can promote adverse consequences on plaque structure and inflammation. For example, knock out or mutation of the MerTk receptor or lactadherin resulted in an accumulation of apoptotic cells, increased size of necrotic cores, but lesion size was either unchanged15 or near doubled.17,18 In contrast, increased macrophage apoptosis by directed deletion of Bcl-XL36 or total phospholipase Cζ37 loss, without manipulation of phagocytosis, led to either no change in plaque and necrotic area36 or a large reduction in both necrotic core and plaque size.37 These discrepancies suggest that accumulation of apoptotic cells per se within a plaque does not drive a similar phenotype, highlighting the need to understand the specific downstream interactions between the apoptotic/necrotic cell type, the “responding” cell, and the local milieu at different stages of plaque development.

This discovery that VSMCs are normally highly efficient phagocytes, clearing apoptotic VSMCs in vivo without the need for macrophages, and that short periods of fat-feeding inhibit VSMC phagocytosis is most surprising. Previous work has only examined presence of apoptotic bodies within established atherosclerosis,7 in part, because of the rarity of apoptosis before plaque development.13 Many mechanisms have been proposed to explain failed clearance within the plaque microenvironment, including competitive inhibition of phagocytic receptors,20 oxidative stress,7 and anti-oxLDL autoantibody binding to apoptotic cells.38,39 However, most of these situations require macrophages, or longer time frames, and therefore should not affect clearance in the present study. Importantly, hyperlipidemia may also in-
hibit phagocytosis in other organs. For example, unclear apoptotic cells within the lymph nodes of Fas ligand mutant gld/ApoE−/− mice were significantly increased on fat-feeding, also suggesting an effect of hyperlipidemia remote from the plaque microenvironment.

IL-1α and IL-1β represent the prototypic cytokines and bind to the same receptor to induce identical biological functions. Both IL-1α and -β can be synthesized by endothelial cells, VSMCs, and macrophages and can induce cytokine production, activation of endothelial cells and macrophages, increased adhesion molecules, proliferation of VSMCs and increased vascular permeability, all processes important during atherogenesis. Indeed, atherosclerosis is reduced in IL-1β knockout mice, whereas IL-1RA–deficient mice develop more neointima after balloon injury. In contrast to IL-1β, the role of IL-1α has been less clear. Our findings suggest that following primary necrosis only IL-1α acts as an endogenous danger signal, whereas after secondary necrosis, both IL-1α and apoptosis-activated IL-1β act in combination. This additive effect following secondary necrosis underscores the adverse consequences of failed apoptotic cell clearance, effectively resulting in a greater proinflammatory potential for a given number of cells than during primary necrosis.

In summary, we have shown that following VSMC apoptosis reduced phagocytosis caused by hyperlipidemia may result in secondary necrosis with release of IL-1α and IL-1β. These cytokines act within the vessel wall on the surrounding viable VSMCs, inducing them to produce proinflammatory cytokines, including IL-6 and MCP-1. Thus, “amplification” of an IL-1–mediated signal from a small quantity of necrotic cells ultimately results in both a local and systemic increase in inflammatory cytokines (Online Figure VIII). Once the remaining apoptotic and necrotic debris has been cleared, the stimulus is removed and serum cytokines return to baseline. We believe the combination of VSMC death and hyperlipidemia may promote an as yet unreported role for IL-1–dependent inflammation during atherosclerosis.

Acknowledgments

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Disclosures

None.

References

23. Clarke MC, Figg N, Maguire J, Davenport AP, Goldmaar M, Littlewood TD, Bennett MR. Apoptosis of vascular smooth muscle cells induces...


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SUPPLEMENTAL MATERIAL
**Detailed Materials and Methods**

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

**Animal Protocols**

Animal experiments were performed under UK Home Office licensing. The minimal mouse SM22α promoter (−445 to +88 relative to transcriptional start site) was cloned into the pEGFP-N1 vector (Clontech) upstream of the hDTR-eGFP sequence. DNA was prepared using the endotoxin-free MaxiPrep kit (Qiagen) and linearised. Pronuclear injection of the vector into oocytes, implantation, and generation of chimeric progenitors was undertaken by Eurogentec (Belgium). SM22α-hDTR mice were born with the expected frequency, and developed normally in the absence of DT. Purified recombinant DT (Quadratech Diagnostics) was prepared in 0.9% saline/0.2% BSA (low endotoxin), sterile-filtered and stored at −80°C until use. To assess phagocytosis in vivo, mice were either left on normal chow (SDS; 801730), or placed on high fat diet (21% fat, 0% cholate, 0.2% cholesterol (SDS; 829100)) for 2 weeks, followed by 3 doses of DT (5ng/g) intraperitoneally, each a day apart. Mice were euthanased under CO₂, blood collected by cardiac puncture, and vessels removed and fixed in 10% neutral-buffered formalin on days -1, 1, 3 and 6 post DT treatment. IL-1 receptor antagonist (IL-1RA; Amgen) or carrier control (Amgen) was administered intraperitoneally (25mg/kg/day) from the first DT injection until sacrifice. Serum cytokine levels were quantified using the mouse inflammation cytometric bead array (Becton Dickinson), and lipids determined using a Dade-Behring Dimension analyser.

**Histology and Immunohistochemistry**

Fixed, washed tissues were embedded in paraffin, and 5µm serial sections cut, deparaffinated and stained with Haematoxylin and Eosin. Antigen retrieval was achieved by boiling in citrate buffer (pH 6.0). The primary antibodies used were: Mac 3 (M3/84; Pharmingen, 1:200); Cleaved caspase-3 (CC3)(9661; Cell Signaling, 1:50). All sections were stained with biotinylated secondary antibodies and detected with ABC reagents and DAB (Vector Laboratories). TUNEL was performed using dUTP-DIG incorporation (Roche), detection with an alkaline phosphatase-conjugated antibody to DIG (Roche), and development with BCIP/NBT (Vector). Images were captured using a BX51 microscope (Olympus), air cooled CCD camera (CoolSnap) and imaging/analysis software (Soft Imaging Systems). Total number of apoptotic VSMCs were counted per whole aortic section and expressed per mm². Counts within mice were made on a minimum of 3 independent planes at least 50µm apart.

**Cell Culture**

Human VSMCs were isolated from aortas of cardiac transplant patients with informed consent and local ethics approval. Cells were cultured in DMEM supplemented with 10U/ml penicillin, 10mg/ml streptomycin, 5mg/ml L-glutamine and 10% FCS. Subconfluent VSMCs were trypsinised and resuspended in serum-free (S/F) DMEM at 7x10⁵ cells/ml. Cell membranes were disrupted by 2 rounds of freeze/thaw in liquid nitrogen in a volume less than 1ml, then stored at -20°C or used at volumes indicated. Apoptotic VSMCs were prepared by incubating with the Fas agonistic antibody CH.11 (100ng/ml; Upstate) and cycloheximide (20µg/ml) for 10 h, and made necrotic by freeze/thaw at 7x10⁵ cells/ml. In addition, necrotic lysates were also prepared by incubating VSMCs with digitonin at RT for 10 mins.

**In vitro phagocytosis assay**

VSMCs were either labeled with cell tracker orange and cultured overnight, or with cell tracker green (Molecular Probes) and apoptosis induced. Green apoptotic bodies were washed, incubated with orange VSMCs for 2 hours, uneaten bodies washed off, cells trypsinised and analyzed by flow cytometry (BD FACScalibur). Where indicated, phagocyte VSMCs were pretreated with fucoidan (100µg/ml), poly-I (100µg/ml), glucosamine (20mM), galactosamine (20mM), or oxLDL (100µg/ml; Biogenesis) for 15 mins at 37°C.
**Cytokine Release Assay**

VSMCs were plated at 15x10^3 cells per well of a 24-well plate, allowed to adhere overnight, and incubated in S/F DMEM for 24 h. Cells were washed with PBS and S/F DMEM added to a final volume of 500µl including all treatments, incubated for 6 h, conditioned media collected, clarified, and assayed by ELISA (PeproTech) for cytokine level according to the manufacturer’s instructions. Where indicated necrotic lysates were treated with protease K (0.2 mg/ml), Dnase (20U/ml), or Lipase (3000U/ml) at 37°C for 30 mins. Where indicated, VSMCs were preincubated with Box A (10µg/ml; HMGBiotech) or IL-1RA (100ng/ml; Amgen), and necrotic lysates preincubated with Hirudin (10U/ml) or neutralising IL-1α/β antibodies (2µg/ml; Peprotech) for 15 mins. Immunodepletion was achieved by covalently crosslinking IL-1α antibody to protein G with dimethylpimelimidate, followed by 2 rounds of depletion at 4°C for 1 h. ‘Sham IL-1α deplete’ controlled for any direct carryover of neutralising antibody from the immunodepletion.

**Glycerol Gradients**

Two continuous 5–25% glycerol gradients were poured in parallel, overlayed with either necrotic VSMC lysate, or native molecular weight markers (Amersham) and ultracentrifuged at 280,000g for 27 h at 4°C in a swing-out rotor. Fractions were collected from bottom to top (heavy to light) from both separations. Necrotic VSMC fractions were assayed for cytokine-inducing activity, whilst marker fractions were subjected to SDS-PAGE and silver staining to allow estimation of peak molecular weight content within each fraction.

**Western Blotting**

Western blotting was performed according to standard laboratory techniques. Control and apoptotic samples were whole cell lysates of control and apoptotic VSMCs, whilst necrotic and secondary necrotic samples were control and apoptotic VSMCs that had been freeze/thawed before lysis, respectively. Samples were loaded on the basis of equal numbers of extracted cells.

**Statistics**

Non-parametric tests were employed for immunohistochemical and morphological analysis, as normality of data distribution could not be guaranteed. Comparison between 3 or more unpaired groups was made with the Kruskall-Wallis test, whilst comparisons between 2 unpaired groups were made with the Mann-Whitney U test (SPSS). Continuous data was analysed by ANOVA (Excel).
Online Figure I: Serum lipid levels in SM22α-hDTR/ApoE−/− mice following two weeks of fat-feeding. Mice were either fat-fed for 2 weeks (black bars) or left on normal chow (grey bars). All mice were administered DT at 5ng/g, and then euthanased 3 days later. Data represent mean±SD, n = 9, * = p <0.0001.
Online Figure II: Cleaved caspase 3 staining of cells only occurs during active apoptosis. SM22α-hDTR/ApoE<sup>−/−</sup> mice were either fat-fed for 2 weeks or left on normal chow. All mice were administered DT at 5ng/g, and then euthanased and vessels collected at the times indicated. Representative longitudinal aortic sections showing that during DT treatment (day -1) cells that are actively undergoing apoptosis are positive for CC3 (arrows). After cessation of DT (day 0) the number of CC3 positive cells rapidly declines, such that at day 3 and 6 no positive cells are detected. The external elastic lamina is highlighted by the dashed line. Scale bars = 50µm.
Online Figure II: Necrotic VSMC-induced release of IL-6 from live VSMCs is by de novo production. Monolayers of normal VSMCs were pre-treated with or without inhibitors of transcription or translation, and then lysates from necrotic VSMCs added. After 6 h. of culture, supernatants were collected and assayed for IL-6 by ELISA. Inhibition of either transcription (Actinomycin D) or translation (Emetine) blocked all cytokine release. Data represent mean ±SD; *p = 0.0003.
Online Figure IV: Necrotic VSMC lysates produced by permeabilisation with digitonin also induce release of IL-6 and MCP-1 from VSMCs. Monolayers of VSMCs were incubated with either digitonin or freeze/thaw-derived necrotic VSMCs lysates as indicated. After 6 h. culture supernatants were collected, debris removed, and assayed for cytokine content by ELISA. Data represent mean ±SD, n=2.
Online Figure V: Necrotic VSMC-derived HMGB-1 or thrombin does not mediate release of IL-6 from VSMCs. Monolayers of VSMCs were incubated with or without lysates from necrotic VSMCs and inhibitors of HMGB-1 (Box A) or thrombin (hirudin) as indicated. After 6 h. culture supernatants were collected, debris removed, and assayed for IL-6 content by ELISA. Data represent mean±SD; NS = no significant difference, n=2.
Online Figure VI: VSMCs exposed to lysates from necrotic VSMCs only release IL-6 and MCP-1. Monolayers of VSMCs were incubated with or without lysates from necrotic VSMCs and an IL-1α neutralising antibody (IL1α pAb) as indicated. After 6 h., culture supernatants were collected, debris removed, and assayed for cytokine content by multiplexed bead array. IL-6 and MCP-1 were the only cytokines released in significant quantities, and their release was IL-1α-dependant. Data represent mean±SD.
Online Figure VII: Hyperlipidemia alone does not increase serum cytokines. Serum inflammatory cytokine concentrations of SM22α-hDTR/ApoE<sup>−/−</sup> mice that were either fat-fed for 2 weeks or left on normal chow. Mice were euthanased, blood collected and serum analysed by multiplexed bead array. No significant difference is found between the two groups for any cytokine, indicating no direct effect of hyperlipidaemia on cytokine levels and no indirect interference with the bead assay. Data represent mean ±SEM, n = 6.
Online Figure VIII: Simplified diagram outlining the novel conclusions of the work. We show that VSMC apoptosis under conditions that reduce phagocytosis, such as hyperlipidemia, results in secondary necrosis with release of IL-1. This cytokine acts on the surrounding viable VSMCs within the vessel wall, inducing them to produce large quantities of the proinflammatory cytokines IL-6 and MCP-1. Thus, release of IL-1 from a small quantity of necrotic VSMCs results in an ‘amplification’ that ultimately produces both a local and systemic increase in inflammatory cytokines. In contrast, VSMC apoptosis under normal conditions does not lead to any inflammation. NC = normal chow; HF = high fat; SR = scavenger receptor.