Multiple Roles for Neutrophils in Atherosclerosis

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Abstract — Because of their rare detection in atherosclerotic lesions, the involvement of neutrophils in the pathophysiology of atherosclerosis has been largely denied. However, over the past couple of years, studies have provided convincing evidence for the presence of neutrophils in atherosclerotic plaques and further revealed the causal contribution of neutrophils during various stages of atherosclerosis. This review describes mechanisms underlying hyperlipidemia-mediated neutrophilia and how neutrophils may enter atherosclerotic lesions. It also highlights possible mechanisms of neutrophil-driven atherogenesis and plaque destabilization. Knowledge of the contribution of neutrophils to atherosclerosis will allow for exploration of new avenues in the treatment of atherogenesis and atherothrombosis. (Circ Res. 2012;110:875-888.)

Key Words: atherosclerosis ■ chemokine ■ granule protein ■ inflammation ■ neutrophil

Atherosclerosis is the primary cause of mortality and morbidity resulting from coronary artery disease, stroke, and peripheral vascular disease. Hyperlipidemia and inflammation represent the two major pillars in the pathophysiology of atherosclerosis.1,2 These two are interconnected as hypercholesterolemia increases circulating monocyte counts and renders these cells more prone for emigration into atherosclerotic lesions.3 Hence, the causal contribution of monocytes and their descendants to atherosclerotic plaque formation, progression, and destabilization has been studied extensively. In addition, the contribution of immune cells such as T lymphocytes, mast cells, dendritic cells, and platelets to atherosclerosis has been firmly established over the years.4,5 However, while being the most abundant white blood cell in the circulation, the neutrophil has received little attention in the pathophysiology of atherosclerosis. Nevertheless, recent advances point at a contributory role of neutrophils during atherogenesis and plaque destabilization. Thus, this review aims at highlighting disturbances of neutrophil homeostasis and function by hyperlipidemia, ultimately promoting atherosclerosis. Furthermore, mechanisms have emerged explaining the importance of neutrophils during very early and later stages of atherosclerosis. It also should be pointed out that because of the lack of original data from atherosclerosis models, many of the mechanistic conclusions herein are extrapolated from microvascular models of inflammation. Furthermore, much of our current knowledge about the potential role of neutrophils in atherosclerosis is based on observations from experimental models in mice. However, it should be pointed out that mouse models have their limitations with regard to modeling plaque destabilization and rupture, and that interventional procedures in mice are of limited availability.4

The Neutrophil: A Proinflammatory Cell

Neutrophils are classically looked at as short-lived, rather crude, and unrefined phagocytes with limited transcriptional capacities whose successful missions are often responsible for significant collateral damage. Much of this view is based on the ability of the neutrophil to release vast amounts of proteolytic enzymes and reactive oxygen species, both of which are important during bacterial infections. However, this understanding has been challenged over the past decade when it was found that neutrophils possess prominent immune-regulatory activities.7-9 Neutrophils do so by release of preformed proteins stored in four different granule subsets. These subsets have different propensities for release with secretory vesicles being mobilized on establishment of neutrophil–endothelial interactions, whereas tertiary granules are released during subsequent neutrophil transendothelial migration. Primary and secondary granules are discharged once the neutrophil has entered the extravascular tissue. Proteins released from neutrophil granule subsets instruct recruitment and activation of monocyte, macrophages, and dendritic cell subsets.10,11 However, a second tool at the immediate command of the neutrophil is lipid mediators, which are produced in a reaction involving oxygenation of arachidonic acid and further processing of the intermediate metabolite leukotriene A4 into the potent chemotaxant leukotriene B4. At sites of inflammation, the life span of the neutrophil is subject to modulation by growth factors and cytokines. Neutrophils eventually undergo apoptosis, which is, per se, an anti-inflammatory process because clearance of apoptotic neutro-
Neutrophils by macrophages unleash signals for resolution of inflammation. However, if the engulfment capacity of macrophages is overloaded, then neutrophils become necrotic and, hence, perpetuate inflammation. A unique cell death termed NETosis has been identified for neutrophils, wherein neutrophils expel nDNA forming a scaffold for extracellular exposure of granule proteins and histones. Interestingly, neutrophil extracellular traps just recently have been identified in association with human and murine atherosclerotic lesions, which may allow speculation about proinflammatory processes this structure may stimulate in atherosclerosis. The extension of the life span of the neutrophil at sites of inflammation also extends the time slot during which neutrophils can launch a transcriptional program. Hence, it should be pointed out that neutrophils are able to synthesize and release chemokines, cytokines, and growth factors.

Identification of Neutrophils in Murine and Human Lesions

Neutrophils classically have been neglected in the pathophysiology of atherosclerosis. Much of this is attributable to their rare detection in atherosclerotic lesions, which may be explained by their relatively short life-span, by their ability to undergo phenotypic changes displaying markers typically expressed on antigen-presenting cells, thus appearing as macrophage-like or dendritic cell-like cells, and, finally, by the lack of sensitive and specific detection methods. This is further complicated by use of staining approaches that are not entirely selective for neutrophils (Table). Over the past years, however, refined staining techniques allowed for sensitive detection of neutrophils in murine and human atherosclerotic plaque specimens. The use of antibodies to Ly6G, an antigen specifically expressed on mouse neutrophils, enabled immunohistochemical detection of neutrophils in early lesions as well as in rupture-prone atherosclerotic lesions. Similarly, staining with antibodies to myeloperoxidase (MPO) in conjunction with Ly6G staining enabled identification of neutrophils in murine atherosclerotic lesions. In early murine lesions, neutrophils were identified in a subendothelial and intimal location. In more advanced plaques, neutrophils localize within the plaque shoulder and in the adventitia. Utilization of Apoe<sup>−/−</sup>/Ly5<sup>−/−</sup>/Cx3cr1<sup>−/−</sup> mice allowed for sensitive intravital detection of interactions between neutrophils and endothelium (Figure 1A). Use of these mice also led to identification of neutrophils within early and advanced plaques. In the latter, accumulation of neutrophils within atherosclerotic lesions was more prominent in regions of high inflammatory activity. This approach can be further refined by use of Apoe<sup>−/−</sup>/Ly5<sup>−/−</sup>/Cx3cr1<sup>−/−</sup>/Fli1<sup>−/−</sup> mice. Injection of a fluorescent antibody to Ly6G allows for simultaneous intravital detection of neutrophil and monocyte interactions with the endothelium in large arteries (Figure 1B).

In humans, CD177 (NB-1) and CD66b are markers restricted to granulocytes, whereof the latter has been used to detect neutrophils in atherosclerotic specimens. Recent analyses of endarterectomy specimens revealed the association of intraplaque neutrophil numbers with histopathologic features of rupture-prone atherosclerotic specimens such as large lipid core and low collagen or smooth muscle cell content. In this study, neutrophils were found in different regions of the plaque, with high numbers in the fibrous cap, the shoulder, the interface to media, and in areas with intraplaque bleeding, and lower numbers were found underneath the luminal endothelium or in the vicinity of microvessels in the plaque. This is in line with earlier reports identifying neutrophils in culprit lesions of patients who had died of acute myocardial infarction or in culprit lesions in patients with unstable angina, but not in patients with stable angina pectoris.

**Table. Strategies for Antibody-Based Identification of Human and Murine Neutrophils**

<table>
<thead>
<tr>
<th>Antibody Clone</th>
<th>Expression Pattern of Antigen</th>
<th>References</th>
<th>Comment</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human CD66b</td>
<td>80H3</td>
<td>Neutrophils, eosinophils</td>
<td>170–172</td>
<td>Detection of neutrophils reported in human plaques and culprit lesions</td>
</tr>
<tr>
<td>Mouse Gr1 (Ly6G/C)</td>
<td>RB6-8C5, NIMP-R14</td>
<td>Neutrophils, inflammatory monocytes</td>
<td>21</td>
<td>Antibodies to Gr1 should be used in combination with antibodies to CD11b or CD115 to discriminate between neutrophils and monocyte subsets</td>
</tr>
<tr>
<td>Ly6G</td>
<td>1A8</td>
<td>Neutrophils</td>
<td>20</td>
<td>Most specific approach for neutrophil detection</td>
</tr>
<tr>
<td>Ly6B</td>
<td>7/4</td>
<td>Neutrophils, inflammatory monocytes, macrophages</td>
<td>173</td>
<td>7/4 antibodies should be used in combination with antibodies to CD11b or CD115 to discriminate between neutrophils and monocyte subsets</td>
</tr>
<tr>
<td>MPO</td>
<td>Various</td>
<td>Neutrophils, monocytes, macrophages</td>
<td></td>
<td>Because of the presence of myeloperoxidase in macrophages, double staining with anti-Ly6G antibodies is recommended</td>
</tr>
</tbody>
</table>
Histological analysis of human carotid endarterectomy specimens suggested that intraplaque hemorrhage could convey neutrophils into the atherosclerotic lesion, leading to its enrichment in neutrophil proteases, which established a potential link between intraplaque hemorrhage, neutrophil infiltration, and plaque fragility.38,39 A recent study also has identified activated neutrophils in human atherosclerotic lesions by use of staining with antibodies to formyl peptide receptor 2 (FPR2; formerly known as FPRL1) and p22phox, as well histological identification of oxidant production and esterase activity.30 However, with the presence of these activity markers in macrophages or mast cells,31–33 it is essential to counterstain with markers that allow identification of neutrophils if conclusions with regard to the cellular origin of the activity shall be drawn.30 In addition, neutrophils have been identified in occlusive thrombi.34,35 Neutrophil extracellular traps as well as neutrophil-derived serine proteases may stimulate coagulation and, thus, offer a functional explanation for neutrophils in thrombus formation.36,37

Neutrophil Granule Proteins Localize in Atherosclerotic Lesions and May Serve as Biomarkers

Much of the neutrophil-dependent proinflammatory activity can be attributed to the release of preformed granule proteins, which are discharged into the surroundings of activated neutrophils.38–40 Expression of granule proteins such as azurocidin, LL-37, α-defensins, and NGAL is largely restricted to neutrophils.39 Interestingly, all of these granule proteins were identified by immunohistochemistry in human atherosclerotic lesions,41–44 suggesting that these proteins might be footprints of neutrophils. However, staining for these granule proteins colocalized with markers of endothelial cells, macrophages, and smooth muscle cells. Because neutrophil granule proteins may be taken-up by other cells on release from neutrophils or transferred onto macrophages during clearance of apoptotic neutrophils, the cellular origin of these granule proteins needs to be firmly established. Granule proteins, however, not only localize in atherosclerotic lesions but also are secreted into the plasma on neutrophil activation.

Hence, several proteins typically expressed and released by neutrophils emerged as possible biomarkers. MPO, for example, is abundantly expressed in neutrophil primary granules and is partially released on neutrophil activation. Through its catalytic activity, MPO contributes to radical formation and subsequently promotes formation of oxidized low-density lipoprotein.45 Studies in patients with established atherosclerosis suggest a positive correlation between plasma MPO levels and the risk of coronary artery disease.46,47 Furthermore, the clinical value of MPO to predict acute myocardial infarction and adverse events during a follow-up period in patients presenting with acute chest pain has been established.48 Similarly, MMP-2 and MMP-9, which are abundant in neutrophil secondary and tertiary granules, were found to be elevated in patients with acute coronary syndrome.49 These observations are further extended by studies indicating a positive correlation between circulating neutrophil counts and the risk for cardiovascular events.50–52 More recently, a direct correlation between the number of circulating neutrophils and lesion sizes in aortic roots of mice fed a high-fat diet has been evidenced,53 firmly suggesting a causal role for neutrophils in atherosclerosis. Thus, recent studies suggest neutrophil activation during atherosclerosis and continuous recruitment of neutrophils at various stages of atherosclerosis; therefore, these lay the basis for investigating mechanisms by which neutrophils contribute to atherosclerotic lesion formation.

Disturbance of Neutrophil Homeostasis by Hypercholesterolemia

Hyperlipidemia, particularly hypercholesterolemia, is considered as an independent risk factor in the development of atherosclerosis and its complications.54 Epidemiological studies have established a strong correlation between elevated total cholesterol levels in serum and morbidity and mortality from myocardial infarction.55 Elevated numbers of circulating neutrophils have been shown to be predictive of cardiovascular events independent of serum cholesterol levels.52

![Figure 1. Detection of neutrophils in large arteries. A](image-url) Adhesion of neutrophils to the external carotid artery in monocyte-depleted Lysmegfp/egfpApoel/–/– mice after 4 weeks of high-fat diet. B, CX3cr1egfp/wtApoel/–/– mice intravenously injected with a PE-conjugated antibody to Ly6G were used to visualize neutrophils (red) and monocytes (green). Mice were fed a high-fat diet for 4 weeks. Recordings were made using an epifluorescence with an integrated beam-splitter to enable simultaneous detection of two wavelengths. Bars in (A) and (B) represent 100 μm.
Consistent with this, recent data from mouse models have provided evidence of a direct correlation between the number of circulating and lesional neutrophils and atherosclerotic lesion size. Neutrophil homeostasis is regulated at various levels, including production in and release from the bone marrow, survival in the circulation, and homing and clearance of senescent neutrophils. The production is, in general, controlled by granulocyte colony-stimulating factor, a myelopoiesis-stimulating growth factor that is enhanced under hypercholesterolemia. Granulocyte colony-stimulating factor itself is primarily induced by tumor necrosis factor and IL-17, the latter of which is the effector of a neutrophil clearance feedback loop. Our study, but also recent findings by others, evidenced increased tumor necrosis factor and IL-17 levels in the plasma of atherosclerotic mice. In a more recent study, a direct mechanistic link between hypercholesterolemia and proliferation of myeloid progenitor cells and, hence, neutrophilia and monocytosis has been identified. In more detail, apolipoprotein E secreted from hematopoietic stem and multipotent progenitor cells became anchored to proteoglycans on their surface, where it interacts with ABCA1, which transports excessive cholesterol from membranes to nascent high-density lipoprotein particles, and ABCG1, which transports cholesterol to mature high-density lipoprotein particles, thereby controlling intracellular cholesterol levels. Murphy et al showed that in hematopoietic stem progenitor cells lacking apolipoprotein E, cholesterol efflux pathways were disrupted and cholesterol accumulated intracellularly, resulting in increased responsiveness to the hematopoietic growth factors IL-3 and granulocyte–macrophage colony-stimulating factor. The net effect was an increase in hematopoietic stem progenitor cell proliferation, neutrophilia, and monocytosis, which accelerated atherosclerosis. Thus, a proteoglycan-bound reservoir of apolipoprotein E on hematopoietic stem progenitor cells controls their proliferation and circulating leukocyte numbers. The bone marrow pool of freshly produced neutrophil expresses high levels of CXCR4 and low levels of CXCR2. Hence, CXCL12, the ligand for CXCR4, exerts signals to retain neutrophils in the bone marrow and to induce return of senescent neutrophils to the bone marrow. Disruption of the CXCL12–CXCR4 axis was shown to induce neutrophilia and increased atherosclerotic lesion sizes. In hyperlipidemia, bone marrow concentrations of CXCL12 and expression of CXCR4 on neutrophils are reduced, hence facilitating neutrophil mobilization and impairing neutrophil homing to the bone marrow. In contrast, hypercholesterolemia increases neutrophilic CXCR2 expression and the circulating levels of its ligand CXCL1, thus promoting release of neutrophils from the bone marrow. Atherosclerotic mice lacking leukocytic CXCR2 not only exhibit neutropenia but also display smaller atherosclerotic lesions. Collectively, hyperlipidemia disturbs the tightly regulated cytokine system controlling neutrophil homeostasis at various levels, ultimately increasing peripheral neutrophil counts.

Role of Neutrophils During Initiation and Progression of Atherosclerosis
Atherogenesis indicates the development of atheromatous plaques in the intima of large arteries. Based on observations from animal experiments and human specimens, dysfunction of endothelial cells covering the luminal arterial surface is considered the very initial stage of atherosclerosis. Endothelial dysfunction is triggered by exposure to irritative stimuli such as hyperlipidemia, high shear forces, and proinflammatory cytokines. Functionally, this leads to expression of endothelial cell adhesion molecules (eg, E-selectin, P-selectin, intercellular adhesion molecule-1), which trigger capture and adhesion of leukocytes. Simultaneous changes in endothelial permeability and the composition of the extracellular matrix beneath the endothelium promote entry and retention of cholesterol-rich low-density lipoprotein (LDL) in the arterial wall. Biochemically modified LDL, namely oxidized LDL, enhances recruitment of leukocytes, many of which are monocytes. These differentiate toward macrophages, which engulf oxidized LDL, thus forming foam cells. In both humans and mice, two subsets of monocytes exist and there is accumulating evidence that hyperlipidemia-mediated monocytopoiesis is restricted to classical (inflammatory, Gr1+) rather than nonclassical (resident, Gr1–) monocytes and that classical monocytes preferentially give rise to foam cells. Among plaque macrophages, the so-called M1 phenotype may prevail. This type of macrophage is characterized by expression of high levels of proinflammatory cytokines such as tumor necrosis factor and IL-1β.

Recent data from mouse models of atherosclerosis indicate that neutrophils accumulate in large arteries soon after commencement of high-fat diet feeding. Lesion sizes positively correlate with circulating neutrophils counts, and depletion of neutrophils with subsequent reduction in lesion sizes provide conclusive evidence for the role of neutrophils at early time points of atherosclerosis. Mechanistic insights from macrovascular models are, at this point, scarce, so that many of the following explanations are extrapolated from microvascular models of inflammation. In addition, alterations in neutrophil phenotype and homeostasis in response to primary risk factors of atherosclerosis in humans paralleling observations from mouse models suggest that neutrophils also may be important in early phases of human atherosclerosis.

Mechanisms of Arterial Neutrophil Recruitment
Several reports indicate that neutrophils are responsible for the majority of transient interactions between leukocytes and endothelial cells covering atherosclerotic lesions. Along with the detection of neutrophils in atherosclerotic lesions, the question rises how neutrophils enter atherosclerotic lesions. Observations from intravital fluorescence microscopy showing adhesion of neutrophils to atherosclerotic lesions and subsequent migration suggest a transluminal route. However, recent work indicates that myeloid cells also may infiltrate large arteries via adventitial or intimal microvessels. There are very few studies available investigating molecular mechanisms of arterial neutrophil infiltration, so that much of the subsequent section is extrapolated from microvascular recruitment models. Although the classical leukocyte recruitment cascade was refined over the past couple of years, much of the original paradigm still holds true. Neutrophils are captured by engagement of selectins such as P-selectin and E-selectin. Mice deficient of P-selectin...
displayed lower macrophage numbers in the plaque and had development of smaller fatty streaks.69 Similar effects on plaque development were observed in E-selectin–deficient mice.70 Because the majority of selectin-mediated transient leukocyte endothelial contacts in atherosclerosis is attributable to neutrophils, it appears likely that some of the protective effect in mice deficient in E-selectin and P-selectin is attributable to impaired arterial neutrophil infiltration.65

After capture, neutrophils roll on the endothelium where they sense chemotactic agents that mediate integrin activation, thus preparing the cell for firm arrest. Classical chemokines are produced in the atherosclerotic lesion or seeded onto the endothelium by cells interacting with the endothelium, such as the platelet. The latter is an important source for C-C motif chemokine ligand (CCL)5, which binds to CCR1 and CCR5 on neutrophils mediating firm adhesion (Figure 2).22 In addition, CXCR2 and CCR2 also mediate neutrophil adhesion to carotid arteries.22 The lipid mediator leukotriene B4 and its high-affinity receptor BLT1 form an important axis for neutrophil activation and recruitment. Deficiency of leukotriene B4 or BLT1 results in clear reduction of atherosclerotic lesion sizes and decreases in lesional macrophage, T-cell, and smooth muscle cell numbers.71,72 In addition, complement compound C5a is a potent chemoattractant for neutrophils. Treatment of atherosclerosis mice receiving high-fat diet with an antagonist to C5a receptor, CD88, results in diminished lesion sizes and reduced lesional lipid accumulation,73 suggesting that cells expressing CD88 promote lesion formation.

Furthermore, endothelial dysfunction during early stages of atherosclerosis is characterized by upregulation of intercellular adhesion molecule-1, which typically mediates neutrophil adhesion in the microcirculation. The vast reduction of atherosclerotic lesions in mice lacking the common \(\beta_2\)-integrin chain CD1874 or its counter-receptor intercellular adhesion molecule-1 \(\delta\) may, at least in part, be attributed to reduced neutrophil extravasation.

**Neutrophils Aggravate Endothelial Dysfunction**

Endothelial dysfunction is characterized by reduced vasodilation and a proinflammatory state displayed as enhanced expression of adhesion molecules and chemokines and, finally, increased leakiness.61 Cardiovascular risk factors such as hyperlipidemia trigger endothelial dysfunction. However, increases in lipid levels not only activate the endothelium but also prime neutro-
Neutrophils Granule Proteins Lure Monocytes Into Atherosclerotic Lesions

Monocytes and monocyte-derived macrophages are abundant in the atherosclerotic plaque and their importance in atherosclerosis is widely acknowledged. Recruitment of monocytes to atherosclerotic lesions is a complex process subject to modification by many soluble mediators with chemotactic activity, including chemokines, activated complement components, and lipid mediators. Analyses of cellular composition of enzymatically digested aortas from neutropenic mice and mice with intact white blood cell counts fed a high-fat diet indicate reduced numbers of classical monocytes and macrophages in the aortas of neutropenic mice, suggesting that neutrophils contribute to lesional accumulation of classical monocytes and their descendants. Although underlying mechanisms have not been identified in the context of atherosclerosis, this has been performed in microvascular models of inflammation. Neutrophil-borne granule proteins hold a central role in neutrophil-dependent monocyte recruitment, wherein granule proteins lacking proteolytic activity exert direct chemotactic activity (Figure 2). LL-37, azurocidin, cathepsin G, and α-defensins were demonstrated to be chemotactic for human and murine monocytes. The relevance of such events in microvascular recruitment in humans becomes evident in patients with specific granule deficiency. These patients lack granule proteins such as α-defensins and LL-37, thus resulting in decreased recruitment of monocytes to skin blister chambers. Interestingly, this defect can be rescued by addition of the supernatant of activated neutrophils from healthy donors, supporting the role of neutrophil granule proteins in monocyte recruitment. PTx sensitivity of these events points at the involvement of G-protein-coupled receptors. Further research elucidated that cathepsin G activates human FPR1 whereas LL-37 acts via FPR2. Also, azurocidin was shown to induce monocyte extravasation via FPRs. In addition to its abilities to attract monocytes, azurocidin is deposited on the endothelium by activated neutrophils, thereby stimulating monocyte adhesion (Figure 2). Through stimulation of chemokine release from monocytes and macrophages, azurocidin and related granule proteins further favor the extravasation of classical monocytes. In contrast to these mechanisms, neutrophil-derived serine proteases (eg, proteinase-3, cathepsin G, neutrophil elastase) act through activation of chemokine proforms. Many cytokines and chemokines and their respective receptors contain putative cleavage sites for neutrophil serine proteases. It therefore is not surprising that many receptors, cytokines, and other molecules have been found to be natural substrates for neutrophil serine proteases. For example, N-terminal cleavage of IL-8 by proteinase-3 and epithelial cell-derived neutrophil-activating protein-78 by cathepsin G release truncated forms of these chemokines that have higher chemotactic activity than the full-length molecules. Similarly, N-terminal modification of MIP-1β (CCL15) by cathepsin G increases its monocyte chemotactic activity many-fold. Recently, it has been shown that activation of chemerin, which is known to attract antigen-presenting cells, can be mediated by neutrophil elastase and cathepsin G through the proteolytic removal of a C-terminal peptide.
Neutrophils Activate Macrophages and Promote Foam Cell Formation

Macrophages are important players throughout all stages of atherosclerosis and macrophages with a proinflammatory M1 phenotype may prevail during later stages of atherosclerosis. These cells are characterized by surface expression of costimulatory molecules (CD40, CD80, CD86), release of proinflammatory cytokines (IL-6, tumor necrosis factor), and expression of reactive oxygen species. Data from studies investigating the relationship between neutrophil degranulation and macrophage maturation suggest that neutrophil secretory products promote a shift toward the M1 macrophage phenotype. For instance, neutrophil-specific granule deficiency, which is characterized by lack of granule proteins such as α-defensins and LL37, exhibits an obvious defect in macrophage maturation, migratory capacity, cytokine gene expression, and phagocytosis in both humans and mice. Using a deductive approach, it was shown that the supernatant of activated neutrophils stimulates formation of reactive oxygen species in macrophages and macrophage phagocytic capacity changes typical for M1 macrophages (Figure 2). Functionally, this phenotypic change led to enhanced phagocytic capacities. Azurocidin and α-defensins were identified as active agents in the supernatant of stimulated neutrophils, of which azurocidin activates macrophages through β3-integrins. Treatment with either protein results in the release of tumor necrosis factor and in lower amounts of interferon-γ from macrophages, which in turn activate macrophages in an autocrine loop to express M1 signature proteins. A similar inter-relation was reproduced in vivo. In a mouse peritonitis model, the phagocytic capacity of macrophages from neutropenic mice is reduced. The majority of the compromised phagocytic capacity can, however, be rescued by local application of azurocidin or α-defensins. Although these data were shown for the phagocytosis of bacteria, they may also hold true for particles relevant to atherosclerosis, such as oxidized LDL. In this context, it has been shown that peritoneal macrophages from mice with neutrophil-specific granule deficiency display reduced scavenger receptors expression along with a diminished ability to accumulate oxidized LDL. Increased expression of scavenger receptors on macrophages after exposure to α-defensins may serve as a mechanistic explanation herein (Figure 2). In addition, neutrophil-borne LL37, which is also lacking in patients and in mice with neutrophil-specific granule deficiency, exhibits similar effects on macrophage phenotype changes as azurocidin and α-defensins, allowing speculation that LL37 contributes to defective foam cell formation in mice with neutrophil-specific granule deficiency. However, α-defensins may aggravate foam cell formation by mechanisms other than stimulation of scavenger receptor expression, namely the interference with extravascular lipid accumulation (Figure 2). Alpha-defensins promote binding of lipoprotein (a) to endothelial cells and smooth muscle cells. Internalization of lipoprotein (a) in these cells is increased in presence of α-defensins; however, it fails to be degraded, thus resulting in intracellular lipoprotein accumulation. Moreover, stable complexes composed of α-defensins and lipoprotein (a) or LDL cannot traverse the endothelial barrier, resulting in lesional lipid accumulation. Activation of endothelial cells and macrophages by α-defensins results in release of reactive oxygen species, which oxidatively modify LDL trapped in the vessel wall. Similarly, oxygen radicals formed via MPO modify apolipoprotein B, induce lipid oxidation, and deplete antioxidants, altogether increasing lipid accumulation within atherosclerotic lesion.

Contribution of Neutrophils to Plaque Destabilization

Rather than a progressive stenosis to a chronically flow-limiting lesion, it has been established that thrombotic complications result from physical disruption of the atherosclerotic plaque. The two major mechanisms involved in atherothrombosis are rupture of the fibrous cap and superficial erosion of the endothelial monolayer. Superficial erosions are related to endothelial cell apoptosis and subsequent desquamation from the plaque surface, whereas plaque rupture is based on the weakening of the fibrous cap. In both instances, reactive oxygen species, protease activity, and cell death are important facilitators of atherothrombosis. Because neutrophils contain large amounts of matrix-degrading proteases, produce vast amounts of oxygen species, and rapidly undergo apoptosis, it is not unlikely that neutrophils promote plaque rupture or erosion. Experimental data indicate that neutrophils infiltrate atherosclerotic arteries not only during early stages but also during later stages, when they were shown to primarily locate in highly inflamed areas. In addition, the accumulation of neutrophils in human atherosclerotic plaques associates with characteristics of rupture-prone lesions and specimens from ruptured or eroded human plaques show distinct infiltrations with neutrophils. Although analyses of human plaque specimens point toward a contributory role of neutrophils in plaque destabilization, direct clinical or experimental evidence is, so far, scarce. Mechanisms by which neutrophils may contribute to plaque destabilization as detailed in the following paragraph are primarily derived from microvascular models of inflammation and require strict experimental confirmation in large arteries. In addition, such mechanisms call for validation in human tissue specimens.

Neutrophil-Derived Reactive Species and Proteases May Promote Endothelial Erosion

Superficial erosion of endothelial cells is attributed to endothelial cell apoptosis and subsequent desquamation. Although reactive oxygen species may be important in the first process, MMP activity relates to the latter (Figure 3). MPO, an enzyme stored in neutrophil primary granules, is released on neutrophil activation by stimuli relevant to atherosclerosis. When released, MPO binds to extracellular matrix and converts chloride anion plus hydrogen peroxide to hypochlorous acid, a potent oxidant and chlorinating species. There is immunohistochemical evidence for the presence of MPO and hypochlorous acid-modified proteins in plaques that have ruptured or undergone superficial erosion. Further work revealed that hypochlorous acid at concentrations relevant to sites of inflammation induces apoptosis in endothelial cells. MPO may further promote survival of neutrophils by ligation of CD11b/CD18, thereby prolonging inflammation. In addition, a recent study points at the importance of neutrophils as a source of reactive oxygen species in vulnerable plaques. Matrix-degrading proteases participate in the degradation of the basement membrane. MMP-2 and MMP-9, which are abundant in neutrophil secondary and tertiary
granules, attack type IV collagen, which is an important member of the subendothelial basement membrane. Hence, neutrophils not only may promote endothelial cell death through radical species but also may favor desquamation of the endothelium by release of MMP-2.

Neutrophils Contribute to Weakening of the Fibrous Cap

The stability of the fibrous cap depends on the balance of synthesis and catabolism of extracellular matrix components. Although little is known about potential mechanisms of neutrophil-mediated interference with matrix synthesis, neutrophils may prominently contribute to matrix degradation through release of proteases (Figure 3). Intraplaque hemorrhage accelerates atherothrombosis,132 and analysis of human carotid endarterectomy samples suggests intraplaque hemorrhage as one entry route for neutrophils into lesions.28 Neutrophils recruited in this way release proteinase-3, neutrophil elastase, MMP-2, and MMP-9.28 In an experimental model of plaque destabilization, the release of neutrophilic MMP-8 and reactive oxygen species was evidenced leading to endothelial cell apoptosis and desquamation.133 Thus, the aforementioned proteases may contribute to degradation of extracellular matrix components. In addition, these proteases exert additional effects that may aggravate plaque destabilization. For example, MMP-9 cleaves and increases the chemotactic activity of CXCL1 and CXCL8, thus amplifying leukocyte tissue infiltration.134 The secretion of MMP-8 is a nonredundant mechanism for cleaving CXCL5 and CXCL8, both of which are major ligands for the neutrophil receptor CXCR2.135 Consequently, chemokine modification by proteases serves as a feed-forward mechanism to promote the initial neutrophil extravasation. In addition, neutrophil elastase activates MMP-9,28,136 hence potentiating its activity, whereas proteinase-3 potently induces endothelial cell apoptosis.137 The importance of proteases in plaque destabilization was repeatedly shown in mouse models of atherosclerosis. Overexpression of auto-activatable pro-MMP-9 greatly reduced plaque stability.138 Similarly, adenovirus-mediated overexpression of MMP-9 disrupted advanced plaques caused by collar implantation.139 In knockout models, the importance of MMP-9 and MMP-8 in lesional matrix degradation was further corroborated.140,141 Surprisingly, mice lacking MMP-2 exhibited an adverse phenotype, which is likely because of reduced smooth muscle cell accumulation and thus is a less stable plaque.142 Although the overall importance of proteases in plaque stabilization is established, the cellular source of these proteins is unclear. There is a large body of data indicating that they primarily derive from macrophages despite the abundance of these proteases in neutrophils.28 However, recent data imply not only that neutrophils secrete proteases within atherosclerotic lesions28

**Figure 3.** Possible mechanisms of neutrophil-driven plaque destabilization. **A**, Myeloperoxidase-dependent oxidative stress and neutrophil-derived matrix metalloproteinases may induce apoptosis in endothelial cells, degradation of the basement membrane, and subsequent endothelial cell desquamation. **B**, Neutrophil-derived matrix metalloproteinases cleave components of the extracellular matrix. **C**, Neutrophils undergo apoptosis and secondary necrosis, which possibly contributes to necrotic core formation. ECM, extracellular matrix; MMP, matrix metalloproteinase; MPO, myeloperoxidase. Illustration credit: Cosmocyte/Ben Smith.
but also that this process can be targeted. In an experimental mouse model, treatment with fluvastatin decreases lesional MMP-9 expression and gelatinolytic activity but increases collagen contents. This is associated with decreases in neutrophil infiltration suggesting a causal role of neutrophils in matrix degradation.

An additional critical feature of unstable plaques is the necrotic core, which contributes to inflammation, thrombosis, proteolytic plaque breakdown, and physical stress on the fibrous cap. Necrotic cores arise from apoptotic cells that are not cleared by macrophages and subsequently undergo secondary necrosis. Smooth muscle cells and macrophages are considered the most frequent source for necrotic cells in advanced atherosclerotic lesions. However, with the recently detected accumulation of neutrophils in advanced human and murine lesions, it is possible that neutrophils may be a major source for apoptotic and necrotic cells in atherosclerosis (Figure 3).

Neutrophils Are Protective in Neointima Formation
Restenosis resulting from neointimal hyperplasia, negative remodeling, and elastic recoil is considered the Achilles heel of interventional cardiology. In animal models of acute vascular injury, such as balloon denudation and wire injury, neutrophils rapidly infiltrate large arteries and disappear after a couple of days. However, the role of neutrophils in neointima formation has been unclear until recently. Lack of $\beta_2$-integrins and P-selectin also are relevant to the recruitment of monocytes, platelets, and progenitor cells, so that deletion of these adhesion molecules does not allow for a precise or exclusive identification of a cellular but rather a molecular target. It was shown that the importance of P-selectin in neointima formation is restricted to platelets. In contrast, blocking mCXCL1 with a neutralizing antibody or deletion of CXCR2, thus targeting neutrophil recruitment to sites of injury, increases neointima formation concomitant with a decrease in endothelial recovery, suggesting a beneficial role of neutrophil during neointima formation. In agreement with this notion, increases in circulating neutrophil counts after granulocyte colony-stimulating factor application reduces neointimal lesions sizes and accelerates re-endothelialization. More direct evidence for the beneficial role of neutrophils in neointima formation comes from a recent study showing decreased re-endothelialization and increased neointima sizes after wire injury in mice rendered neutropenic. Healing after vascular injury is thought to be regulated by circulating endothelial progenitor cells, which limit neointima formation by accelerating re-endothelialization. Hence, a recent study indicates connection between neutrophils, homing of endothelial progenitor cells, and endothelial recovery. LL37 is deposited at the site of arterial injury by activated neutrophils. In this location, LL37 activates circulating endothelial progenitor cells, which home to the site of injury (Figure 4). In the inflammatory environment, LL37 protects endothelial progenitor cells from apoptosis and stimu-
lates release of proangiogenic growth factors, thus promoting endothelial regrowth in a paracrine fashion (Figure 4). This knowledge was translated into an animal model using a neutrophil-instructing biofunctionalized miniaturized stent coated with LL37. This stent reduced in-stent stenosis in a mouse model of atherosclerosis, suggesting that LL37 may promote vascular healing after interventional therapy.

**Perspectives**

Clearly, the addition of the neutrophil as a previously not fully appreciated player in atherosclerosis increases the complexity of cellular interactions in disease pathogenesis, but also harbors valuable strategies for prevention and treatment. Interfering with leukocyte accumulation in atherosclerosis has focused on inhibiting leukocyte recruitment into large arteries. Cell adhesion molecules and chemokines may stand out as possible targets during arterial leukocyte recruitment, providing such interference would not impair host defense. With the emerging notion of the importance of highly specific leukocyte–platelet or leukocyte–endothelial cell interactions in leukocyte recruitment during arterial inflammation, such may become feasible targets. For example, the binding sites for CD40L and CD11b were recently characterized and a specific peptide inhibitor led to reduced atherosclerotic lesion formation and arterial leukocyte infiltration. Chemokines and their receptors may offer another target for treatment of leukocyte recruitment to atherosclerotic lesions. In this context, the recent identification of the importance of platelet-derived CCL5 in adhesion and recruitment of neutrophils to atherosclerotic arteries may prove to be a relevant target. The applicability of such an approach is further strengthened by the CCL5 independence of neutrophil extravasation in models of microvascular inflammation. To limit neutrophil accumulation within atherosclerotic lesions, recent work may shift attention toward the control of leukocyte production and mobilization from the bone marrow.

Treatment with cholesterol-poor phospholipid/apolipoprotein A-I complexes as cholesterol apheresis may act through alternative pathways to limit neutrophil accumulation within atherosclerotic lesions. Recent work may shift attention toward the control of leukocyte production and mobilization from the bone marrow. Treatment with cholesterol-poor phospholipid/apolipoprotein A-I complexes as cholesterol apheresis may act through alternative pathways to limit neutrophil accumulation within atherosclerotic lesions. Recent work may shift attention toward the control of leukocyte production and mobilization from the bone marrow.

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None.

**References**


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