Neutrophils: emerging role in the immunopathology of atherosclerosis
Hartwig, H.

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Neutrophil cell death is associated with features of instability in human atherosclerotic plaques

**Helene Hartwig**, Onno J. de Boer, Allard C. van der Wal, Claire Mackaaij, Carlos Silvestre-Roig, Mat J.A.P. Daemen, Esther Lutgens, Oliver Soehnlein

**Contribution Helene Hartwig**: performed the experiments, analyzed the data and wrote the paper.

In progress
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Background

Atherosclerosis is a chronic inflammatory disease characterized by neointimal lesion formation and luminal narrowing of arteries. This multifactorial inflammatory process results in cerebrovascular and cardiovascular complications associated with high morbidity and mortality. Considering the heightened recognition of the role of neutrophils in atherosclerosis, their continued recruitment throughout all plaque stages and the impact of apoptotic neutrophils in inflammation control, it is likely that targeting of atherosclerosis may be facilitated by interference with neutrophil cell death pathways and clearance of dead neutrophils. Here we studied the nature of neutrophil fate in the different stages of atherosclerosis.

Methods and Results

We performed immunohistochemical analysis of carotid endarterectomy specimens that were classified in early, advanced and complicated lesions. The plaques were stained for the neutrophil specific marker CD177. Additionally, staining for different cell death pathways such as NETosis (anti-citrullinated H3), apoptosis (anti-Caspase3), necrosis (TUNEL+) and autophagy (anti-ATG5) were performed. Increased NETosis, necrosis and autophagy were observed in advanced and complicated plaques as compared to early lesions. Furthermore we could show that inflammatory neutrophil cell death pathways (apoptosis, necrosis, NETosis) correlate with parameters of plaque destabilization such as enlargement of the core and the thinning of the fibrous cap.

Conclusion

We here present a study of the different neutrophil cell death pathways within human atherosclerotic plaques. These results demonstrate that the lesional prevalence of dead neutrophils, in contrast to that of living neutrophils, is strongly associated with traits of plaque instability. These findings may contribute to a better understanding of the role of neutrophils in the progression of atherosclerosis and may allow for design of strategies to interfere with neutrophil apoptosis to halt atherogenesis.
Introduction

Atherosclerosis is considered a chronic inflammation resulting from continued infiltration of lipids and inflammatory cells into the arterial intima leading to formation of atherosclerotic plaques. As atherosclerosis is a dynamic process, early lesions may progress to advanced and finally rupture-prone vulnerable plaque. Vulnerable plaques are characterized by high inflammatory cell content and a large necrotic core (NC), which is covered by a thin fibrous cap (1).

While monocytes and macrophages have dominated the discussion of plaque destabilization over the last decade, recently neutrophils have been introduced into this discussion (2-4). Mechanistically, neutrophils regulate inflammatory processes through release of pre-formed granule proteins or reactive oxygen species (ROS). However, as neutrophils are short-lived cells and may undergo different cell deaths pathways (e.g. apoptosis, autophagy, NETosis) immune-modulatory properties of dead neutrophils possibly stand out as important regulatory mechanisms in atherosclerosis. While apoptosis in early stages of atherosclerosis is considered beneficial, it is fatal at late stages as the uptake of dead cells by macrophages (a.k.a. efferocytosis) is impaired. Hence, apoptotic neutrophils undergo secondary necrosis and are thought to contribute to the pro-inflammatory phenotype and plaque destabilization.

Additionally, epidemiological studies revealed an association between circulating neutrophil counts and the incidence of coronary heart disease (5, 6). Furthermore, neutrophil counts were strongly associated with histopathologic features of rupture-prone plaques, i.e. large lipid core, increased macrophage influx, low collagen and smooth muscle cell content (7). Naruko et al. observed neutrophils in culprit lesions of patients who died of acute myocardial infarction (8). Thus, observations of human studies show a positive correlation between increased neutrophil counts in both blood and atherosclerotic plaques, and cardiovascular risk. In animal studies neutrophils are the dominant cell type to be recruited during advanced atherosclerosis where they co-localize with macrophages in the shoulder region of the plaque indicative of the potential importance of neutrophils during plaque destabilization (9, 10).

In this study, we determined the frequency and location of neutrophils at different stages of human atherosclerosis. Furthermore, we identified the different types of cell death that neutrophils undergo and have correlated these with plaque characteristics.
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Material and Methods

Human Carotid Endarterectomy Specimens and Tissue Processing

Carotid endarterectomy (CEA) specimens (n=79) were obtained from the vascular surgery department of the Academic Medical Center in Amsterdam. Approval was given by the local Ethical Committee. Immediately after removal, specimens were fixed in 10% formalin and processed for paraffin embedding.

Histological and Immunofluorescence staining

To determine the plaque phenotype consecutive sections (4μm) were stained with standard hematoxylin and eosin (H&E) and elastin von Gieson (EVG).

For neutrophil visualization, sections were boiled in citrate buffer (pH6.0, Lab Vision, Fremont, USA) and blocked with Lab Vision™ Ultra V-Block (Thermo Scientific, Fremont, USA). Next, specimens were incubated with monoclonal mouse anti-human CD177 (dilution: 1:2000; Abnova, Taiwan) antibody. Alkaline phosphatase-conjugated goat anti-mouse IgG2a (dilution: 1:50; Southern Biotech, Birmingham, USA) was used as secondary antibody and visualized by Vector® Blue Substrate (Vector Laboratories, Burlingame, USA).

In addition, sections were stained for apoptosis using rabbit Cleaved Caspase3 antibody (dilution: 1:5000; Cell Signaling Technology®, Danvers, USA) using BrightVision poly-horseradish peroxidase-anti-mouse IgG (Immunologic BV, Duiven, The Netherlands) as secondary antibody. Furthermore, staining for autophagy was performed using monoclonal mouse anti-human ATG5 (R&D Systems, Minneapolis, USA) antibody. Horseradish peroxidase-conjugated goat anti-mouse IgG2B (dilution: 1:100; Southern Biotech, Birmingham, USA) was used as secondary antibody. Both stainings were visualized by ImmPACT™ AMEC Red Substrate (Vector Laboratories, Burlingame, USA).

NETosis was shown by staining of neutrophils with CD177 (as described above) using BrightVision poly-horseradish peroxidase-anti-mouse IgG (Immunologic BV, Duiven, The Netherlands) as secondary antibody and visualized by dianminobenzidine. In addition, citrullinated histones were detected via anti-histone H3 antibody (dilution: 1:4000; Abcam, Cambridge, UK). Sections were counterstained with nuclear red.

Necrotic neutrophils were visualized by CD177 (as described above) using Alexa Fluor 647 goat anti-mouse IgG2a (Life Technologies, Bleiswijk, The
Netherlands) as secondary antibody in combination with ApopTag® Red In Situ Apoptosis Detection Kit (Merck Millipore, Darmstadt, Germany). Sections were counterstained with DAPI (Invitrogen, Breda, The Netherlands). Images were acquired with a laser microscope (TCS/SP8, Leica, Wetzlar, Germany) or Philips Scanner (Philips, Best, The Netherlands).

**Atherosclerotic Lesion Analysis**

The H&E and EVG stained sections were scored blinded by two independent, experienced pathologists (M.J.A.P. Daemen and E. Lutgens) with little inter- and intra-observer variability. Plaques were classified as early, advanced and complicated lesions regarding Virmani histopathological classification (11). Next, advanced and complicated lesions were divided into regions of FC, shoulders and core using ImageJ software (National Institutes of Health, Bethesda, MD). Due to variations of the fibrous cap thickness (FCT), the values were assessed by the average of the thickness in 5 separate regions of the cap (one in each shoulder and 3 in equal distance in-between these) and expressed as arbitrary ratio units (AU) of FCT:lesion size (FCT in μm and lesion size area in μm²).

**Statistical Analysis**

Data were represented as mean values ± SD. Statistical analysis of the correlations was done with GraphPad Prism 6 (GraphPad Software, LaJolla, CA). D’Agostino Pearson correlation test was applied. p-values < 0.05 were considered as being statistically significant.

**Results**

**Neutrophil distribution within the plaque**

Human neutrophils have been shown to be present not just in early atherosclerotic lesions but also in later stages of atherosclerosis (9, 12). To assess the presence of neutrophils in early, advanced and complicated plaques we stained lesion sections for CD177, a neutrophil specific cell surface receptor (13). We found that neutrophils were present in increasing numbers from early stages of intimal thickening, to advanced lesions to complicated plaques displaying intraplaque
Table 1. Increase of neutrophil presence with increased stage of atherosclerosis.
The presence of neutrophils was determined via CD177 staining in early, advanced and complicated lesions as absolute and per mm² of the whole plaque area. Sections were classified using the Virmani histopathological classification (11). (early n=11; advanced n=45; complicated n=23).

<table>
<thead>
<tr>
<th></th>
<th>CD177+ cells/plaque</th>
<th>Plaque size (mm²)</th>
<th>CD177+ cells/mm² plaque</th>
</tr>
</thead>
<tbody>
<tr>
<td>early lesions</td>
<td>3 (1-8)</td>
<td>7.6 (0.4-23.4)</td>
<td>1 (1-3)</td>
</tr>
<tr>
<td>advanced lesions</td>
<td>6 (1-23)</td>
<td>12.1 (0.3-40.3)</td>
<td>1 (0-9)</td>
</tr>
<tr>
<td>complicated lesions</td>
<td>11 (1-27)</td>
<td>16.6 (0.2-32.2)</td>
<td>2 (1-19)</td>
</tr>
</tbody>
</table>

Figure 1. Stage-dependent distribution of neutrophils in human atherosclerotic plaques. (A) Schematic representation of the shoulder, cap and core areas within an advanced plaque. (B) CD177+ cells were analyzed dependent on their localization within the plaque, whereas advanced and complicated lesions were divided in shoulder, cap and core regions (early n=11; advanced n=45; complicated n=23).
hemorrhages (IPH) (Table 1). To identify the localization of neutrophils within the plaque, advanced and complicated lesions were subdivided in regions of shoulder, cap and core (Figure 1A). The neutrophils were found to reside in the intima during early stages of atherosclerotic lesion formation, while they primarily located in the core region of advanced and complicated lesions (Figure 1B).

**Neutrophil undergo different cell death pathways within the plaque**

With the limited life span of neutrophils we further aimed at identifying the fate of plaque neutrophils. To detect different types of neutrophil cell death within the plaque, lesions were stained for CD177 and an antibody to ATG5 (to identify autophagy), an antibody to Caspase 3 (to identify apoptosis), or an antibody to citrullinated H3 (to identify NET release). Necrotic neutrophils were identified by co-staining with TUNEL. We observed that the absolute number of NETing, necrotic and autophagic neutrophils increases with the severity of the lesion stage, while the relative number of apoptotic neutrophils decreased. Interestingly, a high amount of plaque neutrophils (early: 59.1% of all neutrophils, advanced: 56.1% of all neutrophils, complicated: 42.9% of all

![Figure 2](image.png)

**Figure 2. Assessment of cell death pathways of lesional neutrophils.** Quantification of CD177+ cells in combination with markers for different cell death pathways in whole plaque area (early n=11; advanced n=45; complicated n=23) (citr.=citrullinated).
neutrophils) did not stain for any of the cell death markers and were hence considered alive. The relative number of living neutrophils decreased also with the increased stage of atherosclerosis (Figure 2).

**Neutrophil contribution to plaque destabilization**

To assess the possible contribution of neutrophils to plaque destabilization, we correlated the total number of plaque neutrophils, the number of neutrophils not stained by markers of cell death, and the number of neutrophils undergoing

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**Figure 3. Inflammatory neutrophil cell death pathways are associated with features of instability.** Correlation between (A) all CD177+ cells/plaque, (B) living CD177+/plaque and (C) dead CD177+ cells/plaque with plaque size (n=45), core size (n=45) and fibrous cap thickness (FCT) (n=39) in advanced lesions (p < 0.05, p < 0.01, p < 0.001 with Spearman correlation test).
inflammatory cell death pathways (NETosis, apoptosis, necrosis) with parameters dictating lesion stability. Rupture-prone plaques are archetypically characterized by exacerbated infiltration of inflammatory cells in combination with a large core covered by a thin fibrous cap (14). Plaque neutrophil numbers were found to positively correlate with plaque size as well as the core size but not fibrous cap thickness (Figure 3A). Similarly, neutrophils considered alive were found to correlate with plaque and necrotic core sizes but not to fibrous cap thickness (Figure 3B). In contrast, a correlation of inflammatory neutrophil cell death pathways with signs of lesion destabilization revealed a positive correlation with plaque size and necrotic core size and a negative correlation with fibrous cap thickness (Figure 3C).

**Discussion**

Atherosclerosis is a chronic inflammatory disease of the arterial wall and the continuous infiltration of leukocyte subsets is a driving force of plaque growth. While a wide range of functions has been shown for various leukocytes including macrophages, T and B cells, the role of neutrophils in the pathogenesis of atherosclerosis has been underappreciated until very recently. The goal of the present study was to determine the frequency and location of neutrophils at different stages of human atherosclerosis. Moreover, we were interested in the identification of the different cell death pathways neutrophils may undergo and we analyzed their potential contribution to plaque instability.

We used CD177 as a specific human neutrophil marker to show their presence within the plaque. CD177 has been reported to be expressed by activated neutrophils and the platelet endothelial cell adhesion molecule-1 (PECAM-1) was identified as a binding partner (13). We showed that early, advanced and complicated lesions exhibit an increase in absolute neutrophil counts as well as per mm2 of plaque size, indicating that neutrophil infiltration increases with an increasing stage of atherosclerosis. Furthermore, we found that CD177+ cells showed a heterogeneous distribution. Neutrophils were predominantly found in the core of advanced and complicated lesions. Almost 74% of our complicated lesions represented intraplaque hemorrhages, which have been shown to contribute to plaque instability and represent an additional route for neutrophils into the lesion next to diapedesis through the luminal site (15, 16). Of note, the amount of CD177+ cells in the cap and the shoulder region of advanced and complicated lesions did not differ significantly.
In contrast to the controlled process of degranulation, neutrophils can also release their granule content as a result of necrosis due to inefficient efferocytosis. For late stages of atherosclerosis is has been demonstrated that uptake of apoptotic cells via macrophages is impaired (17). When not cleared, apoptotic neutrophils undergo secondary necrosis and release their cell content. This leads to tissue damage and contributes to the necrotic core enlargement and increased inflammation in the plaque. In the present study, we could confirm an increased presence of necrotic neutrophils in the core region, whereas absolute numbers of apoptotic neutrophils did not differ between early and complicated plaques. Furthermore it has been demonstrated that NETosis is an essential cell death neutrophils undergo under inflammatory conditions. NET release represents an alternative way to present neutrophil granule proteins to the extracellular space besides degranulation. Studies have demonstrated that NETs trigger activation of plasmacytoid dendritic cells and macrophages and hereby accelerate atherogenesis in mice (18-20). In line, we observed an increase in NETosis with atherosclerotic plaque progression. Besides apoptosis, necrosis and NETosis we analyzed whether plaque neutrophils exhibited autophagy. Autophagy is considered to maintain cell homeostasis (21). We identified an increase in the amount of neutrophils undergoing autophagy throughout the stages of atherosclerosis. However, the amount of these compared to the neutrophils undergoing inflammatory cell deaths was low. Furthermore, it has been reported, that induced autophagy can promote NETosis (22). The correlation between neutrophils undergoing autophagy and NETosis could not be confirmed (data not shown). Interestingly, the majority of plaque neutrophils did not stain for any of the cell death markers and were hence considered alive. Given the short life span of neutrophils this would point towards a high turnover of neutrophils with frequent de novo recruitment into the atherosclerotic lesions. Thus, despite the overall low abundance of neutrophils in the lesions, life or dead neutrophils could considerably shape plaque destabilization.

As described previously, enlargement of the necrotic core and the thinning of the FC are important signs of plaque instability. Neutrophil granule proteins presented via degranulation or NETosis promote further inflammatory cell recruitment leading to a pro-inflammatory phenotype within the plaque. Moreover it has been demonstrated that neutrophils trigger foam cell formation thus contributing to the progression of atherosclerosis (23, 24). A positive
association was observed in our study between the amount of alive, dead or all neutrophils with the size of the plaque and the size of the core. However, it was more interestingly that dead but not alive neutrophils correlated with the thinning of the FC. Mechanical weakening of the FC can be mediated by numerous neutrophil dependent factors, such as MMPs, myeloperoxidase and reactive oxygen species (8, 12, 25, 26).

Taken together, we could show that neutrophils infiltrate into the plaque throughout all stages of atherosclerosis development. Neutrophils were associated with morphological characteristics of plaque vulnerability as the enlargement of the necrotic core. Once neutrophils are at the site of inflammation they undergo different modes of cell death, such as apoptosis, NETosis, autophagy and necrosis. Interestingly, only the dead neutrophils correlated with the FC thinning, implicating their impact in mechanical weakening of the cap. These findings demonstrate that neutrophils, known for their tissue destructive capabilities, have to be considered as important promoters in the progression of atherosclerosis and destabilization of atherosclerotic plaques.

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CHAPTER 4

References


