Neutrophils: emerging role in the immunopathology of atherosclerosis

Hartwig, H.

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Platelet-derived PF4 reduces neutrophil apoptosis following arterial occlusion

Helene Hartwig*; Maik Drechsler*; Dirk Lievens; Birgit Kramp; Philipp von Hundelshausen; Esther Lutgens; Christian Weber; Yvonne Döring; Oliver Soehnlein
(* Equal contribution)

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

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Arterial occlusion results in rapid activation of circulating cells wherein the increase in neutrophils was shown to be a strong predictor of cardiovascular mortality (1). Previous reports identified a delay in neutrophil apoptosis as an important contributor to neutrophilia upon arterial occlusion (2, 3). In this context it was shown that neutrophils from patients with acute myocardial infarction have a more than 50% reduction in neutrophil apoptosis as compared to healthy controls (2). Further mechanistic experiments suggested that platelet-derived soluble factors may control the delay of neutrophil apoptosis (2). However, as it remains unclear how platelets affect neutrophil apoptosis we here examined the effect of platelets and their secretory products on neutrophil apoptosis.

To study the importance of platelets for neutrophil apoptosis we employed a model of acute hind limb ischaemia as an arterial occlusion model. The femoral artery was ligated and 24 hours (h) later both the calf muscle downstream of the ligated artery and blood were harvested. Blood was collected in EDTA coated tubes via retro-orbital puncture. The muscle was enzymatically digested and the tissue homogenate was passed through a cell strainer. Neutrophils in blood and muscle homogenate were quantified by flow cytometry using antibodies to CD45, CD11b, Gr1, and Ly6G (all eBioscience, San Diego, CA, USA). In this model the number of circulating neutrophils positively correlates with the number of neutrophils in the downstream muscle tissue (Figure 1A) where neutrophils may eventually exert deleterious effects. To test the previously suggested role of platelets in neutrophil apoptosis under conditions of arterial occlusion, mice were depleted of platelets by use of rabbit anti-mouse platelet serum (50 μl i.p. per mouse, Accurate Chemicals, Westbury, NY, USA) (4). Treatment in this way efficiently reduces the number of platelets (ctrl: 45.8x10^8 ± 7.0x10^8; platelet depleted: 1.7x10^8 ± 0.2x10^8). Apoptosis was assessed in blood, spleen, and bone marrow neutrophils based on Annexin V binding (see Suppl. Figure 1). In this setup, lack of platelets enhanced neutrophil apoptosis in the blood and spleen, but not the bone marrow (Figure 1B). Interestingly, platelet depletion in mice without femoral artery ligation had no effect on neutrophil apoptosis in the blood, bone marrow, or spleen (not shown).

Platelets alter neutrophil function by direct interaction or by release of preformed granule contents. Preformed platelet granule proteins alter various neutrophil functions such as adhesion and activation (4, 5) and hence we suspected that platelet granule proteins may also have an impact on neutrophil apoptosis. To test the effect of platelet granule proteins on apoptosis of human neutrophils
we cultured human neutrophils in medium (RPMI1640 supplemented with 10% FCS) for 24 h in the presence of CCL5, PF4, serotonin, or TGFβ. These proteins have an α-granule origin and are known to affect neutrophil functions such as degranulation (PF4, TGFβ) and recruitment (serotonin, CCL5) (4–8). Of the proteins tested only PF4 dose-dependently reduced apoptosis of human neutrophils (Figure 1C). In additional experiments we assessed the time-dependent effect of PF4 (Suppl. Table 1). After isolation neutrophil apoptosis steadily increases over time with a low fraction of apoptotic cells within the first 8 h after isolation. Presence of PF4 significantly delays neutrophil apoptosis at 8, 12, and 24 h after isolation, with the fraction of apoptotic neutrophils in presence of PF4 24 h after isolation being equal to the percentage of apoptotic neutrophils 12 h post isolation in absence of PF4. To further temper the notion of the importance of platelet-borne PF4, we used a second, independent approach. We incubated human neutrophils with the supernatant of platelets activated by vigorous shaking. This supernatant, but not the supernatant of resting platelets significantly reduced neutrophil apoptosis (Figure 1D, Suppl. Figure 2). The apoptosis delay through the supernatant of the resting platelets was comparable to the control where the cells underwent spontaneous apoptosis after 24 h. To test the exclusiveness of PF4 in inhibiting neutrophil apoptosis exerted by platelet secretory products PF4 was immuno-depleted. The efficacy of the depletion was confirmed by dot blot analysis (Figure 1E). Of note, depletion of PF4 rendered the platelet supernatant inactive, thus firmly suggesting the importance of platelet-derived PF4 in the control of neutrophil apoptosis.

**Figure 1**: Platelet-borne PF4 reduces neutrophil apoptosis. A) Correlation of circulating neutrophil counts and neutrophil numbers in the calf muscle following ligation of the common femoral artery (24 h). B) Apoptotic neutrophils in the blood, bone marrow, and spleen 24 h after ligation of the femoral artery. Platelets were depleted by injection of anti-platelet serum (50μl). Apoptotic neutrophils were identified based on SSC properties, antibody staining for CD115, CD11b, Gr1, binding of Annexin V, and uptake of 7-AAD. n=8. *p<0.05. t-test. C) Isolated human neutrophils were incubated with CCL5, PF4, serotonin, or TGFβ at the indicated concentrations for 24 h and neutrophil apoptosis was assessed based on Annexin V and 7-AAD staining properties. Apoptotic neutrophils were identified as Annexin V+/7-AAD-. n=4. *p<0.05 compared to ctrl. Kruskal-Wallis test. D, E) Human neutrophils were incubated with the supernatant of resting or activated platelets. In the latter, PF4 was also immune-depleted. Displayed is the quantification of neutrophil apoptosis (D). Depletion efficacy was confirmed by dot blot analysis (E). n=6. *p<0.05. Kruskal-Wallis test. plts, platelets; rPF4, recombinant PF4. F) Anti-PF4 treatment promotes neutrophil survival in the circulation. Mice were treated with an anti-PF4 antibody (i.v., perioperative) or an irrelevant IgG and neutrophil apoptosis was assessed by Annexin V binding. n=7. *p<0.05. Kruskal-Wallis test.
PF4 Reduces Neutrophil Apoptosis

of neutrophil apoptosis. To further link these in vitro findings with our in vivo observation we tested the effect of an anti-PF4 antibody (clone 140910, R&D Systems, Minneapolis, MN, USA; 10 μg/mouse, i.v., perioperative) on the apoptosis of circulating neutrophils following ligation of the femoral artery. In comparison to an irrelevant IgG, the PF4 targeting antibody significantly increased Annexin V binding of circulating neutrophils (Figure 1F). Interestingly, this degree of
increase of neutrophil apoptosis was similar to that found after global platelet depletion suggesting that platelet-dependent inhibition of neutrophil apoptosis is largely PF4-mediated.

PF4 is one of the highest expressed proteins in platelets and stored in the \( \alpha \)-granules, secretory organelles that are packed with proteins (6, 9). Various conditions and factors are able to activate platelets resulting in aggregation and \( \alpha \)-granule release at the same time. Sudden arterial occlusion induces ischemic tissue which in turn provokes platelet activation by several factors including the release of metabolites, mediators and coagulation factors such as ADP, tissue factor and thrombin or by dysfunctional endothelium leading to platelet adhesion (10). Here, we identify the importance of platelet-borne PF4 in inhibition of neutrophil apoptosis, whereas alternative platelet granule proteins such as CCL5, serotonin, and TGF\( \beta \) appear less important. Neutrophil apoptosis represents a control mechanism limiting the toxic potential of these short-lived cells. For example, neutrophils quickly infiltrate the infarcted myocardium and contribute to extensive tissue damage (11). In fact, therapeutic induction of neutrophil apoptosis has proven beneficial in various models of acute inflammatory responses (12, 13). Hence, the here identified importance of platelet-derived PF4 in reducing neutrophil apoptosis may be an interesting target for limiting neutrophil life span, and thereby their tissue damaging properties.

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References


Supplementary Material

Suppl. Figure 1: FACS-based identification of apoptotic neutrophils in blood or spleen. Blood or spleen homogenate was labeled with antibodies to CD45, CD11b, Gr1, and Ly6G and Annexin V was added to identify phosphatidylserine-presenting apoptotic cells.

Suppl. Figure 2: Supernatant of activated platelets inhibits neutrophil apoptosis. Human neutrophils were incubated with the supernatant of resting or activated platelets. In the latter, PF4 was also immune-depleted. Displayed are representative FACS blots.
Suppl. Table 1: Time course of the effect of PF4 on neutrophil apoptosis.
Neutrophils were isolated and cultured for indicated time points in RPMI1640 supplemented with 10% FCS. PF4 (1μg/ml) or vehicle control were added at time point 0 h and neutrophil apoptosis was assessed by flow cytometry. n = 4. * indicates significant difference from ctrl treatment.

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<th>Time</th>
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<tr>
<td>0 h</td>
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<td>1.43 ± 0.22</td>
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<td>12 h</td>
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<td>26.43 ± 21.1*</td>
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<td>24 h</td>
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