Effects of Thrombocytopenia and Shear Rate on Neutrophil and Platelet Deposition on Endothelial and Medial Arterial Surfaces

Yahye Merhi, Jules Y.T. Lam, Lucie L. Lacoste, Jean-Gilles Latour, Robert Guidoin, and David Waters

Arterial wall injury in vivo is associated with localized platelet deposition and vasoconstriction and initiates a sequence of pathological changes that favor the development of atherosclerosis and restenosis. Antiplatelet agents only partially inhibit the platelet-thrombus formation at the site of injury and do not reduce the incidence of restenosis. Several types of cells and several metabolic pathways appear to be involved in thrombosis and the pathological changes that follow arterial wall injury. Mounting evidence suggests that leukocytes, mainly neutrophils, may play an important role in the pathophysiology of ischemic heart disease. However, their contribution to the thrombotic process is unclear. Neutrophils may influence thrombosis by different mechanisms, such as by the release of biologically active substances and by interacting with platelets on the vessel wall. Leukocyte-derived products may be prothrombotic by activating platelet deposition and aggregation. Conversely, substances released after platelet activation can stimulate neutrophil activation and adhesion.

Whereas platelet adhesion to the subendothelium and media and neutrophil adhesion to the endothelium are relatively well understood, the interaction of platelets and neutrophils is now being recognized and how neutrophils interact with the subendothelium and media is still unclear. Whether this interaction is modulated by or occurs independently of platelets is unknown. This study was thus undertaken to assess the interactions of platelets and neutrophils with intact and injured arterial surfaces in ex vivo superfusion chambers under controlled shear rates in normal and thrombocytopenic pigs.

Methods

Animal Preparation

Normal Yorkshire pigs (13-16 kg) obtained from MacDonald College (Montreal, Canada) were anesthetized by an intramuscular injection of 200 mg ketamine (Rogarsetic, Rogar/STB Inc) and 120 mg azaperone (Stresnil, Janssen Pharmaceutica). The pigs were intu-
bated, ventilated mechanically with ambient air, and maintained in an anesthetized state with 0.5% halothane (Fluothane, Ayerst). The electrocardiogram was monitored continuously during the experiment. Catheters introduced into the femoral artery and vein were connected to the ex vivo superfusion flow chambers.

**Experimental Design**

Nonanticoagulated blood was drawn from the femoral artery by means of a peristaltic pump (Cole-Parmer Instrument Co) into 2-mm-internal-diameter flow chambers containing the aortic strips with intact endothelium or media. The cylindrical superfusion Plexiglas chambers were designed to mimic the tube-like shape of the vascular system. They contained a window (2×25 mm) that permitted direct exposure of the endothelium or the media of an aortic strip to flowing blood in the chamber. The aortic media was prepared by lifting and peeling the intima, together with a thin portion of the subjacent media, after longitudinally opening aortas harvested from normal pigs. The exposed media with underlying adventitia was then divided into 35×15-mm strips that were placed inside the superfusion chambers. Strips exposing aortic endothelium were obtained in a similar manner but without removing the intima. For each animal, two aortic strips were used for each superfusion experiment. A duplicate and occasionally a triplicate superfusion experiment was performed in each animal. The mean data of all animals were then recorded for each superfusion time of 3, 5, or 10 minutes and for each shear rate of 427, 853, or 1280 s⁻¹.

Under basal conditions, in vitro neutrophil deposition was determined in eight pigs after 3, 5, and 10 minutes' superfusion at constant blood flow and a shear rate of 853 s⁻¹. Under the same conditions, ⁵¹Cr-platelet deposition in five pigs and simultaneous ¹¹¹In-neutrophil deposition in 18 pigs were measured after 5 minutes' superfusion at blood flow rates of 20, 40, and 60 mL/min, corresponding to shear rates of 427, 853, and 1280 s⁻¹, respectively. In vitro platelet aggregation with ADP and neutrophil aggregation with N-formyl-methionyl-leucyl-phenylalanine (fMLP) were assessed before and after thrombocytopenia.

Thrombocytopenia was induced with an intravenous injection of 5 to 6 mL of a polyclonal rabbit antplatelet serum (1 mL every 15 minutes). Neutrophil deposition was measured in 10 thrombocytopenic pigs at 427, 853, and 1280 s⁻¹ shear rates for 5 minutes' superfusion under the same conditions as during basal measurements. All experiments were performed with nonanticoagulated blood.

Radioactive platelets and neutrophils deposited on the aortic media strips as well as reference blood samples were counted in a gamma counter (Minaxi 5000, Packard Instruments) equipped with a computer and a multinuclide analysis program to correct for the activity spillover of each isotope. A 270- to 350-keV window was used for ⁵¹Cr and a 360- to 480-keV window for ¹¹¹In. Platelet deposition on each aortic strip was calculated from the known radioactivity of each radionuclide in the blood and on the strips and from the known platelet or neutrophil count, which allowed us to calculate the specific activity per cell as detailed previously.¹² Neutrophil deposition was calculated in a similar fashion.

**Platelet deposition**

\[
\text{Platelet deposition} = \frac{({}^{51}\text{Cr cpm in aortic tissue}) \times (\text{No of platelets/mL blood})}{(51\text{Cr cpm/mL blood})}
\]

**Neutrophil deposition**

\[
\text{Neutrophil deposition} = \frac{({}^{111}\text{In cpm in aortic tissue}) \times (\text{No of neutrophils/mL blood})}{(111\text{In cpm/mL blood})}
\]

The neutrophil and platelet depositions are expressed as numbers per aortic tissue, as the tissue surface exposed to flowing blood was constant throughout the experiments because of the use of the same superfusion chamber. Representative sections from the strips of some animals were selected for surface analysis by scanning electron microscopy.

**Isolation and Labeling of Platelets and Neutrophils**

Twenty to 24 hours before the experiments, 43 mL autologous blood with 7 mL acid-citrate dextrose were used to obtain a platelet concentrate.¹² The platelet suspension was incubated with 200 µCi ⁵¹Cr during dual platelet and neutrophil labeling or 500 µCi ¹¹¹In-tropolone (Merck Frosst Canada Inc) at room temperature for 30 minutes. The suspension was then centrifuged to remove unbound ⁵¹Cr or ¹¹¹In. The platelet pellet was resuspended in 5 mL platelet-poor plasma and reinserted into the animal. Platelet labeling has been used extensively in previous studies.¹³⁻¹⁶,²³ Using this technique of platelet labeling, the aggregation responses to ADP of isolated control unlabeled and labeled platelets are comparable.²⁴ On the day of the experiment, 30 mL autologous blood with 10 mL acid-citrate dextrose was used for isolation and radiolabeling of neutrophils according to a modified protocol previously reported.²⁵ After sedimentation with 4% dextran 250, the leukocyte-rich plasma was washed with N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid–Hank's buffer and the cells were suspended in 10 mL of 1% bovine serum albumin solution. The suspension was layered on Ficoll and Monopoly resolving-medium gradients (Flow Laboratories Inc) and centrifuged for 30 minutes at 300g. The neutrophil layer was collected, washed, and incubated with 500 µCi ¹¹¹In-tropolone for 30 minutes at room temperature. The suspension was centrifuged to remove unbound ¹¹¹In, and the labeled neutrophil preparation was resuspended in 5 mL platelet-poor plasma. Neutrophil viability was determined by trypan blue exclusion and the final count was measured before injection.

The average yield of neutrophils obtained from 50 mL blood was 165.6±20.2×10⁹ cells, representing 64.5±2.7% recuperation. The viability of the purified cells as assessed by trypan blue exclusion was 91.0±3.0%, and the labeling efficiency with ¹¹¹In-tropolone averaged 90.1±1.6%. Before injection, a nitroblue tetrazolium reduction function test was performed on the labeled neutrophils, and the activated neutrophils were less than 5% of the total injected. A mean of 340.5±8.5 µCi ¹¹¹In was injected and 1 hour later, the free ¹¹¹In in the circulating blood was only 9.6±1.5% and remained unchanged during the experiment.
Blood samples were collected before each superfusion experiment to determine hematological parameters, including platelet and neutrophil counts, the circulating free $^{51}$Cr or $^{111}$In, and the stability of the radiolabeled cells. Platelet and neutrophil counts were unchanged during control experiments, and the free radioactivity was less than 5% and 10%, respectively. The platelet counts per minute per milliliter of blood, as an index of radiolabeled platelets, remained similar before and at the end of the experiments.

**Antiplatelet Serum Preparation**

A rabbit anti-pig platelet serum was prepared by a modification of the method described for dog neutrophil antiserum preparation. A pure platelet suspension, prepared as described above, was homogenized in complete Freund's adjuvant. Each rabbit received $1 \times 10^8$ platelets injected intradermally on the first immunization and $1 \times 10^8$ platelets injected intramuscularly at the second immunization 10 days later. Ten days thereafter the rabbits were bled; the antiserum was inactivated at $56^\circ$C for 40 minutes and stored at $-70^\circ$C. Injection of 8 to 10 mL of the antiplatelet serum reduced the platelet count by 80%, from $645.2 \pm 34.7$ to $133.3 \pm 22.8 \times 10^6$/mL ($P<.0001$), without affecting leukocyte count ($15.4 \pm 1.8$ before vs $15.6 \pm 1.9 \times 10^6$/mL after; not significant).

**In Vitro Platelet and Neutrophil Aggregation**

A whole-blood aggregometer (type 570, Chronolog Corp) was used to measure aggregation by impedance in samples of fresh blood obtained before and after platelet depletion. Platelet aggregation was induced with 15 $\mu$mol/L ADP, and neutrophil aggregation was induced with 2 $\mu$mol/L FMLP.

**Statistics**

Data are presented as mean±SEM, and a value of $P<.05$ was accepted as being significant. Repeated analyses of variance were used to perform multiple comparisons. When these were significant, intergroup differences were assessed by a Fisher least-significant difference test.

**Results**

**Platelet and Neutrophil Aggregation In Vitro**

Platelet aggregation to 15 $\mu$mol/L ADP and neutrophil aggregation to 2 $\mu$mol/L FMLP in whole blood were related to the blood platelet count (Fig 1). The relationship appeared linear for both platelet and neutrophil aggregation. Both platelet and neutrophil aggregations in whole blood were completely inhibited when the platelet count was below $100 \times 10^6$/mL. There was also a strong positive correlation between neutrophil aggregation and platelet aggregation (Fig 1C).

**Platelet and Neutrophil Deposition Ex Vivo**

Under basal conditions, neutrophil deposition on aortic strips with exposed media increased with time ($P<.05$) (Fig 2). However, on aortic strips with intact endothelium, neutrophil deposition was only 5 to 9 $\times 10^3$ and did not increase with time. The deposition of platelets and neutrophils on normal and damaged aortic strips was measured with increasing shear rates ($427, 853,$ and $1280$ s$^{-1}$) (Fig 3). Platelets did not adhere to aortic strips with intact endothelium, which reflects normal functional endothelium.
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Platelet deposition (x10^6)

\[
\begin{array}{c}
\text{Shear rates (sec}^{-1}) \\
400 & 600 & 800 & 1000 & 1200 & 1400
\end{array}
\]

\[
\begin{array}{c}
\text{Normal artery} \\
\text{Damaged artery}
\end{array}
\]

\[
\begin{array}{c}
\text{n} = 5 \\
\text{X ± SEM} \\
\star \ P < 0.05 \text{ vs normal}
\end{array}
\]

Neutrophil deposition (x10^3)

\[
\begin{array}{c}
\text{Shear rates (sec}^{-1}) \\
400 & 600 & 800 & 1000 & 1200 & 1400
\end{array}
\]

\[
\begin{array}{c}
\text{Normal artery} \\
\text{Damaged artery}
\end{array}
\]

\[
\begin{array}{c}
\text{n} = 18 \\
\text{X ± SEM} \\
\star \ P < 0.05 \text{ vs normal}
\end{array}
\]

Fig 3. Line graphs showing platelet deposition (right panel) and neutrophil deposition (left panel) on normal and damaged aortic strips at shear rates of 427, 853, and 1280 s\(^{-1}\) after 5 minutes' exposure. Both platelet and neutrophil deposition on the damaged aortic strips are shear-rate dependent.

Fig 4. Line graph showing that neutrophil deposition on damaged aortic strips was significantly higher in normal than in thrombocytopenic pigs at shear rates of 427, 853, and 1280 s\(^{-1}\) with 5 minutes' exposure.

Fig 5. Line graph showing neutrophil deposition on normal aortic strips with intact endothelium in normal (n=18) and thrombocytopenic (n=10) pigs at shear rates of 427, 853, and 1280 s\(^{-1}\) after 5 minutes' exposure (mean±SEM; not significant).

Lial thromboreistance. Platelet deposition (x10^6) on aortic strips with exposed media was high and increased significantly with shear rate. Neutrophil deposition (x10^3) was also high and increased with shear rate. Neutrophil deposition on aortic media was thus time dependent and shear-rate dependent and was significantly higher than neutrophil deposition on aortic endothelium, which was not only low but also independent of time and shear rate.

Influence of Thrombocytopenia

Neutrophil deposition on aortic media was inhibited by platelet depletion at the three shear rates tested (P<.05 for each) (Fig 4). Neutrophil deposition was shear-rate dependent at normal platelet levels but became independent of shear rate with thrombocytopenia.

However, adhesiveness to normal endothelium was preserved before and after thrombocytopenia. Neutrophil deposition to intact aortic strips with normal endothelium was not significantly affected by thrombocytopenia relative to normal controls (Fig 5). If anything, there was a nonsignificant trend (P=.1 at shear rates of 427 and 853 s\(^{-1}\)) for neutrophil adhesion to increase after thrombocytopenia.

Electron Microscopy

The quantitative platelet and neutrophil data were confirmed by qualitative scanning electron photomicrographs (Fig 6), which showed that exposure of the aortic media to normal flowing blood led to the formation of a thrombotic matrix composed of platelets, leukocytes, red blood cells, and fibrin. Thrombocytopenia inhibited the development of this thrombotic matrix; only a few cellular elements and leukocytes adhered to the exposed media.

Discussion

This study demonstrated for the first time that circulating neutrophils are deposited on the exposed media of a damaged artery in a manner that is dependent on the time of exposure and the shear rates of the flowing blood and that this interaction is reduced by platelet...
depletion. The interaction of neutrophils with the exposed media is similar to that of platelets; however, neutrophil deposition appears to be modulated by the extent of platelet deposition itself.

The receptors implicated in the adhesion and aggregation of platelets and the pathophysiological significance of these interactions with the vessel wall have been well characterized. It has been shown that platelet deposition is modulated by the nature of the surface exposed (mild or severe injury) and is regulated by the interactions between platelet glycoprotein (Gp) receptors GpIIb/IIIa, GpIib, and Gpla; von Willebrand factor; fibronectin; collagen; and other procoagulant proteins found in the plasma and on the exposed vessel surface. Platelet activation and deposition amplify the thrombotic process and the vasoconstrictive response after arterial injury. The vasoconstrictive effect of platelets is related to the secretion of potent vasoactive substances such as thromboxane A2, serotonin, and ADP. Antiplatelet agents do not completely eliminate mural thrombus formation and vasoconstriction after angioplasty and have little effect on restenosis, which supports the concept that the pathophysiological events after arterial injury are controlled by multiple cell types and metabolic pathways. Increasing evidence suggests that neutrophils can modulate vascular tone and influence the activation of platelets and the development of ischemic heart disease. Stimulated neutrophils express receptors such as the CD18 glycoprotein complex, which is implicated in the adhesion of neutrophils to the intracellular adhesion molecule-1 found on activated endothelial cells. However, the mechanisms for the interaction of neutrophils with the injured vessel wall are less well known. It is possible that neutrophil deposition to the subendothelial matrix may occur by a CD11/CD18-dependent pathway. This mechanism can mediate aggregation of neutrophils and their addition to a variety of matrix proteins and subendothelial compounds. A second mechanism for neutrophil deposition to the subendothelium may occur indirectly through platelet deposition. The adhesion and activation of platelets stimulate the release of vasoactive and chemotactic products for neutrophils and may also expose a member of the selectin family receptor, the granule membrane protein-140 (GMP-140), which in turn can fix neutrophils and may contribute to neutrophil deposition. This receptor is also implicated in leukocyte interactions with stimulated endothelium. The comprehension of these mechanisms requires further study and may be essential for the pharmacological inhibition of neutrophil deposition and its sequelae.

In this study, neutrophil deposition was assessed without prior stimulation. Under dynamic flowing blood conditions, neutrophils interact to a small extent with the intact endothelium. The minimal neutrophil deposition that did occur was not influenced by the time of exposure (Fig 2) or by the shear rate (Fig 3). Also, the intact endothelium provides a thromboresistant surface to platelet deposition (Fig 3), even in the presence of increasing shear rate. It is known that platelets do not adhere to the intact endothelium. However, stimulation of the endothelium by viruses or by thrombin and inhibition of prostacyclin production can cause platelets to adhere to the endothelium, either by a direct interaction or indirectly by an interaction with neutrophils via a selectin-mediated mechanism. The thrombo-resistance observed in our study likely suggests that the endothelium was not in an activated state. However, neutrophil deposition on the exposed media of the damaged aorta was abundant, and it increased significantly with time and with increases in flow or shear rates. But similarly to neutrophils, platelets accumulated on exposed media in a shear rate-dependent manner.

Not only did neutrophils and platelets interact with the vessel wall, but also neutrophil aggregation and the neutrophil–vessel wall interaction appeared to be further modulated by platelet deposition. This conclusion is supported by the demonstration that platelet depletion reduced both the shear rate–dependent neutrophil deposition on exposed aortic media and the neutrophil aggregation to FMLP in whole blood. Antiplatelet serum produces a severe thrombocytopenia with a marked decrease in the serum thromboxane B2 level. In our study, this occurred with an 80% reduction in the peripheral platelet count and no significant change in leukocyte count and was accompanied by complete
inhibition of platelet aggregation to ADP. More importantly, the adhesion of neutrophils to normal aortic strips with intact endothelium was not significantly affected by platelet depletion (Fig 5), which indicates that antiplatelet serum did not interfere directly with neutrophil adhesion. If anything, neutrophil adhesion to the endothelium was enhanced, although not significantly. Scanning electron microscopy confirmed these findings, which showed extensive platelet deposition on the exposed media in normal pigs but only an organized matrix of fibrin devoid of platelets, with few neutrophils, after thrombocytopenia. However, the absence of platelet deposition in the thrombocytopenic pigs unmasked a persistent and low level of neutrophil deposition that was independent of shear rate. This finding suggests that neutrophils and the exposed media may interact in a specific, direct way, independent of platelets. At the low shear rate, neutrophil deposition on the injured vessel was four times higher than on the vessel with intact endothelium and increased at the high shear rate to 12 times that on the normal vessel, which suggests that both platelets and shear rates may modulate the neutrophil–vessel wall interaction under normal blood flow conditions. The mechanism and the nature of this interaction are as yet unknown. It is possible that the neutrophil–vessel wall interaction, which is modulated by platelets, may be due perhaps to the exposure of GMP-140 receptors (P-selectins) from activated platelets.

The pathophysiological implication of platelet and neutrophil interactions in mural thrombus formation, vasocostriction, and the development and progression of atherosclerosis and restenosis may be amplified by the activation of these blood elements. Shear rates are high at sites of arterial stenoses, and with plaque rupture (spontaneously or by angioplasty), the thrombogenic media is also exposed. Both of these factors may combine to increase platelet and neutrophil interactions with the vessel wall.1–4 In our study, the lesion produced by lifting and peeling the intima and a thin portion of the subjacent media mimics a severe or deep injury by exposing the arterial media to flowing blood and is comparable to a type III injury as classified by Fuster et al.29 This type of injury is characterized by damage to both the intima and media, in addition to endothelial denudation, and may occur after coronary angioplasty or during spontaneous atherosclerotic plaque rupture. Activated platelets can fix the circulating neutrophil, probably via exposure to the GMP-140 receptors.21 In this environment, neutrophils may be implicated in thrombogenesis by the secretion of platelet-activating factor, proteolytic enzymes, free radicals, and leukotrienes.6–10 These substances may participate in subsequent vessel injury, in the recruitment of other platelets, in the stimulation of smooth muscle cell proliferation, and in the enhancement of the vasocostrictive response to injury.

In summary, this study demonstrated that platelets and neutrophils interact with the exposed media of the damaged arterial wall. The extent of both platelet deposition and neutrophil deposition increased with increasing shear rates. Importantly, platelet deposition appears to modulate neutrophil deposition. The mechanism of these interactions and their pathophysiological implications after arterial injury require further investigation.

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References


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