Neutrophil Implications in Platelet Deposition and Vasoconstriction After Deep Arterial Injury by Angioplasty in Pigs

Yahye Merhi, PhD; Lucie L-Lacoste, MSc; Jules Y.T. Lam, MD

Background Experimental studies in vitro suggest that neutrophils can modulate platelet function and vasomotor responses. In the present study, the interactions among neutrophils, platelets, and arterial responses to injury in vivo were evaluated. The acute thrombotic and vasoconstrictive responses of porcine carotid arteries to balloon injury in vivo were evaluated in three groups of animals: neutropenic pigs treated (n=11) or not treated (n=12) with aspirin and healthy untreated control pigs (n=15). Neutropenia was achieved by treatment with cyclophosphamide (50 mg/kg, 4 days before the experiment), which decreased circulating leukocyte count by 92% and almost abolished neutrophil aggregation to N-formylmethionyl-leucyl-phenylalanine without affecting blood platelet count, hematocrit, hemoglobin concentration, or whole blood platelet aggregation to ADP. 

Conclusions Neutrophils can influence the vasoconstrictive response at the site of endothelial injury in vivo. In addition to platelets, neutrophil interaction with the injured vessel wall may be implicated in the pathophysiological response to arterial injury in vivo. (Circulation. 1994;90:997-1002.)

Key Words: platelets • neutropenia • arterial injury • vasoconstriction

Platelet and leukocyte interactions with the intact and injured arterial wall have been implicated in thrombogenesis, inflammation, vasospasm, atherosclerosis, and restenosis. The response to an acute arterial injury in vivo by the formation of a mural thrombus and a localized vasoconstrictive response are largely influenced by platelet adhesion and activation. The extent of platelet deposition is modulated by the severity of arterial injury and is correlated positively with the injury-related vasoconstrictive response, and both platelet deposition and vasoconstriction are inhibited by aspirin. Although the inhibition of platelets in these studies or the depletion of platelets in other studies using thrombocytopenic animals have suggested an important role for platelets in the arterial responses to injury, these interventions do not totally inhibit the arterial vasoconstrictive response occurring at the site of endothelial injury, thereby suggesting the existence of a platelet-independent pathway mediating vasoconstriction in vivo. Other cellular elements such as neutrophils are also believed to participate in the pathogenesis of thrombosis by activating platelets through the release of neutrophil-derived products such as platelet-activating factor (PAF), free radicals, proteolytic enzymes, and leukotrienes. These products secreted by activated neutrophils can amplify the arterial response to injury. We have previously demonstrated that similar to platelet interactions with the injured vessel wall, neutrophil adhesion to injured arteries is time and shear rate dependent and is reduced after thrombocytopenia. Increasing evidence also suggests that neutrophils are implicated in vascular tone regulation. Activated neutrophils may also enhance the generation of thromboxane A2, a potent platelet-derived vasoconstrictor. These findings suggest a close interaction among platelets, neutrophils, and the injured arterial wall.

Neutrophil implication in ischemic heart disease and reperfusion injury and in many inflammatory processes has been studied using a variety of strategies including the use of anti-inflammatory drugs, neutralization by antiserum or by filtration, and the use of monoclonal antibodies to the adhesive receptors on neutrophils. Recently, a nonimmunological method using cyclophosphamide treatment has been used to produce a progressive and severe degree of neutropenia. This latter strategy of neutrophil depletion, with or without concomitant aspirin treatment, was used in the present study to determine the implication of neutrophils in platelet deposition and in the vasoconstrictive response following acute balloon arterial injury in pigs.

Methods Animal Preparation and Experimental Design

We used 38 Yorkshire pigs (mean weight, 17.9±0.6 kg) prepared according to the Canadian Council on Animal Care Regulation. Four days before the angioplasty, 12 pigs (neutropenic group) were treated with 50 mg/kg cyclophosphamide...
TABLE 1. Hematological Parameters of the Three Groups of Animals

<table>
<thead>
<tr>
<th>Group</th>
<th>Control</th>
<th>Neutropenic</th>
<th>Neutropenic Plus Aspirin</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>15</td>
<td>12</td>
<td>11</td>
</tr>
<tr>
<td>Platelets, ×10^10/mL</td>
<td>712.2±30.3</td>
<td>613.2±46.8</td>
<td>635.2±87.1</td>
<td></td>
</tr>
<tr>
<td>Leukocytes, ×10^3/mL</td>
<td>19.0±1.8</td>
<td>1.6±0.5</td>
<td>1.4±0.3</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Erythrocytes, ×10^6/mL</td>
<td>4.3±0.2</td>
<td>3.9±0.2</td>
<td>4.0±0.2</td>
<td>NS</td>
</tr>
<tr>
<td>Hemoglobin, g/L</td>
<td>89.4±2.1</td>
<td>84.6±3.0</td>
<td>84.1±2.7</td>
<td>NS</td>
</tr>
<tr>
<td>Hematocrit, %</td>
<td>24.9±1.0</td>
<td>21.3±1.0</td>
<td>21.8±1.2</td>
<td>NS</td>
</tr>
</tbody>
</table>

(Sigma Chemical) in 30 mL of sterile water injected intravenously through the ear vein. In 11 other pigs, cyclophosphamide treatment was followed immediately by an oral dose of 80 mg aspirin on day 1 and 40 mg on days 2, 3, and 4 (neutropenic plus aspirin group). Fifteen pigs receiving no active drug therapy served as controls. Platelet aggregation in response to 15 μmol/L ADP in whole blood and neutrophil aggregation in response to 2 μmol/L N-formyl-methionyl-leucyl-phenylalanine (FMLP), as well as the hematological parameters of the pigs, were determined before the initiation of treatments and 4 days later, before the balloon dilatation procedure. On the morning of the experiment, anesthesia was induced by intramuscular injection of 200 mg ketamine (Rogarsetic, Rogar/STB Inc) and 120 mg azaperone (Stresnil, Janssen Pharmaceuticals). The pigs were intubated, ventilated mechanically with ambient air, and maintained in an anesthetized state with 0.5% halothane (Fluothane, Ayerst).

Isolation and Labeling of Platelets

In all animals, 100 mL of autologous blood collected in 15-mL acid citrate dextrose was used for isolation and radiolabeling of platelets with ^51^Cr (Merck Frost) as previously described. Briefly, a platelet-rich plasma was obtained by differential centrifugation of the blood sample. This platelet suspension was washed and then incubated with 400 μCi ^51^Cr for 40 minutes. The suspension was then centrifuged to remove unbound ^51^Cr, resuspended in plasma, and reinfused into the animal. The labeling efficiency of platelets with ^51^Cr averaged 52.7±5.2% in the control group, 46.0±3.9% in the neutropenic group, and 48.9±2.9% in the neutropenic plus aspirin group (P=NS), and a mean of 197.6±24, 183.0±14.6, and 194.5±11.8 (P=NS) μCi of ^51^Cr-labeled platelets was injected respectively into the control, neutropenic, and neutropenic plus aspirin groups. Radiolabeled platelets were allowed to circulate for at least 2 hours before angioplasty. The free circulating unbound ^51^Cr was <5% throughout the experiments. The platelet count per milliliter of blood, as an index of radiolabeled platelets, remained similar before and at the end of the experiments in all groups.

Carotid Arterial Injury

Carotid arterial injury by balloon angioplasty was begun approximately 2 hours after the injection of the radiolabeled platelets and was performed using a 7F polyethylene balloon dilatation catheter (size, 8 mm×3 cm, Meditech Inc). After a single bolus of heparin (100 USP IU/kg), the catheter was advanced under fluoroscopic control through the right femoral artery into the left and the right common carotid arterial segments between the fifth and fourth cervical vertebrae. Five inflations were performed at 6 atm pressure, each for 30 seconds with 60-second intervals between inflations. The vasoconstrictive response localized at the site of the distal tapering end of the balloon, where endothelial injury occurred without any balloon stretching of the arterial wall, was quantified using angiograms obtained before and after the dilatation. The vasoconstrictive response was defined as the lumen diameter after dilatation and expressed as percentage of lumen diameter before dilatation.

Quantification of Platelet Deposition

Immediately after the angioplasty procedure, the carotid arteries were fixed in situ with a buffered solution of 2% glutaraldehyde and 1% paraformaldehyde perfused anterogradely. The dilated portion was divided into two segments, and the internal diameter and length of each segment were measured with an electronic calliper to determine the surface area (cm²). A distal uninjured segment was also measured. After surface measurements, the radioactivity of each segment as well as that of reference blood samples was counted for 5 minutes in a gamma counter (Minaxi 5000, Packard Instruments) equipped with a computer and a multiscintillation analysis program (COMPSHERE). With known circulating platelet count and radioactivity in blood and on the arterial segments, platelet (×10^10) deposition per centimeter squared of the dilated and deeply injured or intact uninjured segments was calculated as detailed previously.2,3,9

Pathological Analysis

After radioactivity counting, we prepared sections of each segment for histological analysis by light microscopy (after Movat pentachrome staining). The presence of deeply damaged arteries characterized by lesions that extended beyond the internal elastic lamina and exposed the media to the circulation was confirmed by two observers. Deep injury was found in 26 of 30 dilated arteries in the control pigs, in 20 of 24 dilated arteries in the neutropenic pigs, and in 19 of 22 dilated arteries in the neutropenic plus aspirin pigs (P=NS among groups).

Statistical Analysis

Values are given as mean±SEM. Intragroup analyses were performed using a paired Student's t test. Intergroup differences were analyzed by one-way ANOVA, and multiple comparisons were determined using Fisher PLSD test. Statistical significance was defined as P<.05.

Results

Characteristics of Animals

Immediately before carotid arterial injury by balloon dilatation, hematological parameters were obtained for the three groups of pigs (Table 1). Neutropenia achieved by cyclophosphamide treatment is characterized by a 92% reduction in circulating leukocyte count in both neutropenic and neutropenic plus aspirin groups. Among the circulating leukocytes remaining after cyclophosphamide treatment, there were no quantifiable neutrophils; the remaining cells were 99% mononuclear as differentiated by Wright-Giemsa stain. Blood platelet and erythrocyte counts and hematocrit and hemoglobin concentrations were statistically un-
changed after treatments and were similar among groups.

**Platelet and Neutrophil Aggregation**

Baseline neutrophil aggregation in response to FMLP was statistically similar in all groups, as shown in Fig 1. After cyclophosphamide treatment in both neutropenic and neutrophilic plus aspirin groups, neutrophil aggregation in response to FMLP was almost completely abolished (*P<.001) compared with before the treatment. In contrast, in the neutrophilic group platelet aggregation in response to ADP was unaffected by cyclophosphamide treatment compared with the control group (*P=NS), but aggregation was significantly reduced (by 50%) in the neutrophilic plus aspirin animals.

**Platelet Deposition**

Platelet deposition at the site of deep arterial injury in the various groups of animals is shown in Fig 2. Relative to control, platelet deposition was not significantly different in the neutrophilic animals (39.8±7.6 versus 45.1±7.0×10⁶/cm² for control group, *P=NS). However, treatment with aspirin in combination with neutropenia significantly reduced platelet deposition to 19.5±4.0×10⁶/cm² (*P<.05 versus control or neutropenic group). The uninjured arterial segments with intact endothelium were resistant to platelet thrombus formation in all groups (Fig 3); platelet deposition on uninjured segments was less than 0.5×10⁶/cm² and was not modified by treatment with cyclophosphamide or aspirin.

**Vasoconstriction**

The vasoconstrictive response localized at the distal edge of the dilated area, where selective endothelial denudation without arterial wall stretching was induced by the tapering end of the balloon, is presented in Fig 4. In the control animals, the degree of vasoconstriction was 46.3±2.9%. This was significantly reduced by 41% to 27.2±4.1% in the neutrophilic group (*P<.05) and by 66% to 15.6±3.0% in the neutrophilic plus aspirin group.
group (P<.05 versus control or neutropenic group). The absolute diameters of the arteries before angioplasty were similar for all groups (Table 2). After dilatation, the mean diameter was reduced from 4.0±0.07 to 2.1±0.1 mm in the control group, from 4.2±0.11 to 3.1±0.2 mm in the neutropic group, and from 4.1±0.08 to 3.6±0.1 mm in the neutropenic plus aspirin group. The balloon-to-artery ratio, determined angiographically by dividing the diameter of the balloon during dilatation by the diameter of the artery before dilatation, also was statistically similar for the groups (P=NS).

**Discussion**

Carotid arterial wall injury induced at the site of the maximally inflated balloon at 6 atm pressure produces a histologically severe arterial lesion that exposes the arterial media to flowing blood. This arterial injury is analogous to the deep or type III injury as classified by Fuster et al. and is characterized by rupture of the internal elastic lamina, exposing the highly thrombogenic medial components to the circulation. Cellular blood elements such as platelets interact with the injured vessel wall, leading to the acute formation of a mural thrombus. Secretion of potent vasoactive substances, such as thromboxane A₂, serotonin, ADP, and PAF, from the activated adherent platelets may contribute further to the arterial vasoconstrictive response occurring at the site of endothelial injury in vivo. Other cellular elements, such as neutrophils, also can interact with the injured arterial wall. We have previously shown a close interaction between platelets and neutrophils after arterial injury ex vivo in superfusion flow chambers and in vivo after balloon dilatation. The results of the present study show that neutrophils can influence the pathophysiological events occurring during acute arterial wall injury, especially the vasomotor responses of the arterial wall at the site of endothelial injury.

Selective neutrophil depletion was successfully produced by the cyclophosphamide treatment, which resulted in severe neutropenia without affecting blood platelet and erythrocyte counts and the hemoglobin or hematocrit. Platelet function was not directly affected, as demonstrated by the platelet aggregation response to ADP, which was similar before and after neutropenia. In addition, platelet deposition at the site of deep arterial injury was similar in the control and neutropenic groups of animals, and platelet interaction with the uninjured arterial segments with intact endothelium was low and not influenced by cyclophosphamide treatment. Although platelet number and function were unchanged, the induction of severe neutropenia was accompanied by an almost complete inhibition of neutrophil aggregation to FMLP and by a 41% reduction in the arterial vasoconstrictive response at the site of endothelial injury. Although inhibition of platelet function can inhibit the injury-related vasoconstrictive response, the present study shows that this vasoconstrictive response in vivo can also be inhibited by marked depletion and inhibition of neutrophil function without affecting platelet count or function. These results provide experimental evidence implicating neutrophils in the vasoconstrictive response associated with endothelial injury in vivo and extend observations showing the vasomotor influences of neutrophils when exposed to arterial rings in organ bath experiments. A further 25% reduction (P<.05) in the vasoconstrictive response was observed when platelet function was inhibited with aspirin in conjunction with production of neutropenia. Aspirin treatment plus neutropenia was also associated with almost 50% significant reduction in platelet aggregation and deposition. Aspirin alone, without concomitant neutropenia, has previously been shown to decrease platelet deposition and reduce the associated arterial vasoconstrictive response in this porcine model.

Activation of neutrophils has also been associated with the pathophysiological events occurring after balloon angioplasty in humans. Neutrophil adhesion and stimulation following arterial injury may contribute to vascular tone regulation directly by the secretion of vasoactive substances such as oxygen metabolites, PAF, proteolytic enzymes, and leukotrienes. These substances can also be produced through interactions among neutrophils, platelets, and the vessel wall. Experimental data suggest that human neutrophils release an endothelium-derived relaxing factor—like material that causes relaxation of human internal mammary artery and saphenous vein rings. However, canine neutrophils have been shown to induce an endothelium-dependent contraction of arterial rings. Thus, depending on the nature of the neutrophil preparation and of the animal model used, neutrophils can exercise a dilator or constrictor response on vascular rings. In our study, the predominant effect of neutrophils in vivo

**TABLE 2. Balloon-to-Artery Ratio and Diameter of Arteries Before and After Dilatation in the Three Groups of Animals**

<table>
<thead>
<tr>
<th>Group</th>
<th>Balloon-to-Artery Ratio</th>
<th>Diameter, mm</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Control</td>
<td>1.30±0.03</td>
<td>4.0±0.07</td>
</tr>
<tr>
<td>Neutropenic</td>
<td>1.27±0.02</td>
<td>4.2±0.11</td>
</tr>
<tr>
<td>Neutropenic plus aspirin</td>
<td>1.22±0.02</td>
<td>4.1±0.08</td>
</tr>
</tbody>
</table>

*P<.001 vs before.
†P<.05 vs other groups.
was vasoconstrictor because neutropenia was associated with inhibition rather than amplification of the arterial vasoconstrictive response to injury without any effect on platelet functions. In addition, our group26 used the same porcine model and recently demonstrated that a 5-lipoxygenase inhibitor can decrease significantly neutrophil adhesion and the vasoconstrictive response associated with arterial injury in vivo. In addition, activated neutrophils have been shown to contract atherosclerotic arteries through hydroxyl radical,10 thromboxane A₂,29 or prostaglandin E₂ generation. Recently, we have demonstrated9 that neutrophils can interact with the damaged artery directly or indirectly through platelet deposition by cell-cell interactions implicating (probably) P-selectin receptors. Independent of neutrophils, platelets have been implicated in arterial vasoconstriction through the secretion of potent vasoactive substances such as thromboxane A₂ and serotonin at the site of arterial injury.1,13 In the present study, aspirin treatment decreased platelet aggregation and deposition and inhibited the arterial vasoconstrictive response. However, it is possible that the combined interactions between platelet and neutrophil enhance the vasoconstrictive response. Whether a cooperative transcellular metabolism between these blood cells exists in this model is unclear, but cooperative mechanisms have been implicated in the production of thromboxane A₂,12 leukotrienes,31 hydroxyeicosatetraenoic acids,32 and PAF.33 Also, the vasoconstrictive leukotriene C₄ can be synthesized by platelets from neutrophil leukotriene A₄ through a steal phenomenon.11 In the absence of neutrophils, the production of many of these vasoactive substances can be decreased or even completely inhibited and may contribute to attenuate the vasoconstrictive response associated with arterial injury by balloon dilatation. Further investigations are needed to determine which components of neutrophils or platelet and neutrophil interactions modulate vascular tone in vivo.

In summary, the present study demonstrated that neutrophil depletion that does not affect circulating platelet count or function significantly decreased the vasoconstrictive response associated with arterial injury. This is the first direct experimental evidence implicating neutrophils in vascular tone regulation at the site of endothelial injury in vivo. Inhibition of neutrophil and platelet function may provide a way to limit many of the adverse pathophysiologic events associated with arterial injury in vivo.

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