Urokinase Receptor (uPAR, CD87) Is a Platelet Receptor Important for Kinetics and TNF-Induced Endothelial Adhesion in Mice

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Background—Urokinase plasminogen activator receptor (uPAR, CD87) is a widely distributed 55-kD, glycoprotein I-anchored surface receptor. On binding of its ligand uPA, it is known to increase leukocyte adhesion and traffic. Using genetically deficient mice, we explored the role of uPAR in platelet kinetics and TNF-induced platelet consumption.

Methods and Results—Anti-uPAR antibody stained platelets from normal (+/+ ) but not from uPAR−/− mice, as seen by fluorescence-activated cell sorter analysis. 51Cr-labeled platelets from uPAR−/− donors survived longer than those from +/+ donors when injected into a +/+ recipient. Intratracheal TNF injection induced thrombocytopenia and a platelet pulmonary localization, pronounced in +/+ but absent in uPAR−/− mice. Aprotinin, a plasmin inhibitor, decreased TNF-induced thrombocytopenia. TNF injection markedly reduced the survival and increased the pulmonary localization of 51Cr-labeled platelets from +/+ but not from uPAR−/− donors, indicating that it is the platelet uPAR that is critical for their response to TNF. As seen by electron microscopy, TNF injection increased the number of platelets and polymorphonuclear neutrophils (PMNs) in the alveolar capillaries of +/+ mice, whereas in uPAR−/− mice, platelet trapping was insignificant and PMN trapping was slightly reduced. Platelets within alveolar capillaries of TNF-injected mice were activated, as judged from their shape, and this was evident in +/+ but not in uPAR−/− mice.

Conclusions—These results demonstrate for the first time the critical role of platelet uPAR for kinetics as well as for activation and endothelium adhesion associated with inflammation. (Circulation. 1999;99:3315-3321.)

Key Words: platelets • tumor necrosis factor • plasminogen activators

Platelets are essential for hemostasis. After damage of a blood vessel, they bind to the exposed basement membrane by use of several adhesion molecules, where they become activated and trigger the hemostatic cascade. During activation, platelets change their shape and their expression of surface molecules and eventually release their granular content.

In addition, platelets also bind to the endothelium, a phenomenon frequently observed in vivo in association with various types of inflammation, including fibrogenesis and atherosclerosis. Tumor necrosis factor (TNF), the cytokine recognized as the central mediator of inflammation, has been reported to promote platelet adhesion to venular endothelial cells and alveolar capillaries. However, the effects of TNF on platelet adhesion are variable, depending on the mode of administration. In the Shwartzman type of reaction, elicited by sequential TNF injections, platelet consumption, evidenced by thrombocytopenia, and trapping are associated with thrombosis. This platelet consumption is presumably due to the capacity of TNF to increase the production of tissue factor. In contrast, in the reaction elicited by a single intravenous (IV) injection of TNF, platelet consumption is to a large extent coagulation-independent, because it is not associated with fibrinogen consumption and is not attenuated by heparin. Our recent work indicates that TNF-induced thrombocytopenia is not exerted by a direct effect of TNF on platelets but rather by an effect on the TNF receptor 1 (TNFR1) of cells other than platelets, leading to the production of platelet agonist(s), which are immediately responsible for the thrombocytopenia. One of the agonists involved in the thrombocytopenia induced by a single IV injection of TNF is serotonin, released by mast cells. Because TNF is extremely pleiotropic, it is likely that its influence on platelets is mediated by several different molecules whose importance varies depending on the model and mode of administration. Platelet agonists are indeed numerous (see review in Reference 13), and the list includes proteases such as thrombin, cathepsin, and plasmin.

We have been interested in the possible role of plasminogen activators (PAs) and plasmin in inflammation and have...
observed that in some cases, platelet consumption and trapping induced by TNF are attenuated in urokinase plasminogen activator–deficient (uPA−/−) mice. However, these results were difficult to interpret, because TNF does not raise the plasmatic activity of uPA. Recent reports concerning the role of uPA receptor (uPAR) in leukocyte migration have shown that uPA binding to this receptor can not only polarize proteolysis on the surface of leukocytes but also influence adhesion by plasmin-independent mechanisms. uPAR is widely distributed and has been described on leukocytes, endothelial cells, and megakaryocytes.

In the present report, we explored the role of uPAR in platelet kinetics as well as in platelet localization elicited by a single intratracheal (IT) injection of TNF in genetically deficient mice. We found that uPAR is expressed on platelets and that it has a profound effect on platelet kinetics as well as on TNF-induced platelet activation and endothelium adhesion.

Methods

Mice

C57BL/6j (normal, +/+ and uPA−/−, tissue PA (tPA)−/−, and uPAR−/− genetically deficient mice isolated on the C57BL/6×129 background) were obtained from P. Carmeliet, Belgium, and bred in our animal facility.

Bleeding Time and Platelet Aggregation

Bleeding time was recorded after a 2-mm cut on the tail. Platelet microaggregation in whole blood was performed as described elsewhere.

Mouse Recombinant TNF

Mouse recombinant TNF-α (TNF) was a kind gift from B. Allet, Glaxo IMB, Geneva. TNF or its saline control was injected IV in a volume of 0.1 mL in the retro-orbital sinus or IT, also in a volume of 0.1 mL. In the latter case, mice were anesthetized with Enflurane (Abbot No. 9406, Enflurane), the trachea was briefly exposed, the lung was fixed, and the lung was fixed by IT instillation of glutaraldehyde (2% in 0.1 mol/L cacodylate buffer, pH 7.0) and processed for embedding in epoxy resin. A section was taken in the left lobe, across the hilum. Thin sections were prepared from 2 blocks per mouse from the parenchyma. Thin sections were examined with a Philips 400 electron microscope (EM) at 60 kV. Cells within alveolar capillaries were examined and RBCs, platelets, and PMNs counted. On each block, ≥100 RBCs were counted. RBCs were used as a neutral indicator of blood stasis, and sequestration was evaluated by the platelet/RBC and PMN/RBC ratios.

To evaluate platelet activation, we measured the largest and smallest platelet diameters on EM micrographs at 6000- to 12 000-fold magnification. Twenty or more intracapillary platelets were examined for each mouse. Nonactivated platelets are disk-shaped, and the majority of sections thus appear as ellipses, with a ratio between the largest and smallest diameters of ≈3, as described elsewhere. During the early phase of activation, they become more spherical, and sections appear as circles, with the ratio between the large and small diameters approaching 1. This ratio was used as an “index of eccentricity” to evaluate platelet activation.

Statistical Evaluation

Groups of values were compared by the nonparametric Mann-Whitney U test.

Results

uPAR Is Expressed on Platelets

Platelets from +/+ or uPAR−/− mice were incubated with a rabbit anti-mouse uPAR antibody or with nonimmune rabbit IgG as a control. The fluorescence intensity obtained with a nonimmune rabbit IgG was similar for either type of platelet, whereas the anti-uPAR IgG shifted the peak of fluorescence intensity in +/+ but not in uPAR−/− platelets (Figure 1).

Effect of uPAR Deficiency on Platelet Counts, Bleeding Time, and Platelet Aggregation In Vitro

Platelet counts are known to be the most variable of the blood elements. In normal mice, we observed values ranging between 850×10⁶ and 1300×10⁶ platelets/μL of blood from the retro-orbital sinus in different experiments. Counts in +/+ and uPAR−/− were within that range.

The tail bleeding time is influenced by defects in both coagulation and platelet function. In anesthetized mice at room temperature, the bleeding time was 15±4 minutes for either +/+ or uPAR−/− mice.
Platelet aggregation in response to thrombin in a whole-blood assay was similar for the blood of +/+ and uPAR2/2 donors (not shown).

Role of uPAR in Platelet Survival
To explore more directly the role of platelet uPAR, we compared the circulation of 51 Cr-labeled platelets from +/+ or uPAR2/2 donors injected into isogenic +/+ hosts. As seen in Figure 2, platelets from uPAR2/2 donors survived longer than those from +/+ donors in a normal +/+ recipient. In Figure 2, the survival was expressed in percentage of the injected cpm. Platelet collection and manipulation involve the risk of eliciting some platelet activation that might, after injection, result in an immediate elimination from the circulation. To deal with this possible bias, we also used the cpm measured in 0.05 mL of blood 0.5 hours after injection as 100% and expressed the following values in % of this starting point, and the figure was similar to that shown in Figure 2A. Thus, whatever the mode of evaluation, a significant difference was evident between platelets from +/+ and uPAR−/− donors in 5 different experiments.

The platelet population might contain tiny contaminants of leukocytes or RBCs (<1/10 000), which might contribute some radiation to the platelet population. To evaluate whether this contaminant can bias the survival evaluation, the platelet population was treated with thrombin in vitro immediately before platelet injection. In this case, the radiation detectable in the circulation 1 hour after injection was <1/105 of the injected radiation, indicating that the injected radiation was essentially carried by thrombin-responsive elements.

Because uPAR is expressed in endothelial cells, we also explored the role of the host uPAR in platelet survival. As seen in Figure 2B, platelets from +/+ donors survive much longer in uPAR−/− than in +/+ recipients. These results indicate that both the host and the platelet uPAR contribute to reduce the platelet life span.

TNF-Induced Thrombocytopenia in Aprotinin-Treated Mice and in uPA-, tPA-, and uPAR-Deficient Mice
Administration of aprotinin (500 μg IV) significantly (P<0.05) attenuated the thrombocytopenia induced by an IT instillation of TNF; 6 hours after TNF injection, the platelet counts were 980±90, 510±70, and 720±120 for controls, saline/TNF-treated, and aprotinin/TNF-treated mice, respectively (mean±SD, n=6).

The thrombocytopenia induced by an IT instillation of TNF was explored in uPAR-deficient mice. As can be seen in a representative experiment (Figure 3), the injection of TNF resulted in a decrease in platelet counts from ≈1100×10^3 down to 650×10^3 /μL, resulting in a loss of ≈40% of the circulating platelets in +/+ mice 6 hours later, whereas in the uPAR−/−, the loss was on the order of 20%. Injection of saline IT (and the necessary surgery) raised the platelet counts, as seen 6 hours after injection (Figure 3). TNF-induced thrombocytopenia was also attenuated in uPA−/− but not in tPA−/− mice (not shown).
Thrombocytopenia induced by a TNF injection in +/- or uPAR-/- mice. Saline or TNF was injected IT at 10 AM, and aliquots of blood were collected at various times thereafter for platelet counts. Results are mean (SD, n=5) of values observed in a representative experiment.

**Effect of TNF on the Survival and Pulmonary Localization of Labeled Platelets From +/- or uPAR-/- Donors**

Labeled platelets from +/- or uPAR-/- donors were transferred into +/- recipients, which were subsequently injected with saline or TNF IT. As evident by the radiation measured in the blood 4 hours after IT injection, TNF markedly reduced the survival of platelets from +/- donors, whereas it had no significant effect on platelets from uPAR-/- donors (Figure 4).

Concomitantly, injection of TNF significantly increased the pulmonary localization of labeled platelets from +/- but not from uPAR-/- donors (Figure 4).

**Evaluation by EM of TNF-Induced Platelet and PMN Trapping in the Alveolar Capillaries: Effect of uPAR on Platelet Activation and Trapping**

IT instillation of TNF (10 μg) markedly increased the number of platelets and polymorphonuclear neutrophils (PMNs) within the alveolar capillaries, as seen by EM 4 hours after injection (Figure 5). At that time (Table), the number of platelets and PMNs in the capillaries from +/- mice was increased 3- to 4-fold compared with the saline-injected controls. The relative frequency of platelets was also markedly increased, from 0.1 and 0.07 for the blood of the retro-orbital sinus of saline- and TNF-injected mice, respectively, up to 0.98 in the alveolar capillaries. Injection of saline itself (and the necessary surgery) also increased the number of PMNs and platelets within the alveolar capillaries compared with untreated controls (Table). In uPAR+/+ mice, TNF still increased the number of PMNs, whereas it had no significant effect on platelet localization.

During activation, platelets lose their disk shape, become more spherical, and produce pseudopodia. In the saline-injected controls, the majority of platelets are presumably in transit, and their eccentricity was accordingly ≈3, similar to that seen in nonactivated platelets (Table and Figure 5). In TNF-injected +/- mice, the eccentricity was significantly decreased, indicating that platelets are in an early phase of activation. In uPAR+/+ mice, the change in eccentricity was not significant.

Thus, within the alveolar capillaries of TNF-injected +/- mice, there was an increase of both the relative number of platelets and their state of activation. As seen in Figure 5, platelet trapping is not associated with contact with the basement membrane due to severe endothelial damage or with the presence of fibrin. Platelet-endothelium contacts were not frequently observed in the plane of section. However, in rare micrographs, platelets with pseudopodia sectioned within their axes were observed, and these manifested at their extremity an area of intimate contact with the endothelium (Figure 5C). Because the number of pseudopodia per activated platelet is small (2 to 4 per platelet, as seen by scanning EM of lightly activated platelets in vitro), the probability of seeing a pseudopodium sectioned along its axis by transmission EM is very low. Thus, the rarity of these events on transmission EM does not exclude the possibility that they are important for platelet binding.

**Discussion**

In the present report, we provide evidence that uPAR (CD87) is an important receptor for platelet kinetics, activation, and endothelium binding associated with inflammation.

Using a rabbit anti-mouse uPAR antibody, we detected the uPAR on mouse platelets by fluorescence-activated cell sorter (FACS) analysis. A polyclonal antibody might raise doubt concerning the nature of the product recognized, but because there was a clear difference in the fluorescence intensity obtained with platelets from +/- and uPAR-/- donors, this indicates that a product of the uPAR gene must be present on platelets. In humans, the uPAR and its mRNA have been detected in megakaryocytic cells.

The regulation of platelet number and platelet kinetics in mice or humans is incompletely understood (eg, see review in Reference 28). Platelet number is regulated in part by platelet production, ie, the growth of megakaryocytes, for which thrombopoietin appears to be the most important factor. On the other hand, cytokines such as TNF reduce platelet life span and might therefore play a role in platelet kinetics in
normal mice, because this cytokine or its messenger RNA is detectable in normal individuals. The present report provides evidence that both platelet and host uPARs contribute to reduce the survival of platelets in the circulation. It remains to be explored whether platelet production is also modified in uPAR\(^2/2\) mice.

TNF exerts a variety of effects on platelets that vary depending on the mode of administration. The present work was performed with single local (IT) TNF injection, a model that has the advantage, compared with IV injection, that the site of platelet consumption is localized and can therefore be examined directly. This TNF-induced thrombocytopenia was attenuated in uPAR\(^{-/-}\) mice and in uPA\(^{-/-}\) mice, but not in tPA\(^{-/-}\) mice. It was also attenuated by the plasmin antagonist aprotinin, which suggests that plasmin contributes to this thrombocytopenia. In vitro, aprotinin has been reported to decrease platelet-endothelium adhesion.

The functional importance of the uPAR, and more precisely of the platelet uPAR, was documented directly by the comparison of the TNF-induced localization of labeled platelets transferred in \(+/+\) recipients. Platelets from uPAR\(^2/2\) donors, when present in \(+/-\) recipients, were indeed unable to respond to a local TNF injection, in contrast to those of \(+/+\) donors (Figure 4). Because the uPAR is present on a

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**Figure 5.** TNF-induced platelet trapping in alveolar capillaries. Alveolar capillaries from saline- (A) or TNF-injected \(+/+\) mice (B and C). In A, platelet sections are ellipsoidal; in B, sections are more spherical. C, platelet with a pseudopodium sectioned longitudinally. At tip (arrow) of pseudopodium, endothelial and platelet membranes are blurred. Magnification: A, \(\times 6000\); B, \(\times 10000\); C, \(\times 17000\). p indicates platelet; r, red blood cell; and i, interstitial cell.
### Cell Trapping in the Alveolar Capillaries

<table>
<thead>
<tr>
<th>Mice</th>
<th>Treatment (IT)</th>
<th>PMN/RBC Ratio</th>
<th>Platelet/RBC Ratio</th>
<th>Platelet Eccentricity</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/+</td>
<td>None</td>
<td>0.01 (0.01)</td>
<td>0.04 (0.01)</td>
<td>ND</td>
</tr>
<tr>
<td>+/+</td>
<td>Saline</td>
<td>0.09 (0.03)</td>
<td>0.23 (0.04)</td>
<td>3.1 (1.1)</td>
</tr>
<tr>
<td>+/+</td>
<td>TNF</td>
<td>0.27 (0.07)</td>
<td>0.98 (0.18)*</td>
<td>2.2 (1.0)*</td>
</tr>
<tr>
<td>uPAR −/−</td>
<td>TNF</td>
<td>0.14 (0.03)</td>
<td>0.13 (0.08)†</td>
<td>2.9 (1.1)‡</td>
</tr>
</tbody>
</table>

ND indicates not determined.

Mice were euthanized 4 hours after injection. The lungs were fixed, and the alveolar capillaries were examined by transmission electron microscopy. The numbers of RBCs, platelets, and PMNs were counted. Results are the mean (SD) of the PMN/RBC and platelet/RBC ratios observed in 5 individual mice. Eccentricity is the ratio between the largest and smallest platelet diameter seen in tissue sections.

*P<0.01 vs. the saline-injected group.
†P<0.01 vs. the TNF-injected +/+ group.
‡P=0.5 vs. the saline injected +/+ group.

wide variety of cells, including endothelial cells, this type of experiment demonstrates that it is the platelet uPAR that is critical for TNF-induced localization.

After IT TNF instillation, thrombocytopenia is to a large extent due to platelet sequestration within the lung capillaries, which can be observed and quantified by EM. Platelets were not observed in a state of aggregation and degranulation or in association with fibrin, as is the case during hemostasis or the local Shwartzman reaction, elicited by 2 sequential TNF injections. Platelets not only were increased in number but also manifested evidence of activation, suggested by a more spherial shape. Rarely, pseudopodia connected to the platelet body were observed. These showed intimate contact with the endothelium, suggesting that they are the sites by which platelets are retained in the alveolar capillaries. In uPAR −/−, TNF had almost no effect on platelet activation and localization, indicating that this receptor is critical for both platelet activation and endothelium adhesion. Instillation of TNF also increased the number of PMNs within the capillaries, and this trapping was slightly attenuated in uPAR −/− mice, similar to what has been described previously in the mouse peritoneum. Other receptors have been documented to contribute to the TNF-induced adhesion of platelets to the venular endothelium, notably P-selectin, glycoprotein IIB/IIa, and CD18.

As a whole, our experiments demonstrate that the uPA-uPAR system is involved in platelet kinetics and in TNF-induced platelet-endothelium adhesion associated with inflammation. The platelet uPAR thus appears to be a platelet receptor whose function is restricted to inflammation, because its deficiency has no effect on hemostasis or platelet homotypic aggregation. The uPAR might act in part by the localization of plasmin, a platelet agonist, on the platelet surface, as suggested by the effect of aprotinin. In addition, uPAR might also act by increasing the function of other platelet integrins, notably β1, β2, and β3-integrins, a mode of action documented for leukocytes. This latter possibility is suggested by the fact that uPA deficiency or aprotinin injections have less influence on platelet localization and trapping than the uPAR deficiency.

### Acknowledgments

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### References


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