Platelet membrane glycoprotein IV (GPIV) is a cell-surface glycoprotein that has been proposed as a receptor for collagen. Recently, it has been shown that platelets with the Nak-negative phenotype lack GPIV on their surface, whereas donors with this phenotype are healthy and do not suffer from hemoragic disorders. In this study, we compared Nak-negative platelets with normal platelets in adhesion to collagen types I, III, IV, and V and the extracellular matrix of endothelial cells (ECM) under static and flow conditions. No differences in platelet adhesion and subsequent aggregate formation on the collagens types I, III, and IV were observed under static and flow conditions. Adhesion of both homozygous and heterozygous Nak-negative platelets to collagen type V was strongly reduced under static conditions. Collagen type V was not adhesive under flow conditions. No difference in platelet adhesion to ECM was observed, which suggests that GPIV is not important in adhesion to subendothelium, for which ECM may serve as a model. These results indicate that GPIV is not a functional receptor for collagen under flow conditions.

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coverslips were incubated with 1% human albumin in phosphate-buffered saline (PBS; 10 mmol/L sodium phosphate pH 7.4, 150 mmol/L NaCl) for 1 hour.

**Purity of the Collagens**

As a check on the quality of the collagens and on cross-contamination with other major collagens and/or plasma proteins, the collagen type I, III, IV, and V preparations were tested in enzyme-linked immunosorbent assay (ELISA) on the presence of collagen type I, III, and IV and the presence of von Willebrand factor (vWF) and fibronectin (FN). Collagens were also tested in a more sensitive inhibition ELISA and with Western blotting for the presence of vWF or FN could be detected in an ELISA using a polyclonal antibody directed against vWF (Dakopatts, Glostrup, Denmark) and an MoAb directed against FN (CLB-HEC 140; Dr J. van Mourik, CLB, Amsterdam, The Netherlands).

**Static Adhesion Assay**

Static adhesion experiments were performed in a 24-well plate (Costar, Cambridge, MA) using circular coverslips (Knittel Gläser, Braunschweig, Germany) with a surface area of 1.1 cm². Platelet-rich plasma (PRP) was prepared by centrifugation, 120g, 10 minutes, 22°C. Platelet count was adjusted to 200,000 platelets/μL with platelet-poor plasma (PPP) and 400 μL was added per well and incubated for 45 minutes at 22 to 24°C. At the end of this period, coverslips were removed and stained as described below.

**Perfusions**

Perfusions were performed in a parallel plate rectangular perfusion chamber, the characteristics of which have been described by Sakariassen et al.²⁴ Duplicate coverslips were inserted in the chamber. Fifteen milliliters of whole blood was prewarmed at 37°C for 5 minutes and then recirculated through the chamber for 5 minutes at wall shear rates of 300/s and 1,600/s. Subsequently, 15 mL of prewarmed HEPES-buffered saline (HBSS; 10 mmol/L HEPES, 150 mmol/L NaCl, pH 7.35) was drawn through the chamber to wash the coverslips.

Before and after perfusion, samples of the perfusate were taken to measure single platelet disappearance (SPD), as a check on platelet clumping due to handling of blood, as described.²⁵ For routine perfusions, SPD values of 20% were found and no differences in SPD values were observed between control and Nak-negative blood.

After performing the static adhesion assay or after perfusion, the coverslips were removed, rinsed, with HBSS, fixed in 0.5% glutardialdehyde, dehydrated in methanol, and stained with May-Grünwald/ Giemsa as previously described.²⁶ Platelet adhesion was evaluated with a light microscope, at 1,000 × magnification, connected to an Image analyzer (AMS 40-10; Saffros, Walden, UK). Platelet adhesion was expressed as the percent of surface covered with platelets.

**Statistical Analysis**

Statistical analysis was performed with Student’s t-test and considered significant at P < .05. Results, obtained in two independent experiments, are presented as the averages of the individual data.

**RESULTS**

**Static Adhesion**

Adhesion of control platelets under static conditions was observed to collagen types I, III, IV, and ECM. All collagens induced platelet aggregate formation under static conditions (Fig 2), whereas ECM induced platelet adhesion and spreading. No significant differences in adhesion of platelets of the homozygous Nak-negative donors to collagen types I, III, IV, and ECM was observed (Table 1). A significant decrease (P < .01) in adhesion was observed with both homozygous and heterozygous Nak-negative platelets to collagen type V. No aggregate formation was observed when homozygous Nak-negative platelets were used. The remaining adhesion of homozygous Nak-negative platelets consisted of contact platelets, whereas the adhesion of heterozygous Nak-negative platelets consisted of platelets with pseudopods and some small aggregates (Fig 3).

**Adhesion Under Flow Conditions**

All adhesive collagens induced aggregate formation under flow conditions, whereas perfusion over the ECM induced platelet adhesion and spreading. Perfusion at shear rate 300/s of blood with the heterozygous Nak-negative phenotype over collagen type I resulted in normal aggregate formation and platelet coverage (data not shown). No differences in platelet adhesion (Tables 2 and 3) or morphology were observed when blood with the
DISCUSSION

The purpose of this study was to investigate the role of GPIV in platelet adhesion to collagen under static and flow conditions.

Fig 2. Morphology of platelet adhesion under static conditions to human collagen type I. (A) Adhesion of normal platelets, normal aggregate formation; (B) adhesion of platelets of a homozygous Nak⁻-negative donor.

Homzygous Nak⁻-negative phenotype was perfused over collagen types I, III, IV, and ECM at shear rates 300/s and 1,600/s with en-face evaluation, and no differences in aggregate formation could be detected (not shown). No platelet adhesion to collagen type V was observed for the normal control or Nak⁻-negative platelets at shear rates of 1,600/s, which is in agreement with previous observations.²²

Table 1. Adhesion of Nak⁻-Negative Platelets Under Static Conditions

<table>
<thead>
<tr>
<th>Surface</th>
<th>Control Platelets</th>
<th>Nak⁻-Negative Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagen type I</td>
<td>10.9 ± 0.4</td>
<td>10.2 ± 1.1</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>15.6 ± 0.8</td>
<td>20.1 ± 1.5</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>16.2 ± 0.5</td>
<td>17.1 ± 1.7</td>
</tr>
<tr>
<td>Collagen type V</td>
<td>8.6 ± 1.4</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>ECM</td>
<td>17.0 ± 1.0</td>
<td>18.4 ± 1.1</td>
</tr>
</tbody>
</table>

Data are expressed as percent area (mean ± SEM) covered by platelets in three controls and two Nak⁻-negative phenotypes. Platelets were from donors with the homozygous Nak⁻-negative phenotype. Platelet coverage on collagen type V using platelets with the heterozygous phenotype was also decreased: 1.3% ± 0.1%.

Fig 3. Morphology of platelet adhesion under static conditions to human collagen type V. (A) Adhesion of normal platelets, normal aggregate formation; (B) adhesion of platelets of a heterozygous Nak⁻-negative donor, adhesion of dendritic platelets, reduced aggregate formation and platelet coverage; (C) adhesion of platelets of a homozygous Nak⁻-negative donor, only adhesion of contact platelets, reduced platelet coverage.
In accordance with the results of Tandon et al., who used washed platelets, we found with PRP that static adhesion of Nak- negative platelets to collagen was normal after 45 minutes of incubation. Unexpectedly, in view of the defect in initial adhesion reported by Tandon et al, we found no abnormality in adhesion to collagen types I, III, and IV from flowing blood after 5 minutes. Also, no abnormality was observed in adhesion of Nak- negative platelets to ECM under flow conditions. One may speculate that the shear stress to which platelets are subjected in flowing blood accelerates the interaction of other receptors, GPIa-IIa in particular with collagen, eliminating the necessity for GPIV.

A strong decrease in platelet adhesion to collagen type V under static conditions was observed. Adhesion of platelets with the homozygous as well as the heterozygous Nak- phenotype was decreased by 85%. Homozygous Nak- negative platelets were completely unable to form aggregates, whereas platelets with the heterozygous phenotype were only able to form some small aggregates. The reason why adhesion of platelets of the heterozygous phenotype to collagen type V was almost absent is unclear. Platelets probably need a critical number of GPIV receptors that is not reached in the heterozygous phenotype for adhesion to collagen type V. Interestingly, the heterozygous platelets also hardly aggregate in response to collagen type V. A major difference in morphology was found between the two phenotypes: the adhesion of platelets with the homozygous phenotype consisted of contact platelets, which indicates that no platelet activation had taken place. The residual adhesion of platelets with the heterozygous phenotype consisted of dendritic platelets and some small aggregates, which indicates that some platelet activation had taken place, but that it was insufficient to induce normal aggregate formation. These results show that GPIV is essential for platelet spreading and aggregate formation induced by collagen type V, which agrees well with results from aggregation studies. Such a role is also in accord with the observation that static adhesion to collagen type V is partially cation independent and GPIV does not require cations for its function.

GPIV is not the only receptor involved in adhesion to collagen type V. Recent studies from our laboratory showed that platelet adhesion to collagen type V was completely inhibited by an MoAb directed against GPIa, indicating that GPIa-IIa (VLA-2, α2β1-integrin) is also a receptor for collagen type V. The same antibody also blocked adhesion of platelets with the homozygous phenotype consisting of contact platelets, which indicates that no platelet activation had taken place. The residual adhesion of platelets with the heterozygous phenotype consisted of dendritic platelets and some small aggregates, which indicates that some platelet activation had taken place, but that it was insufficient to induce normal aggregate formation. These results show that GPIV is essential for platelet spreading and aggregate formation induced by collagen type V, which agrees well with results from aggregation studies. Such a role is also in accord with the observation that static adhesion to collagen type V is partially cation independent and GPIV does not require cations for its function.

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Table 2. Adhesion of Nak- Negative Platelets at Shear Rate 300/s

<table>
<thead>
<tr>
<th>Surface</th>
<th>Control Platelets</th>
<th>Nak- Negative Platelets</th>
</tr>
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<tbody>
<tr>
<td>Collagen type I</td>
<td>18.3 ± 0.5</td>
<td>19.3 ± 0.5</td>
</tr>
<tr>
<td>Collagen type III</td>
<td>23.9 ± 0.8</td>
<td>21.8 ± 1.1</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>15.5 ± 1.1</td>
<td>14.2 ± 1.0</td>
</tr>
<tr>
<td>Collagen type V</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ECM</td>
<td>26.1 ± 1.7</td>
<td>22.9 ± 1.5</td>
</tr>
</tbody>
</table>

Data are expressed as percent area (mean ± SEM) covered by platelets in three controls and two Nak- negative phenotypes.

The biologic relevance of the decreased static adhesion of platelets to collagen type V remains to be established. Collagen type V as fibrillar collagen may be present in mixed fibrils together with collagen types I and III, and it is conceivable that interaction via GPIV may enhance the interaction of platelets with such fibrils.

In conclusion, our results indicate that GPIV is not important in platelet adhesion to collagen types I, III, and IV under flow conditions. Results obtained with ECM under flow conditions indicate that GPIV is not important in platelet adhesion to the vessel wall under physiologic conditions. These results explain the observation that individuals with the Nak- negative phenotype are healthy and do not suffer from bleeding disorders.

ACKNOWLEDGMENT

We thank Martin J.W. Jisseldijk for his expert technical assistance.

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Platelet adhesion to collagen and endothelial cell matrix under flow conditions is not dependent on platelet glycoprotein IV

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