Platelet-monocyte complexes in touch with the endothelium
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Chapter 6

Human platelet antigen-1a alloantibodies reduce endothelial cell spreading and monolayer integrity

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Abstract
Maternal human platelet antigen (HPA)-1a alloantibodies causing neonatal alloimmune thrombocytopenia can bind also to endothelium, via the β3-integrin (CD61). The aim of this study was to investigate the effect of HPA-1a Abs on endothelial cell function, with emphasis on monolayer integrity. We used a CD61 mAb as a model for the HPA-1a alloantibodies and confirmed the results with purified IgG fractions from HPA-1a alloimmunized women. The effect of these antibodies was examined by monitoring the adhesion, spreading, and monolayer integrity of primary HUVECs with conventional adhesion assays as well as Electrical Cell-substrate Impedance Sensing. We found that both the mAb CD61 and the HPA-1a antibodies caused a significant reduction in HUVEC spreading. Moreover, addition of the mAb CD61 and the HPA-1a antibodies prior to or following formation of a stable endothelial monolayer negatively affected endothelial monolayer integrity, which was accompanied by a redistribution of junctional proteins. Inhibiting Rho kinase as well as the Rac1 GTPase also reduced the endothelial monolayer integrity. Our data suggest that HPA-1a alloantibodies have a direct effect on endothelial cell spreading and monolayer integrity, which may contribute to the increased bleeding tendency in children with neonatal alloimmune thrombocytopenia.

Introduction
Neonatal alloimmune thrombocytopenia (NAIT) occurs at an estimated frequency of 1/1000 live births and results from maternal immunization against fetal human platelet antigens (HPA), inherited from the father, primarily HPA-1a. Maternal IgG alloantibodies against HPA-1a cross the placenta and can bind to fetal HPA-1a-positive platelets, causing immune-mediated fetal platelet destruction. The consequences can range from mild thrombocytopenia to massive intracranial hemorrhage (ICH), both postnatal and as early as after 16 weeks of pregnancy. The HPA-1a polymorphism results from a mutation from cytosine to thymidine at position 196 of the gene for platelet glycoprotein (GP) IIIa (β3-integrin), causing the substitution of proline for leucine at amino acid 33 of the protein. The β3-integrin appears on the cell surface of platelets as part of the GPIIb/IIIa-complex, but also on vascular endothelial cells (ECs) in the αvβ3-integrin complex. Since it is known that ECs express the β3-integrin carrying the HPA-1a antigen, it was suggested that ECs, like platelets, may be involved in the pathophysiology of alloimmune thrombocytopenia and that vascular damage could be an important aspect of this disease. The integrin αvβ3 is involved in blood vessel development during vasculogenesis and angiogenesis. The αvβ3 integrin is constitutively expressed in large vessels, but also in the microvascular endothelium from skin and lung. The surface expression of αvβ3 on ECs increases during an inflammatory response and during angiogenesis, induced by, among others, vascular endothelial growth factor and basic fibroblast growth factor. The integrin is equally distributed on both the luminal and basolateral surface of the cells. ECs in culture often express the αvβ3 integrin, located on both the abluminal and luminal surfaces. The αvβ3 integrin binds the Arg-Gly-Asp (RGD) sequence in a variety of ligands, such as vitronectin (Vn), fibronectin (Fn), fibrinogen, Von Willebrand factor, thrombospondin, osteopontin and collagen.

We made use of electric cell-substrate impedance sensing (ECIS), which has been used to monitor cell attachment and spreading, monolayer integrity and wound healing. We
examined cell adhesion, spreading, and monolayer integrity in real-time in primary human endothelial cells upon treatment with a CD61 mAb or with HPA-1a alloantibodies from maternal sera. Our results show that the CD61 mAb as well as the HPA-1a Abs reduce EC spreading and impair EC monolayer integrity. Thus, HPA-1a Abs could well contribute significantly to the bleeding tendency associated with NAIT through their binding to the αvβ3 integrin on ECs.

Materials and methods

Antibodies

The following Abs were used: CD31 (CLB-HEC/75, IgG1), CD61 (CLB-thromb/1, C17, IgG1), control IgG1, CD31/FITC, control IgG/FITC (Sanquin Reagents, Amsterdam, The Netherlands); CD29 (P4C10, β1-integrin) (Gibco BRL, Breda, The Netherlands); FAK (Upstate Millipore, Lake Placid, NY, USA); β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA, USA); VE-cadherin (Cayman, Ann Arbor, MI, USA); VE-cadherin/FITC (Bender MedSystems, Vienna, Austria); CD31/APC (eBioscience, San Diego, CA, USA); goat-anti-rabbit-Ig conjugated to Alexa 488, goat-anti-mouse-Ig conjugated to Alexa 488 (Molecular Probes Invitrogen, Leiden, The Netherlands); anti-human IgG/FITC (Sigma-Aldrich Chemie, Steinheim, Germany); and control IgG/APC (BD Biosciences, Erembodegem, Belgium). CD61 F(ab’)_2 fragments were prepared by pepsin (Sigma-Aldrich Chemie, Steinheim, Germany) digestion, residual whole mAb and Fc fragments were removed with Protein-G Sepharose (GE Healthcare Biosciences, Uppsala, Sweden).

Endothelial cells

Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins as described and maintained in Medium 199 supplemented with 300 μg/ml glutamine, 100 U/ml penicillin, 100 U/ml streptomycin (all from Gibco Invitrogen, Paisley, Scotland), 20% (v/v) heat-inactivated fetal calf serum (Bodinco, Alkmaar, The Netherlands), 50 μg/ml endothelial mitogen (Biomedical Technologies, Stoughton, MA, USA) and 100 μg/ml heparin (Sigma-Aldrich, Steinheim, Germany).

Serum samples and purification of IgG

Maternal serum samples were selected from a panel of mothers with NAIT due to anti-HPA-1a Abs (n=8) and from one woman who developed HPA-1a antibodies, with high titers maintained for years, after an episode of post-transfusion purpura. The patients’ characteristics are listed in Table 6.1. Also nine control samples, seven sera from donors with blood group AB, one from a patient with HPA-3a Abs (recognizing an epitope expressed by GPIIb, CD41) and one with HPA-5b Abs (recognizing an epitope expressed by GPIa, CD49b) were collected. The latter two served as negative controls. HPA-3a and HPA-5b Abs can cause NAIT, but are not reactive with CD61. Samples containing HPA Abs were obtained from immunized women after informed consent.

Immunoglobulin was purified from the serum samples by affinity chromatography over a protein-G column (Protein-G Sepharose). After elution from the column the fractions were dialyzed in phosphate buffered saline (PBS), and the IgG concentration was measured by NanoDrop (ND-1000; NanoDrop Technologies, Wilmington, DE, USA). The purity of the IgG

105
was assessed by SDS-PAGE, followed by coomassie staining. The presence of HPA-1a Abs in the maternal sera and purified total IgG fraction was detected by the monoclonal antibo- 
dspecific immobilization of platelet antigens (MAIPA) test. Titers varied from 1:8 to 1:512 (Table 6.1).

Table 6.1. Clinical characteristics and flow cytometric data of maternal serum samples with HPA-1a antibodies.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Titer in serum</th>
<th>Titer in purified IgG</th>
<th>Binding to endothelium</th>
<th>Platelet count at birth</th>
<th>Complication</th>
<th>Treatment after birth</th>
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<tr>
<td>1</td>
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<td>42,000</td>
<td>8</td>
<td>petechiae</td>
<td>Ivig</td>
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<tr>
<td>2</td>
<td>512</td>
<td>64</td>
<td>31,000</td>
<td>2</td>
<td>hematomas, ICH</td>
<td>Ivig</td>
</tr>
<tr>
<td>3</td>
<td>256</td>
<td>128</td>
<td>80,000</td>
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<td>not known</td>
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<tr>
<td>4</td>
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<td>64</td>
<td>29,000</td>
<td>24</td>
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<td>Tx</td>
</tr>
<tr>
<td>5</td>
<td>256</td>
<td>64</td>
<td>23,000</td>
<td>12</td>
<td>hematomas</td>
<td>Tx, pred</td>
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<tr>
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<td>64</td>
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<td>&lt;10</td>
<td>hematomas</td>
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<tr>
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</tr>
<tr>
<td>9</td>
<td>64</td>
<td>128</td>
<td>60,000</td>
<td>identified upon episode of PTP</td>
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<td></td>
</tr>
</tbody>
</table>

*aTiter of antibodies measured by MAIPA, for example 1 in 128.

bData are mean fluorescence intensity measured by flow cytometry, see also figure 6.6A.

cData *10^7/L.

dICH = intracranial hemorrhage, PTP = post transfusion purpura.

eIvig = intravenous immunoglobulins, Tx = trombocyte transfusion, pred = prednisone.

**Adhesion assay**

Flat-bottom Maxisorp 96-well plates (Nunc, Roskilde, Denmark) were coated with Fn (10 ng/ml) (Sanquin Reagents, Amsterdam, The Netherlands) o/n at 4°C. After coating, the wells were washed with PBS and then blocked with 4% (v/v) human serum albumin (Sanquin Reagents, Amsterdam, The Netherlands) at 37°C for 30 minutes. HUVECs were labeled with calcein-acetoxymethyl ester (Molecular Probes Invitrogen, Leiden, The Netherlands). The indicated Abs were added to the cells in the Fn-coated 96-well plates (30,000 cells per well). After 60 minutes at 37 °C, non-adherent cells were removed by washing twice with PBS, and adherent cells were lysed in 0.5% (v/v) Triton X-100 for 10 minutes at room temperature. Plates were then read on a microplate fluorescence plate reader (Tecan GENios plus, Tecan Group Ltd, Maennedorf, Switzerland) at excitation wavelength 485 nm and emission wavelength 525 nm. The adherent ratio (%) was calculated as follows: (fluorescence from experimental sample)/fluorescence from control-untreated cells-sample) × 100%.

**Transendothelial electrical resistance measurement**

HUVEC were seeded at 1×10^5 cells per well (0.8 cm²) for analysis of seeding, and 0.6×10^5 cells per well for analysis of stable monolayers on Fn- or Vn-coated electrode arrays (8W10E, unless stated otherwise; Applied Biophysics, Troy, NY, USA). Measurements of transeendothelial electrical resistance (TER) were performed in real time by means of an ECIS system (ECIS, Applied Biophysics, Troy, NY, USA) at 37°C, 5% CO₂. Briefly, the small measuring electrode and the larger counter electrode were connected to a phase-sensitive lock-in amplifier,
and a 1-V, 4000-Hz signal was supplied by a 1-MΩ resistor to approximate a constant current source of 1 μA. The in-phase and out-phase voltages between the electrodes were monitored in real time, followed by conversion to scalar measurements of transendothelial resistance. Spreading of the cells upon treatment and seeding was analyzed at the maximal resistance value, around 1 hour after seeding. For analysis of HUVEC monolayers, the average resistance over 5 hours was compared. The effect of treatment of stable HUVEC monolayers was analyzed by comparing the average resistance before treatment, set to 100%, to the average resistance after treatment. Occasionally, upon addition of IgG Ab a transient increase in resistance was observed. As measured with 8W10E electrodes, HUVEC monolayer values read around 1000 ohm, with 8W1E around 6000 ohm. This last value is higher compared to that measured with the 8W10E electrodes, as resistance is measured over a smaller area.

For the wound healing assay HUVEC monolayers grown on Fn-coated 8W1E electrodes were subjected to a high field current of 3 V at a frequency of 40 kHz for 15 seconds. Such an electrocution results in the death of cells covering the electrode, leaving the rest of the monolayer intact. Subsequently, the increase in TER resulting form cells migrating into the wound was recorded in real time. When indicated, TER was normalized by subtracting the resistance at time t=0 from every resistance value in the successive time points. The resistance values differed around 8% between wells.

The used ROCK inhibitor Y27632 and Rac1 inhibitor NSC23766 were purchased from Calbiochem (San Diego, CA, USA).

**Immunofluorescence staining**

HUVECs were seeded on glass coverslips. After treatment, cells were fixed with 3.7% (w/v) formaldehyde for 10 minutes at room temperature and permeabilized with 0.1% (v/v) Triton X-100 for 1 minute if necessary. Thereafter, cells were incubated with the indicated primary Abs, followed by incubation with a goat-anti-rabbit-Ig or goat-anti-mouse-Ig conjugated to Alexa 488 (1:400 dilution). F-actin was visualized with Texas Red-X phalloidin or Alexa 488 phalloidin (1:200 dilution; Molecular Probes Invitrogen, Leiden, The Netherlands). Images were recorded with a Zeiss LSM 510 confocal laser scanning microscope with a Plan-Neofluar 40x/1.3 or 63x/1.4 oil objective and appropriate filters. Image acquisition was performed and fluorescence distribution profiles were created with LSM 510 software (Carl Zeiss, Jena, Germany). The mean maximal cell diameter was measured by Imagepro AMS 6.1 software (Media Cybernetic, Breda, The Netherlands).

**Flow cytometry**

HUVEC monolayers were washed with PBS containing 1 mM Ca²⁺, 1 mM Mg²⁺, and 0.5% (w/v) bovine serum albumin and were incubated with VE-cadherin/FITC and CD31/APC mAbs. After 30 minutes incubation at 4°C the cells were washed with PBS and detached by 5 mM ethylenediaminetetraacetic acid (EDTA) in PBS. For HPA-1a Abs binding analyses, cells were detached before incubation with purified IgG from patients and control sera. Cells were analyzed by flow cytometry (FACS Canto, Becton Dickinson, San Jose, CA, USA).

**Statistical analysis**

All results were expressed as the mean ± SEM of the indicated number of independent experiments. Differences between two groups were analyzed by two-tailed Student’s t-test.
\( p \) values <0.05 were considered to be statistically significant. Pearson correlation coefficients were calculated for data in Figure 6.6B and 6.7C.

**Results**

*CD61 antibody reduces adhesion and spreading of HUVEC*

The HPA-1a Abs bind to endothelium via the \( \beta3 \)-integrin (CD61)\(^8\). We used a CD61 mAb (clone C17\(^28\) 10 \( \mu \)g/ml) as a model for the HPA-1a Abs, and analyzed the effect of the anti-CD61 mAb on the adhesion, spreading, and cell-cell contact of ECs. HUVEC, seeded in the presence of the CD61 mAb or F(ab')\(_2\) fragments thereof, showed an approximately two-fold reduction in adhesion compared to the control (56% and 54%, respectively, Figure 6.1A). A CD31 mAb, used as a control, had no influence on adhesion, in contrast to the \( \beta1 \)-integrin blocking mAb (P4C10), which reduced adhesion to 22%.

Subsequently, the effect of the CD61 mAb on HUVEC spreading was analyzed by (immuno)fluorescence microscopy. CD61 mAb-treated cells showed significantly less spreading than control-treated cells (Figure 6.1B). Moreover, cells treated with the CD61 mAb showed an increase in cortical actin compared to control cells. We could not detect a significant increase in the overall number of actin stress fibers, suggesting that the reduction in cell spreading is not the result of increased contractility. Quantification of the spreading response showed that the average diameter of CD61-treated cells was 36 \( \mu \)m ±4.0 (mean ±SEM n=5) and of CD61 F(ab')\(_2\)-treated cells was 43 \( \mu \)m (±7.6), compared to 58 \( \mu \)m (±5.1) of control-treated cells (Figure 6.1C). Since focal adhesions are important structures regulating cell adhesion and spreading we studied the distribution of the focal adhesion kinase (FAK) in control and CD61-treated HUVECs. However, we found no gross differences in the overall distribution of FAK between CD61-treated and isotype control-treated cells (Figure 6.1D).

To quantitatively study the spreading of the cells in real-time, we seeded HUVEC on Fn-coated electrodes and measured electrical resistance by ECIS\(^20\). When cells adhere and spread, an initial peak in the electrical resistance, induced by maximal spreading, was observed. After initial adhesion and spreading, cells form a confluent monolayer and the TER stabilizes (Figure 6.2A). In the presence of the CD61 mAb, a dose-dependent reduction in the electrical resistance values at maximal spreading, at \( t=20-100 \) minutes, and also in the TER of the monolayer (at \( t=10-15 \) hours), was observed (Figure 6.2A). The specificity of the response was confirmed by the use of the CD31 mAb, which also binds to ECs, but induces no change in TER (Figure 6.2B). The effect of the CD61 mAb was also not Fc-receptor mediated, since F(ab')\(_2\) fragments of the CD61 mAb induced similar changes in TER reduction as seen with the complete CD61 mAb. Since the \( \beta3 \)-integrin binds to different ligands\(^19\) we analyzed the effect on spreading and TER of the CD61 mAb following seeding of the cells on Fn- or Vn-coated electrodes. However, we found no differences between the effects on cells seeded on Fn- or Vn-coated electrodes (Figure 6.2C).

From these results we conclude that the CD61 mAb impairs adhesion and spreading of HUVECs. Moreover, addition of the CD61 mAb results in the formation of an electrically more permeable HUVEC monolayer.
HPA-1a Abs affect endothelial integrity

Figure 6.1: Reduced adhesion and spreading in the presence of CD61 antibody. (A) HUVECs, labeled with calcein, were seeded on Fn and allowed to adhere for 1 hour in the presence of isotype control (IgG) mAb, CD31 mAb, CD61 mAb, F(ab’)2 fragments of CD61 mAb, or CD29 (β1-integrin blocking) mAb. The adhesion of control cells was arbitrarily set to 100% as described in Materials and methods. Data are mean ±SEM, n=3-6. *Significantly different from IgG, p<0.05. (B) HUVECs were seeded on Fn-coated glass coverslips in the presence of isotype control (IgG) or CD61 mAb. After 1 hour, cells were fixed and stained for F-actin. The data are representative images from 8 independent experiments. Bar, 20 μm. (C) HUVECs were seeded on Fn-coated glass coverslips in the presence of isotype control mAb (IgG), CD31 mAb, CD61 mAb, or F(ab’)2 fragments of CD61 mAb. Cells were fixed after 1 hour, stained for F-actin, and the diameter of at least 100 cells per condition was measured. Data are mean ±SEM, n=3. Representative images are shown. *Significantly different from IgG, p<0.05. Bar, 100 μm. D, HUVECs were seeded on Fn-coated glass coverslips in the presence of isotype control (IgG) or CD61 mAb and were fixed after 1 hour. Cells were stained for F-actin (red) and immuno-stained for FAK (green). Bar, 20 μm. Boxes are magnified in the right panels. Bar, 5 μm.
CD61 antibody increases permeability of an established HUVEC monolayer

The effects on endothelial integrity, described in Figure 6.2, were observed when cells were seeded in the presence of the CD61 mAb. To test whether the CD61 mAb also affected established endothelial monolayers, we examined the effect of the mAb on the integrity of HUVEC monolayers with a stabilized electrical resistance, as measured by ECIS. Treatment with increasing concentrations of the CD61 mAb caused the TER to drop in a dose-dependent manner (Figure 6.3A). This effect was biphasic: a rapid loss of resistance within 20-40 min was followed by a more gradual reduction of monolayer resistance over the next 10-15 hours. A similar reduction in TER was also seen when F(ab’)2 fragments of the CD61 mAb were added, while the control CD31 mAb had no effect (Figure 6.3B). This again underscored the Fc-independent and CD61 mAb-specific effect on ECs. Also the coating (Fn or Vn) made no difference in the effect of CD61 mAb on the TER of a stable monolayer (Figure 6.3C). From these data it can be concluded that the CD61 mAb impairs the electrical resistance of a stable HUVEC monolayer.

Increased contractility could explain the effect of the CD61 mAb on the transendothelial resistance. The GTPase RhoA and its downstream effector Rho kinase (ROCK), key mediators of EC contractility, are important regulators of endothelial cell-cell contact and have been associated with increased permeability and reduced TER 21,29. To test whether ROCK mediates the loss of TER induced by the CD61 mAb, we used the well established ROCK inhibitor, Y27632. The Y27632 inhibitor was added to the HUVEC monolayer one hour prior to addition of the CD61 mAb. However, inhibition of ROCK already by itself reduced the TER of a stable HUVEC monolayer (Figure 6.3D), mimicking the effect of the CD61 mAb. In line with this, pretreatment of HUVEC monolayers with Y27632 followed by addition of the CD61 mAb reduced monolayer TER even further (Figure 6.3D). Thus, blocking the Rho-Rho-kinase pathway augmented rather than reversed the loss in TER, induced by the CD61 mAb.

Since the treated cells showed reduced spreading, suggestive for reduced Rac1 activity, we next explored whether inhibition of Rac1 function might be involved in the reduced monolayer integrity which followed CD61 Ab treatment. We therefore added the pharmacological Rac1 inhibitor NSC23766 30 to a stable HUVEC monolayer and monitored the TER. Addition of NSC23766 induced a rapid and significant loss of TER (Figure 6.3E), which was qualitatively comparable to the effects observed with the CD61 Ab. Thus, inhibition of Rac1 activity may underlie the loss of TER in EC monolayers, treated with the CD61 Ab. The fact that the effect of the NSC compound was transient, compared to the effects we observed with the antibodies, is most likely due to a more rapid breakdown of the Rac1 inhibitor.

Endothelial monolayer integrity and TER are significantly dependent on VE-cadherin, which is concentrated at adherens junctions 31. Since CD61 mAb induced a decrease in TER, we explored whether the organization of adherens junctions was altered after CD61 mAb incubation. Immunofluorescence analysis of HUVEC monolayers incubated with the CD61 mAb showed no induction of F-actin stress fibers (Figure 6.4A). However, the levels of β-catenin (Figure 6.4B) and VE-cadherin (Figure 6.4C) at cell-cell contacts were decreased, which is in agreement with the decrease in TER. In contrast to β-catenin and VE-cadherin, CD31 showed no change in junctional distribution upon CD61 mAb treatment (Figure 6.4D). The distribution of CD61 itself, which was detected over the cell surface as well as at cell-cell contacts, was also not influenced by the CD61 mAb (Figure 6.4E). Flow cytometric analysis of VE-cadherin and CD31 showed no change in cell-surface expression upon incubation with the
CD61 mAb (Figure 6.4F), indicating that there was no increased internalization of VE-cadherin. These data suggest that the CD61 mAb induces a relocalization of VE-cadherin from the cell-cell junctions to a more diffuse distribution over the cell membrane, leading to a reduction of monolayer integrity.

**Figure 6.2: CD61 antibody impairs HUVEC spreading and monolayer integrity.** (A) TER of HUVECs was measured following seeding on Fn-coated ECIS electrodes in the presence of increasing doses of isotype control (IgG; only maximal dose is shown in the line graph) or CD61 mAb. Dashed line indicates time point for analysis of TER at maximal spreading, shown in the left bar graph. Grey box indicates the time frame (t=10-15 hour) used for analysis of average TER of the monolayer, shown in right bar graph. Open bars, IgG mAb; closed bars, CD61 mAb. (B) TER of HUVECs was measured after seeding on Fn-coated ECIS electrodes in the presence of isotype control mAb (IgG), CD31 mAb, CD61 mAb, or F(ab’)_2 fragments of CD61 mAb and was analyzed for spreading (left panel) and monolayer formation (right panel), as in A. The TER of control, untreated cells, was set to 100%. Data are mean ±SEM, n=7-15. *Significantly different from IgG, \( p<0.01 \). (C) HUVECs were seeded on Vn (open) or Fn (closed) coated ECIS electrodes in the presence of isotype control (IgG) or CD61 mAb and were analyzed for spreading (left panel) and monolayer formation (right panel), as in A. The TER of control, untreated cells, was set to 100%. Data are mean ±SEM, n=5-6. *Significantly different from IgG, \( p<0.005 \).
Figure 6.3: CD61 antibody reduces HUVEC monolayer resistance. (A) TER of a stable HUVEC monolayer cultured on Fn-coated ECIS electrodes was measured prior to and following treatment with increasing doses of isotype control (IgG; only maximal dose is shown in the line graph) or CD61 mAb. Arrow indicates time point of mAb treatment. Bar graph (right panel) indicates the difference in average TER before (left grey box indicates time frame) and after (right grey box indicates time frame) mAb treatment. Open bars, IgG mAb; closed bars, CD61 mAb. (B) TER was measured prior to and following treatment of HUVEC monolayers on Fn-coated ECIS electrodes, with isotype control mAb (IgG), CD31 mAb, CD61 mAb or F(ab’)_2 fragments of CD61 mAb. The average TER after treatment is expressed as percentage of average TER before treatment, as indicated in A, set to 100%. Data are mean ±SEM, n=3-8. *Significantly different from IgG, p<0.05. C, TER was measured prior to and following treatment of HUVEC monolayers cultured on Vn (open) or Fn (closed) coated ECIS electrodes treated with isotype control (IgG) or CD61 mAb. Data are mean ±SEM, n=3-4. *Significantly different from IgG, (continued on next page)
CD61 antibody impairs re-formation of a stable monolayer following wound healing
The αvβ3 integrin plays an important role in EC migration and wound healing. Therefore, we investigated the effect of the CD61 mAb on EC migration in a model based on wound closure. A HUVEC monolayer, cultured on an ECIS electrode, was locally wounded by electrocution of the cells located at the electrode (diameter 250 μm). Subsequent wound closure by surrounding cells was monitored by ECIS, which represents migration of cells into the wound, followed by subsequent restoration of monolayer integrity.

We treated endothelial monolayers with CD61 or control mAb directly after wounding and monitored wound closure by ECIS (Figure 6.5). The slope of the curve, as an indicator for the speed of migration during wound closure, was not significantly different between CD61 mAb and control treated cells (data not shown). This suggests that the migration of the ECs was not significantly affected by the CD61 mAb. However, the restoration of monolayer integrity following wound closure was impaired in the presence of the CD61 mAb, compared to control. This reduced monolayer integrity was similar as seen in HUVEC monolayer formation upon seeding in the presence of the CD61 mAb and in stable HUVEC monolayers treated with the CD61 mAb (Figures 6.2 and 6.3). Thus, the CD61 mAb appears to primarily affect formation of stable cell-cell contact, rather than EC migration.

Effect of maternal HPA-1a antibodies on HUVEC
In addition to CD61 mAb, we used sera from allo-immunized women with HPA-1a Abs and control sera with no HPA Abs., or with HPA-3a or HPA-5b Abs. To exclude effects of components in the sera other than the Abs, we purified the IgG with protein-G sepharose columns. The ability of the HPA-1a Abs to bind to HUVEC was confirmed by flow cytometry (Figure 6.6A). The anti-HPA-1a-containing purified IgG fractions showed a donor-dependent level of binding to HUVEC, and significantly higher levels of binding than IgG fractions from control donors (Figure 6.6A). Although not statistically significant, a positive correlation was observed between the binding of the HPA-1a Abs to HUVEC and the titer of the purified IgG (R=0.54) (Figure 6.6B).

Figure 6.3 (continued from previous page)
*p<0.05. D, TER was measured prior to and following treatment of HUVEC monolayers cultured on Fn-coated ECIS electrodes treated with Y27632 (Y27) 1 hour prior to the treatment with isotype control (IgG) or CD61 mAb. A representative result from 7 independent experiments is shown (left panel). Arrows indicate time points of addition of Y27632 or mAb, respectively. The average TER before (left grey box indicates time frame) and after (right grey box indicates time frame) Y27632 and mAb treatment was compared. Bar graph (right panel) represents the average TER after treatment. Data are mean ±SEM, n=7. *Significantly different from IgG, p<0.05. **Significantly different from IgG Y27, p<0.01. E, TER was measured of stable HUVEC monolayers cultured on Fn coated ECIS electrodes. Cells were treated with increasing doses of NSC23766 (NSC) and analyzed prior to and following treatment. Representative results from 3 independent experiments are shown (left panel). Arrow indicates time point of NSC treatment. Bar graph (right panel) indicates the average TER after treatment (time point indicated by dashed line) expressed as percentage of average TER before treatment (grey box indicates time frame) set to 100%. Data are mean ±SEM, n=3. *Significantly different from 0 μM NSC, p<0.05.
Figure 6.4: Reduced junctional VE-cadherin upon CD61 antibody treatment. (A-E) HUVEC monolayers were treated with isotype control (IgG) or CD61 mAb for 20 hours, fixed and immuno-stained for F-actin (A) β-catenin (B), VE-cadherin (C), CD31 (D), or CD61 (E). Fluorescence intensity profiles along the indicated arrow are shown below the images and serve as a semi-quantitive means to underscore the differences in junctional staining. The data are representative images from 3 independent experiments. Bar, 20 μm. (F) HUVEC monolayers were treated with isotype control (IgG) or CD61 mAb for 20 hours, immuno-stained with VE-cadherin/FITC and CD31/APC (filled) or IgG/FITC and IgG/APC (open), detached with EDTA, and analyzed by flow cytometry. The data are representative images from 3 independent experiments.
HUVEC monolayers, cultured on Fn-coated ECIS arrays were incubated with increasing doses of HPA-1a Abs (Figure 6.6C). TER decreased in a dose-dependent manner. In subsequent experiments we used a concentration of 1 mg/ml of total IgG. No statistically significant difference was found in the adhesion of cells treated with HPA-1a Abs compared to control IgG treatment (Figure 6.6D). However, cells treated during seeding on Fn with HPA-1a Abs showed reduced spreading compared to cells treated with control IgG (HPA-1a, mean diameter of 51 μm (± 2.0); control, 66 μm (± 2.5), data are mean ±SEM, 9 patients and 5 controls) (Figure 6.6E).

Next, cells were seeded on Fn-coated ECIS electrodes and incubated, during seeding, with HPA-1a Abs or control IgG. The TER at maximal spreading after incubation with HPA-1a Abs was not significantly reduced compared to control IgG treated cells (Figure 6.7A). However, the TER of the monolayers in the presence of HPA-1a Abs was lower compared to the control IgG (81% (±3.6) vs. 99% (±3.8), data are mean ±SEM, 8 patients and 8 controls) (Figure 6.7A). Subsequently, stable HUVEC monolayers on Fn-coated electrodes were treated with HPA-1a Abs or control IgG. The TER after addition of the HPA-1a Abs was reduced compared to monolayers treated with control IgG (71% (±4.7) and 87% (±3.9) data are mean ±SEM, 9 patients and 9 controls, respectively) (Figure 6.7B). This effect of the IgG fractions on the endothelial monolayer correlated significantly with the ability of the Abs to bind to HUVEC (Figure 6.7C).

From these results we conclude that HPA-1a Abs from allo-immunized women impair spreading and cell-cell contact of ECs. This effect correlates with the ability of the Abs, in a concentration-dependent fashion, to bind to ECs and may well be directly related to the pathogenesis of bleeding tendency in NAIT.

Figure 6.5: CD61 antibody impairs restoration of monolayer integrity after wounding. TER was measured prior to and following treatment of stable HUVEC monolayers cultured on Fn-coated 1E ECIS electrodes. HUVECs were left untreated (control) or treated with isotype control (IgG) or CD61 mAb directly after wounding (arrow). Representative results from 4 independent experiments are shown (left panel). The average TER before (left grey box indicates time frame) and after (right grey box indicates time frame) mAb treatment was compared. Bar graph (right panel) represents the average TER after treatment expressed as percentage of average TER before treatment, set to 100%. Data are mean ±SEM, n=4. *Significantly different from IgG, p<0.05.
Figure 6.6: HPA-1a antibodies from allo-immunized women reduce spreading and monolayer integrity of HUVECs. (A) HUVECs were incubated with IgG from controls (IgG, open) or patients (HPA-1a, filled) and with anti-human IgG/FITC, and were analyzed by flow cytometry. Representative results from at least 5 independent controls or patients are shown. Dot plot (lower panel) shows the individual mean fluorescent intensity (MFI) and the average (line) of IgG from 5 controls (IgG; closed circles, no HPA; open circle, HPA-5b; open square, HPA-3a) and 9 patients (HPA-1a). *Significantly different from IgG, p<0.01. (B) The binding to HUVECs of purified IgG from patient sera was analyzed with respect to the HPA-1a titer. Regression analysis indicates a non-significant positive correlation between HPA-1a titer and the binding of IgG to HUVECs (R=0.54, p=0.13). (C) TER of a stable HUVEC monolayer cultured on Fn-coated ECIS 1E-electrodes was measured during treatment with increasing doses of IgG from a control (IgG; only maximal dose is shown in the line graph) or a patient (HPA-1a). Use of the 1E-electrode commonly results in additional fluctuation in the resistance values and a high value of relative resistance (see also the Materials and methods section). Arrow indicates time point of Abs addition. (D) HUVECs, labeled with calcein, were seeded on a Fn coating and allowed to adhere for 1 hour in the presence of IgG from controls (IgG; closed circles, no HPA; open circle, HPA-5b; open square, HPA-3a) or patients (HPA-1a). Line indicates average. (E) HUVECs were seeded on Fn-coated glass coverslips in the presence of IgG from controls (IgG) or patients (HPA-1a). Cells were fixed after 1 hour, stained for F-actin, and the diameter of at least 100 cells per condition was measured. Representative images are shown. Bar, 100 μm. Dot plot (right panel) shows the diameter of cells treated with IgG from controls (IgG; closed circles, no HPA; open circle, HPA-5b; open square, HPA-3a) or patients (HPA-1a). Line indicates average. *Significantly different from IgG, p<0.01.
Discussion

Patients with NAIT suffer from thrombocytopenia, bleedings, and in some cases even ICH. The thrombocytopenia may well explain the bleeding disorder, but may not be the only cause. HPA-1a maternal Abs recognize a platelet antigen, expressed by the β3-integrin (CD61), which is also expressed on other cell types, including ECs. Since β3-integrins on ECs are important for angiogenesis and vascular repair, it is not unlikely that some pathophysiologic characteristics of alloimmune thrombocytopenia are caused by effects of HPA-1a Abs on the vascular endothelium and that systemic vascular damage could underlie part of the NAIT-associated pathology. To investigate this, we used patient-derived anti HPA-1a Abs as well as a mAb to human β3-integrin to test for effects on endothelial integrity.

Our data show that both the mAb to CD61, which is known to be a blocking Ab for platelet aggregation and binding to fibrinogen, as well as the patient-derived, purified IgGs comprising HPA-1a Abs, negatively regulate endothelial integrity. For these studies, we made use of a sensitive technique, based on real time, non-invasive analysis of TER. The Abs consistently caused a 25-30% decrease in transendothelial resistance. The effect was observed for prolonged periods of time and was already detectable with 0.5-1 mg/ml IgG containing HPA-1a Abs, which is well below normal IgG concentrations in human plasma (~10 mg/ml). The CD61 mAb binds to the β3-integrin, albeit to another epitope than the HPA-1a Abs, indicating that the effects observed are not epitope specific.

It is important to underscore that the effects of the CD61/HPA-1a Abs on TER may appear relatively mild when compared to, e.g., thrombin. However, whereas thrombin-induced leakage resolves in a few hours, the effects of the Abs did not reverse for periods of up to 40 hours (data not shown). Moreover, in our hands the effects of the CD61/HPA-1a Abs are faster and more pronounced than those of, e.g., TNF-α or vascular endothelial growth factor, which cause a gradual loss of endothelial permeability, as measured by Transwell-based assays, only several hours to days after addition to preexisting monolayers. Finally, we show that the reduction in transendothelial resistance by the CD61/HPA-1a Abs is not only observed when these are added to ECs upon seeding, but also when added to pre-existing, stable endothelial monolayers. This indicates that the Abs to CD61 impair formation of new as well as steady-state endothelial cell-cell contacts and thus may affect the intact vasculature. So, the effects of the CD61/HPA-1a Abs on primary human endothelial monolayers are likely physiologically significant and may well be an important cause of the bleeding disorders occurring in patients with NAIT.

Endothelial cell attachment and spreading on Vn is mediated by the αvβ3. Therefore, HPA-1a Abs might block the adhesion and spreading of endothelial cells. In contrast to the effect of the CD61 mAb, we observed no effect of the HPA-1a Abs on cell adhesion, but there was a clear effect on cell spreading as quantified by microscopy (Figure 6.6D-E). This is in line with the notion that the majority of HPA-1a Abs have no β3-integrin blocking capacity. The Abs appeared to have a signaling rather than a blocking effect, also since the subcellular matrix, be it Fn, Vn, or poly-L-lysine, made no difference in the CD61 mAb effects detected by ECIS (Figure 6.2 and data not shown). Therefore assuming that integrin-mediated adhesion is not involved in these effects, this would suggest that CD61 compromises endothelial cell spreading and integrity through the regulation of cell signaling, rather than by blocking αvβ3. In line with this notion, HPA-1a Abs have been shown to trigger platelet activation upon binding to...
platelets. Thus, HPA-1a Abs likely regulate intracellular signaling in HUVECs, which results in reduced spreading and monolayer integrity.

A defect in cell spreading could be easily caused by increased activation of the RhoA GTPase, which controls acto-myosin-based contractility. However, the defect in spreading, induced by the CD61 mAb, was not accompanied by formation of actin stress fibers or a contractile morphology. In contrast, the cells displayed clearly a rim of cortical actin. In addition, the distribution and size of the focal adhesions, which are increased following RhoA activation, was not significantly altered after incubation with the CD61 mAb.

The RhoA-Rho kinase pathway has been associated with increased permeability and reduced TER. However, blocking the RhoA target ROCK in endothelial monolayers did not impair the loss of TER induced by the CD61 mAb. In fact, the ROCK inhibitor induced a similar response, i.e. a reduction in TER, suggesting that the effect of the CD61/HPA-1a Abs may even involve inhibition of RhoA signaling. Recently, van Nieuw-Amerongen et al. showed that there are local differences in ROCK activity within the cell, suggesting a dual role for ROCK in the regulation of endothelial barrier function; ROCK is required for proper barrier activity, but also mediates cell contraction and barrier disruption upon activation. Similar to our results, Arg-Gly-Asp (RGD) peptides, activating β1- and β3-integrins, were found to increase endothelial permeability, in the absence of reorganization of the actin cytoskeleton.

Alternatively, the CD61 or HPA-1a Abs may act independently of RhoA and impair spreading and cell-cell contact by other means, e.g. through the inhibition of Rac1 activity. Preliminary data indicate that Rac1-GTP levels were reduced following incubation with the CD61 mAb, but the low basal levels of active Rac1 in endothelial monolayers preclude a proper biochemical analysis of this effect. However inhibition of Rac1 by a pharmacological inhibitor resulted in increased endothelial permeability, suggesting that the CD61 and HPA-1a Abs may act by negatively regulating Rac1 activity.

The loss in monolayer integrity induced by the CD61 mAb is accompanied by the dislocalization of VE-cadherin and β-catenin from the cell-cell contacts. Since total surface expression of VE-cadherin was not reduced, this indicates that it is the distribution of VE-cadherin at cellular contacts that is affected through the β3-integrin. Loss of VE-cadherin from adherens junctions was also demonstrated upon αβ3 integrin ligation by Vn-coated beads, which induce focal clustering of the αβ3 integrin. Loss of junctional VE-cadherin through redistribution over the cell surface is known to cause increased endothelial permeability. Integrins can influence cell-cell contacts by inducing contractility that disrupts cadherin-mediated adhesions or by inducing signaling that modulates the cadherin/catenin complex. The observation that CD61 was found, by immunostaining, at endothelial cell-cell junctions (Figure 6.4) suggests that one of the functions of β3-integrins in endothelial monolayers is to regulate VE-cadherin-based cell-cell contacts. Previously, only Radder et al. have studied the effect of HPA-1a Abs on ECs. They showed that maternal sera with HPA-1a Abs did not affect the expression of intercellular adhesion molecule-1, vascular cell adhesion molecule-1, and tissue factor, the release of Von Willebrand factor and interleukin-8 or the monolayer integrity evaluated by light microscopy. The discrepancy with our results may be explained by the use of the more sensitive ECIS technique.
Figure 6.7: HPA-1a antibodies increase monolayer permeability. (A) TER was measured following seeding of HUVECs on Fn-coated ECIS electrodes in the presence of medium (control) or IgG from controls (IgG) or from patients (HPA-1a). Representative results from 8 independent controls or patients are shown. Dashed line indicates time point for analysis of TER at maximal spreading, shown in left bar graph. Grey box indicates the time frame taken for analysis of average TER of the monolayer, shown in right bar graph. Data are mean ±SEM, 8 controls, 8 patients. *Significantly different from IgG, p<0.01. (B) TER was measured of stable HUVEC monolayers cultured on Fn-coated ECIS electrodes. Cells were either left untreated (control) or treated with IgG from controls (IgG) or from patients (HPA-1a). Representative results from 9 independent controls and 9 patients are shown (left panel). Arrow indicates time point of Abs treatment. Bar graph (right panel) indicates the average TER after treatment expressed as percentage of average TER before treatment, set to 100%, as in Figure 6.3B. Data are mean ±SEM, 9 controls, 9 patients. *Significantly different from IgG, p<0.05. C, The binding capacity to HUVECs of purified IgG from control and patient sera was analyzed with respect to the effect on monolayer TER, as in B. Regression analysis indicates a significant negative correlation, R=-0.58, p=0.03.
HPA-1a Abs are by far the most common cause of severe alloimmune thrombocytopenia associated with ICH. Incompatibility to HPA-5b (expressed by GPIa, CD49b), another relative common cause of NAIT, causes a milder disease. Cases with ICH have also been described for the less common platelet antigen incompatibilities, such as HPA-3a (expressed by GPIIb, CD41), in which endothelial activation will not be relevant.

In hemolytic disease of the newborn due to anti-rhesus D, the titer of Abs correlates with disease severity. The predictive value of the titer of HPA-1a Abs for severity of NAIT is not clear. We found no clear correlation between Ab titer and its binding to or effect on ECs. However, a significant correlation was seen with the binding to the endothelium and the effect on the barrier function of the endothelium. This could be explained by a difference in affinity due to heterogeneity of the HPA-1a alloantibodies.

We conclude from this study that HPA-1a Abs not only induce platelet degradation but also compromise vascular integrity. These effects may well contribute to the increased bleeding tendency in children with NAIT.

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