Increased Gs signalling in platelets and impaired collagen activation, due to a defect in the dystrophin gene, result in increased blood loss during spinal surgery

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Controversy exists regarding the cause of the significantly increased blood loss during spinal surgery in Duchenne muscular dystrophy (DMD) patients compared with similar surgery in other patients. DMD is caused by a mutation in the cytoskeletal dystrophin, which binds to extracellular matrix laminin and which has been described as a G-protein-coupled receptor. We hypothesized that disturbed cytoskeleton organization in DMD patients would alter Gs protein and collagen signalling in platelets, leading to dysfunctional platelets and a haemorrhagic tendency during surgery. In the present study, we found that platelets and skin fibroblasts, respectively, express the Dp71 and Dp116 dystrophin isoforms. Absent or decreased expression of these isoforms in DMD patients correlates with significant Gsα upregulation. Moreover, dysfunctional dystrophin in these cells is accompanied with increased Gs signalling and higher cAMP levels after Gs stimulation. Functional analysis showed that DMD platelets responded slower to collagen with an extensive shape change in the aggregometer and with a significantly reduced platelet adhesion to coated collagen under flow. The decreased collagen activation was shown to result from both Gs activation and cytoskeletal disruption and not from decreased expression of platelet membrane receptors or impaired von Willebrand factor (vWF) activity. In conclusion, DMD platelets have a disorganized cytoskeleton and manifest Gs hyperactivity and reduced platelet collagen reactivity. Their increased bleeding during surgery will, at least partly, result from the increased platelet Gs activity after the release of natural Gs agonists as prostacyclin during surgery and an ineffective reactivity to collagen.

INTRODUCTION

Duchenne muscular dystrophy (DMD; OMIM 310200) is a fatal X-linked recessive disorder, with an incidence of about one in 3500 newborn males. It is caused by a mutation in the dystrophin gene. Approximately 60% of dystrophin mutations are large insertions or deletions that lead to a reading frame shift downstream, whereas ~40% are point mutations or small reading frame rearrangements. In general, DMD mutations cause premature translation termination, leading to total loss of full-length dystrophin protein.

Boys with DMD usually present between 3 and 5 years old, with a waddling gait and difficulty in climbing stairs, due to progressive degeneration of muscle. Most common fatal complications are respiratory failure (due to intercostal muscle weakness and early scoliosis) and cardiac failure with cardiomyopathy and/or cardiac conduction abnormalities. To ameliorate the patients’ quality of life, early surgical fusion of the spine...
is the treatment of choice (1). Although DMD patients do not bleed spontaneously, we and several other investigators have noted that they bleed more during spinal surgery than do patients with other underlying disorders, including other neuromuscular disorders (2–5). However, conflicting data have been reported on the possible cause for the surgery-related bleeding diathesis in DMD. When blood vessels are injured, haemostatic mechanisms are switched on. These mechanisms are classically divided into two steps: (i) primary haemostasis, including the vascular response and platelet function, and (ii) secondary haemostasis, which initiates almost simultaneously and involves the activation of clotting factors (6). Because no association between DMD and clotting factor deficiencies or PT or aPTT prolongation has been described, the increased bleeding tendency probably results from impaired primary haemostasis. Previous studies, in DMD patients and mice, have indeed shown an in vitro platelet dysfunction, i.e. an attenuated ristocetin-induced aggregation or decreased platelet adhesion to glass beads (7) and to collagen (8). The platelet dysfunction was explained by a deficiency of glycoprotein (GP)IV (7) or by the absence of the Dp71Δ116 dystrophin isoform in platelets (8). However, others could not confirm these hypotheses. Moreover, Noordeen et al. (3) concluded that the blood loss was due to vascular smooth muscle dysfunction. In a recent retrospective study, Turturro et al. (5) also described an increased blood loss without platelet hypofunction and suggested that impaired vessel reactivity caused the haemostatic dysfunction in DMD patients.

This study was performed to explore the role of G-protein signalling pathways in the platelet function of DMD patients. Dystrophin is found in an assembly with several (glyco)proteins, i.e. the dystrophin glycoprotein complex (DGC). Linking the extracellular matrix and the actin cytoskeleton, the DGC mainly provides structural integrity to the muscle membrane (9–11). Recently its role as a signalling complex has been studied more extensively (11–14). Hence, Zhou et al. (12) described that interactions between Gsα, Gβγ and the DGC component syntrophin occur in response to laminin-α,dystroglycan binding. They showed that absence of dystrophin in myoblasts contributed to decreased laminin binding to α,dystroglycan, weakened Gsαβγ-syntrophin interactions and, consequently, increased Gsα activity via enhanced GTP binding.

Increased Gsα signalling in platelets can result in an increased bleeding tendency, since cAMP inhibits platelet function. Indeed, we previously demonstrated in patients with an insertion in the extra-large variant of Gsα, XLαs, that the Gs agonist-induced Gs function in their platelets is associated with a trauma- or surgery-related (but no spontaneous) bleeding problem (15). The increased bleeding tendency was caused by platelet hypersensitivity to prostacyclin, released from the injured vessel wall; prostacyclin increases cAMP via Gsα stimulation and inhibits platelet function (16).

Therefore, we investigated whether the aberrant DGC in DMD platelets could be involved in dysregulation of the Gs pathway in DMD platelets. In addition to the analysis of aberrant dystrophin expression, Gsα expression and Gs function were studied in platelets and skin fibroblasts from DMD patients and normal controls.

Different other functional platelet tests were performed in DMD patients to evaluate their overall platelet reactivity.

RESULTS

Patient description and platelet morphology

Out of 86 DMD patients, followed in our paediatric department, 13 patients have undergone spinal surgery so far. Despite extensive packed cell transfusions, their mean fall in haemoglobin was 6.47 g/dl (SEM 0.44 g/dl). All patients had normal coagulation parameters, a normal platelet count and routine platelet functional tests were normal.

A group of 17 patients was studied in more detail. They presented with a clinical picture consistent with DMD and the diagnosis was confirmed by the absence of dystrophin on muscle biopsy in four patients and/or by mutation analysis in the others, based on multiplex ligation-dependent probe amplification (MLPA) (Table 1). In two patients (DMD2 and DMD8), denaturing high performance liquid chromatography (DHPLC) screening and direct sequencing were also performed. Blood was taken from 15/17 patients and skin fibroblasts from 5/17 patients, after informed consent by their parents. In none of our patients, spontaneous bleeding tendency was known. Electron microscopy of their platelets was normal or showed only minor abnormalities, as a slightly more prominent open canalicular system (Fig. 1). All patients had normal-sized platelets with a normal content of alpha and dense granules. At the time of blood sampling, eight patients were on corticosteroids. However, none of them had taken aspirin-like medication during the last 10 days.

Dystrophin isoforms in platelets and skin fibroblasts

Control platelets express Dp71, whereas fibroblasts express Dp116 as shown by immunoblot analysis and confirmed by reverse transcriptase (RT)–PCR and sequencing (Supplementary Material, Fig. S1). Dp71 expression in platelets from DMD patients (n = 14), measured via quantitative densitometry, was significantly decreased (P = 0.008) in comparison to controls (n = 7) (Fig. 2A and C). Although there was a variable expression pattern of GP Ibα (Fig. 2C), there was no statistical difference in GP Ibα expression between DMD patients and controls (Fig. 2B). A similar variation in expression pattern of β-actin was also observed (Fig. 2D). Furthermore, the expression of Dp71 varied largely between different patients (Fig. 2C) but was always decreased when compared with controls. No straightforward correlation between the nature of the mutation and the Dp71 expression level could be identified. In addition, immunostaining qualitatively showed a decreased expression of the Dp116 dystrophin isoform in skin fibroblasts from both DMD patients tested (Fig. 3). No other known dystrophin isoforms could be immunologically detected nor amplified from RNA in platelets or fibroblasts.

Gsα signalling in platelets and skin fibroblasts of DMD patients

The expression of Gsα in DMD platelets (n = 14) was significantly increased (P = 0.03) compared to controls (n = 7) (Fig. 2A and C). This finding was also confirmed qualitatively via immunostaining of fibroblasts, showing a remarkable increase of Gsα in DMD fibroblasts (n = 2) (Fig. 3).
Table 1. Genetic mutation in dystrophin gene and availability of muscle biopsy data

<table>
<thead>
<tr>
<th>Patient</th>
<th>Mutation (MLPA +/- DHPLC)</th>
<th>Biopsy</th>
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<tr>
<td>DMD1</td>
<td>Deletion exon 20–34</td>
<td>No</td>
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<tr>
<td>DMD2</td>
<td>Deletion exon 46</td>
<td>Yes</td>
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<td>DMD3</td>
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<td>Deletion exon 45</td>
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</tr>
<tr>
<td>DMD5</td>
<td>Deletion exon 47–50</td>
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</tr>
<tr>
<td>DMD6</td>
<td>Duplication exon 13–19</td>
<td>Yes</td>
</tr>
<tr>
<td>DMD7</td>
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</tr>
<tr>
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<td>Deletion exon 47–50</td>
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<td>DMD16</td>
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</tr>
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<td>DMD17</td>
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We next investigated whether the increased Gsα expression is associated with enhanced Gs activity. The Gs function in platelets from DMD patients was determined via stimulation of a Gs-coupled receptor with the prostacyclin analogue iloprost, prior to measuring cAMP levels. Inhibition of phosphodiesterase through addition of 3-isobutyl-1-methylxanthine (IBMX, 100 μM) immediately after blood drawing showed normal basal cAMP levels but a significantly faster increase of platelet cAMP after Gs stimulation in five DMD patients (DMD3, DMD7, DMD11, DMD12, DMD15) compared with four normal controls: 21.8+/−1.3 pmol/10^9 platelets in DMD patients versus 13.4+/−2.7 pmol/10^9 platelets in controls, 5 min after Gs stimulation (P = 0.03) (Fig. 4A). Also in the absence of IBMX, normal basal cAMP levels were found but again a significantly faster increase in cAMP was seen after Gs stimulation in four DMD patients (DMD4, DMD13, DMD14, DMD15) compared with three other controls (P = 0.04) when lysis buffer was added at 60 s to stop Gs stimulation (Supplementary Material, Fig. S2). We also studied the Gs function in platelets via stimulation of the Gi-coupled platelet receptor P2Y12 with adenosine diphosphate (ADP). Gi stimulation by addition of ADP (1 μM) prior to Gs stimulation in the presence of IBMX neutralized the difference in cAMP levels between DMD patients (n = 5) and normal controls (n = 4) (Fig. 4B), indicative of a normal Giα activity in DMD patients. To further evaluate the functional importance of elevated intracellular cAMP levels in platelets, we have studied the phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) in IBMX-treated blood samples. Protein kinase A is known to play a predominant role in VASP phosphorylation and subsequently platelet inhibition (17). We found that although VASP phosphorylation was not significantly different between DMD patients (n = 6) and controls (n = 5), DMD platelets tend to have an increased VASP phosphorylation after Gs activation (P = 0.09 after 1 min of Gs stimulation). There were no differences seen in the basal VASP phosphorylation, which was absent in both groups (Fig. 4D).

To further substantiate the Gs hyperfunction in DMD platelets, we also studied Gs function in fibroblasts from five DMD patients (DMD2, DMD5, DMD8, DMD11, DMD13). After stimulation of the Gs-coupled β2-adrenergic receptor with isoproterenol, cAMP levels in DMD fibroblasts increased markedly compared with controls (Fig. 4C) in the presence of IBMX. This difference was statistically significant when lysis buffer was added after 5 min to stop Gs stimulation (P = 0.035).

Functional platelet tests in DMD patients

To explore the impact of the abnormal cytoskeleton on the overall platelet function in DMD patients, we performed different functional platelet tests. Platelet ATP secretion after stimulation with Horm collagen and ADP was normal in DMD patients (n = 13) (Supplementary Material, Table S1). We also found no significant differences in standard platelet aggregations (n = 12) induced with ADP, collagen, thromboxane analogue, arachidonic acid and ristocetin (n = 7). Although DMD platelets aggregate after stimulation with low doses of Horm collagen (0.5 μg/ml and 1 μg/ml), a retarded collagen response with a more pronounced shape change was noted in all DMD patients (n = 7; Fig. 5A). This difference in response was not observed in the convulxin-induced aggregation indicative of a normal GPV1 function (Fig. 5A). In addition, we also performed platelet aggregations after pre-incubation of platelet-rich plasma (PRP) with a cytoskeleton-disrupting agent, cytochalasin B.
to investigate whether the disorganized cytoskeleton would directly contribute to the platelet reactivity towards collagen in DMD patients. At the concentration used, cytochalasin B slightly delayed platelet aggregation with collagen in normal controls. In contrast, in DMD patients, the delay was more pronounced and the aggregation response to low collagen concentration was significantly impaired (Fig. 5B). This inhibitory effect of cytochalasin on platelet aggregation in normal controls was described for collagen but not for thromboxane (18). We did not observe an inhibitory effect of cytochalasin on the ADP-induced platelet aggregation of normal controls or DMD patients (data not shown).

Since platelets from DMD patients show a pronounced shape change after collagen activation and an increased reactivity towards cytochalasin B, the role of the platelet cytoskeleton was further studied via platelet adhesion experiments under static and flow conditions. Platelet spreading under static conditions on fibrinogen (n = 3) or on collagen (n = 2) was not significantly different between DMD platelets and control platelets. In contrast, when tested under flow conditions, DMD platelets (n = 12) showed a reduced adhesion on collagen, both at 2700 s⁻¹ (P = 0.015) and at 1300 s⁻¹ (P = 0.038) compared to control platelets (n = 12) (Fig. 6A and B). Moreover, cytochalasin B treatment of whole blood from normal controls almost completely inhibited platelet adhesion to collagen (data not shown). Adhesion studies on coated fibrinogen showed no differences between DMD platelets (n = 2) and control platelets (n = 2) (data not shown). To exclude the possibility of differences in expression of the major GP membrane receptors involved in platelet adhesion under flow, their expression was compared between DMD patients and normal controls. Fluorescence-activated cell sorting analysis showed a normal expression of GPIbα, GPIIb/IIIa, GPIV, GPIa/IIa and GPVI in DMD patients (Supplementary Material, Table S2). As platelet adhesion at 2700 s⁻¹ also necessitates the interaction of vWF, its expression and function was also studied in the platelet function analyser (PFA100), as well as vWF antigen determinations and ristocetin-cofactor assays. These tests were also normal in our patients (Supplementary Material, Table S3). Finally, a causal role for Gs hyperfunction in attenuating platelet adhesion was demonstrated by adding the Gs agonist prostaglandin E1 (PGE1) to normal blood (n = 5) prior to perfusion. This procedure strongly impaired the platelet adhesion and aggregate formation (P = 0.008) (Fig. 6C).

DISCUSSION

Although DMD patients have no spontaneous bleeding diathesis, a markedly increased blood loss during spinal surgery has been documented in these patients. No association between DMD and coagulation disorders has been described. Several other hypotheses have been put forward to explain
this extensive surgery-induced blood loss but none of them have been confirmed unequivocally (3,5,7,8). Hence, the recent increase in life expectancy and the higher frequency of major surgery at increasing age because of pronounced scoliosis warrants better characterization of haemostasis in DMD.

DMD is caused by a mutation in dystrophin, which has recently been described as a G-protein-coupled receptor (12,13). Since a short isoform of dystrophin, Dp71D110, has been identified in the platelet cytoskeleton (8), we hypothesized that disturbed organization of the cytoskeleton in DMD patients would alter Gs signalling in platelets, potentially leading to an increased bleeding tendency during spinal surgery.

All patients in this study had a normal platelet count and none of them had a history of spontaneous bleeding or coagulation abnormalities. The Dp71 expression in our patients’ platelets was decreased to a variable degree. However, no obvious correlation between Dp71 expression and the underlying mutation could be found. We used GPIbα as internal loading control since flow cytometric analysis showed a normal GPIbα expression. GPIbα is localized in the actin cytoskeleton and therefore its expression pattern is variable in different individuals but the mean expression was similar when comparing normal and DMD individuals. A similar observation was made by using a β-actin antibody. In DMD platelets, we found a significant elevated protein expression level of Gsα, normal basal cAMP levels but stimulation of the Gs pathway resulted in a faster and larger increase of cAMP. The Gs hyperfunction in DMD patients could also be confirmed in fibroblasts and was associated with a higher expression of Gsα in both DMD platelets and fibroblasts. As cAMP is known to stimulate the phosphorylation of VASP (17,19), we have studied the basal and Iloprost-stimulated VASP phosphorylation in IMBX-treated blood samples from DMD patients and normal controls. Although there was no significant difference between both groups, DMD platelets tend to have an increased VASP phosphorylation after Gs activation. There were no differences seen in the basal VASP phosphorylation, which was absent in both groups. The role of Gs via stimulation of the Gi-coupled receptor P2Y12 by ADP was also investigated. ADP is known to stimulate P2Y12 and thereby inhibits cAMP formation. We did not observe a different platelet Gi function between DMD patients and controls after stimulation of P2Y12 in the absence (time point 0) and presence of Gs agonists (Iloprost at 60 and 300 s). DMD patients have a platelet Gs hyperfunction similar to that we previously described in patients with an insert in XLαs and increased trauma- or surgery-related bleeding tendency (15). In the latter group, however, Gs hyperfunction is much more pronounced, which could be explained by Gsα upregulation and the additional effect of increased XLαs signalling in those patients, due to lost affinity between XLαs and ALEX.

To investigate the impact of the Gs hyperfunction on the overall platelet reactivity in DMD patients, we performed several functional platelet tests. Several authors have described impaired aggregation responses for DMD platelets to agonists, such as ristocetin (7), ADP and epinephrine (8), but others could not confirm these abnormalities (5). Since different agonist concentrations were used throughout these
studies, such may explain the different observations. In our patients, platelet aggregations after stimulation with high doses of several agonists were all normal. However, low concentrations of Horm collagen induced a delayed aggregation response with a more pronounced shape change in DMD platelets compared with control platelets, indicative of an abnormal cytoskeletal reorganization rather than resulting from Gs hyperfunction since patients with an XLαs insert did not show such a retarded collagen aggregation with pronounced platelet shape change (Kathleen Freson, personal communication). We therefore hypothesized that a disruption of the cytoskeleton via the use of actin depolymerization agents as cytochalasin B would mimic the effect of disrupting the dystrophin cytoskeleton. Cytochalasin B treatment of normal platelets indeed resulted in a decreased platelet response towards collagen, both in perfusion and aggregation studies. Moreover, the effect of cytochalasin on collagen activation of DMD platelets was much more pronounced than that found for normal platelets, resulting in an almost absent collagen activation for DMD platelets. We did not observe any effect of cytochalasin B on platelet activation in DMD and normal platelets after stimulation with ADP. Remarkably, platelet spreading on Horm collagen and fibrinogen under static conditions did not show significant differences between DMD patients and normal controls. In contrast, during perfusion studies of whole blood over surfaces of collagen, we found attenuated adhesion of DMD platelets at 2700 s⁻¹ and at 1300 s⁻¹ compared with that of control platelets. These findings agree with those of Austin et al. (8), who described impaired adhesion under static conditions of thrombin-activated platelets isolated from mdx3cv-mice compared with wild-type platelets, suggesting a role for Dp71Δ110 in the relation between the cytoskeleton and platelet signalling. Furthermore, platelet collagen adhesion with and without pre-incubation with prostin in normal controls also significantly decreased adhesion after Gs stimulation, confirming at least a partial causal role for Gs hyperfunction in attenuating collagen adhesion. In addition, cytoskeleton disruption in normal platelets by adding cytochalasin B to whole blood prior to perfusion also decreased platelet collagen adhesion. The abnormal cytoskeleton structure and not expression or functional differences of membrane receptors in DMD platelets result in a delayed collagen reactivity and a Gs hyperfunction with decreased platelet adhesion to collagen under flow conditions. Several receptors play a role in platelet adhesion in vivo, but the expression of the most important membrane GPs, including the collagen receptors GPla/IIa and GPVI, appeared normal in DMD patients. Platelet adhesion in vivo strongly depends on the local shear rates. At higher shear rates, interaction of the platelet receptor complex GPIb/V/IX with immobilized vWF takes place first (6) and induces a mild platelet activation which is probably enough to activate

Figure 4. cAMP measurements and VASP phosphorylation. Data are presented as mean ± SEM. P-values of the observed differences between DMD patients and normal controls are indicated. (A) Measurement of cAMP levels (basal, 1 and 5 min after Gs stimulation with Iloprost, in the presence of IBMX) in platelets of DMD patients (DMD3, DMD7, DMD11, DMD12, DMD15) and normal controls (n = 4). (B) Measurement of cAMP levels (basal, 1 and 5 min after Gs stimulation with Iloprost, in the presence of IBMX) in platelets of DMD patients (DMD3, DMD7, DMD11, DMD12, DMD15) and controls (n = 4). Platelets were pre-incubated with ADP prior to Gs stimulation. (C) Measurement of cAMP levels (basal, 1, 2 and 5 min after Gs stimulation with isoproterenol) in fibroblasts of DMD patients (n = 5) and normal controls (n = 3). (D) IBMX treated platelets were stimulated with Iloprost (2 ng/ml) for 0, 1 or 5 min and analysed by immunoblot analysis. Basal VASP phosphorylation was absent in all samples. Gs stimulation caused an increase in VASP phosphorylation as shown for patients DMD11 and DMD7 and a normal control. This blot is representative for similar observations in 6 DMD patients and five controls.
the integrins necessary for stable platelet adhesion to collagen fibres (20,21). vWF expression and function was studied using the PFA100 and determining vWF antigen and ristocetin-cofactor activity: these were all normal in DMD patients.

In conclusion, our results demonstrate the presence of an intrinsic platelet dysfunction, which is the direct result of dysfunctional Dp71 in the cytoskeleton of DMD platelets. We confirmed this dysfunctional Gs pathway in DMD fibroblasts with decreased Dp116. Because of the disorganized dystrophin-containing cytoskeleton, DMD patients seem to have a significantly enhanced Gsα expression and an inducible Gs hyperfunction, whereby natural Gs agonists as prostacyclin released during surgery increase platelet Gs activity leading to increased blood loss during spinal surgery. In addition, the cytoskeleton disorganization in DMD platelets results in a decreased collagen response. We suggest that DMD patients, despite a normal platelet count, would benefit from a platelet transfusion prior to surgery rather than from packed cell transfusions.

MATERIALS AND METHODS

Electron microscopy
The platelet-rich fractions of citrated blood were immediately fixed overnight in 2.5% glutaraldehyde, 0.1 mol/l phosphate buffer (4°C). After centrifugation at 800g for 10 min, a condensed pellet of platelets was formed. Post-fixation was carried out in 2% osmium tetroxide, 0.1 mol/l phosphate buffer and dehydration in graded series of ethanol. Afterwards, the pellets were embedded in epoxy resin. Ultrathin sections were cut, stained with uranyl acetate–lead citrate and examined on a Zeiss EM 10 electron microscope. Images were recorded digitally with a Jenoptik Progress C14 camera system operated using Image-Pro express software.

Platelet aggregation and ATP secretion
Blood was anticoagulated with 3.8% (wt/vol) trisodium citrate (9:1). PRP was obtained after centrifugation at 150g for 15 min and platelet count was adjusted to 250×10⁹ platelets/l with autologous platelet-poor plasma. Aggregation was performed on two dual-channel Chrono-Log aggregometers (Chronolog Corp.). Horm collagen (0.5 μg/ml, 1 μg/ml, 2 μg/ml; Nycomed Arzneimittel), convulxin (100 ng/ml, 25 ng/ml; Pentapharm), ADP (2.5 μM, 5 μM; Sigma), ristocetin (0.5 mg/ml, 1.2 mg/ml; Kordia), arachidonic acid (1 mM) and the thromboxane A₂-analogue U46619 (TXA₂, 1.33 μM; Sigma) were used as agonists. To investigate the role of a cytoskeleton-disrupting agent on platelet function, PRP was incubated with cytochalasin B (50 μM; Sigma) 5 min prior to stimulation with Horm collagen (0.5 μg/ml, 1 μg/ml, 2 μg/ml) or ADP (2.5 μM, 5 μM). To study ATP secretion, platelet aggregation and secretion were recorded in real time at 37°C with stirring after stimulation with Horm collagen (2 μg/ml).
and ADP (10 μM). ATP secretion was determined by measuring the release of ATP using luciferin/luciferase reagent (Kordia).

cAMP detection in platelets
Citrated PRP at 250 × 10^9 platelets/l was obtained as described above and incubated with the Gs agonist Iloprost (1 ng/ml; Schering) in the presence or absence of IBMX (100 μM; Aldrich-Janssen Chimica). To study the Gi pathway, ADP (1 μM) was added 5 min prior to Gs stimulation with Iloprost, again in the presence or absence of IBMX. We arrested the reaction at different time points by adding 12% trichloroacetic acid and measured platelet cAMP using a cAMP enzyme-immunoassay (GE Heathcare).

Culture and cAMP detection in fibroblasts
Skin fibroblasts were obtained via punch biopsy from the volar side of the upper arm. Fibroblasts were cultured in DMEM/F12 (Invitrogen), at 37°C in a 5% CO2 humidified incubator. Cells were grown to 100% confluency before cAMP measurements. Patient or control fibroblasts were stimulated with the Gs agonist isoproterenol (10 μM; Calbiochem) in the presence of a phosphodiesterase inhibitor (IBMX, 400 μM). At different time points, addition of a lysis buffer supplied with the kit stopped the reaction. The cAMP levels were measured using the enzyme-immunoassay mentioned above.

Immunostaining
Skin fibroblasts were seeded on chamber slides and cultured for 2 days as above. They were fixed using Cytoskelfix™ Cell Fixative (Tebu-Bio) according to manufacturer’s protocol. Cells were stained with a monoclonal anti-Gsα antibody made in our laboratory (22) or a commercial anti-dystrophin antibody (Santa Cruz Biotechnology Inc). A FITC-conjugated goat-anti-mouse and goat-anti-rabbit secondary antibody (DAKO) was used, respectively. Nuclei were stained using Vectashield® mounting medium with DAPI (Vector Laboratories). The slides were immediately analysed using a Zeiss CLSM510 confocal microscope.

Immunoblot analysis and VASP phosphorylation
To obtain total protein fraction from platelets and fibroblasts, cells were lysed in solubilisation buffer [PBS containing 1% ipegal CA-630 (Sigma), 1 mmol/l EDTA, 2 mmol/l DTE, one protease inhibitor cocktail tablet/50 ml] and subjected to three freeze–thaw cycles. Lysates were cleared of insoluble debris by centrifugation at 16 100 g for 10 min at 4°C and protein concentrations were determined using the Bio-Rad Protein Assay (Bio-Rad). Protein fractions (100 μg, 25 μg and 10 μg for dystrophin, Gsα and GPIbα, respectively) were mixed with 5% SDS reducing sample buffer, separated by SDS/PAGE acrylamide gels and transferred to Hybond ECL-nitro-cellulose membrane (Amersham, Pharmacia

Figure 6. Platelet adhesion studies. Platelet adhesion on collagen was analysed at 2700 and 1300 s⁻¹. (A) Pictures after 5 min of perfusion on collagen shown for DMD3 and a normal control, being representative for 12 patients and 12 controls. (B) Mean surface area coverage (percentage) of platelets after a perfusion time of 5 min. The statistical significance of observed differences between DMD patients (n = 12) and controls (n = 12) is indicated above each pair of bars. Error bars indicate mean ± SEM. (C) Mean surface area coverage (percentage) of platelets in controls (n = 5) with or without addition of prostan prior to perfusion. The statistical significance of observed differences is indicated above each pair of bars. Error bars indicate mean ± SEM.
Biotec). The blots were blocked for 1 h at room temperature in Tris-buffered saline with Tween-20 supplemented with 5% non-fat dry milk. Incubations overnight (4°C) with primary antibody (Gαs, dystrophin, GPIbα or β-actin) and with HRP-conjugated secondary antibody (3 h at room temperature) were also performed in Tris-buffered saline with Tween-20 supplemented with 5% non-fat dry milk. Anti-Gαs and anti-GP Ibα antibodies were described previously (22); the anti-β-actin antibody was purchased from Santa Cruz Biotechnology Inc. Staining was performed with the western blotting ECL detection reagent (Amersham Biosciences). Expression was quantified by measuring the density of the bands (corrected for background), using the Image J software (National Institutes of Health).

To study phosphorylation of VASP, IBMX (100 μM) had been added to the blood samples immediately after drawing. Three hundred microlitres of citrated PRP at 250 × 10⁹ platelets/l was stimulated with Iloprost (2 ng/ml) for 1 or 5 min. A negative sample without addition of Iloprost was also prepared. Samples were immediately centrifuged at 16 100 g for 1 min at room temperature and the pellet was resuspended in sampling buffer (10 mM Tris pH 8.0, 1 mM EDTA, 5% SDS, 5% β-mercapto-ethanol, 2 mM NaF, 2 mM NaVO₃, Bromphenol blue, one protease inhibitor cocktail tablet/50 ml). Proteins were separated on an SDS/PAGE acrylamide gel and further processed as described above. We used the commercial anti-VASP239 antibody from Santa Cruz Biotechnology Inc. as primary antibody. Expression was quantified using the Image J software as described above.

Perfusion studies

Glass cover slips of 24 × 50 mm were coated with calf skin collagen type III (Sigma), dissolved in 1 mg/ml in 50 mM acetic acid. Alternatively, human fibrinogen (100 μg/ml; Sigma) was used to coat the cover slips. They were kept in a dark and wet box at 4°C overnight.

The perfusion chamber consisted of the coated cover slip and a silicon rubber gasket designed with a conically shaped flow path. Maintaining a flow rate of 4 ml/min, shear rate ranged from 1300 to 2700 s⁻¹ throughout the flow path.

Tyrode buffer containing 1% human albumin (HSA) was aspirated at 37°C for 5 min to warm and perfuse the chamber. Meanwhile, the blood was kept at 37°C. Whole blood, drawn on low molecular weight heparin (enoxaparin, 50 μg/ml), was perfused and reperfused through the perfusion chamber for 5 min. The cover slip was washed with Tyrode buffer containing 1% HSA and fixed with PFA 1%, for 4 min each. Cover slips were then removed from the perfusion chamber, rinsed in Tyrode buffer and dried on air prior to May-Grünwald-Giemsa staining. To study the effect of prostaglandins on platelet adhesion, PGE₁ (Prostin®, 100 ng/ml; Pfizer) was added ex vivo to whole blood of normal controls 20 min prior to perfusion. Cytochalasin B (50 μM) was added ex vivo to whole blood of normal controls 5 minutes prior to perfusion to investigate the effect of disruption of the cytoskeleton on collagen adhesion. Platelet adhesion was assessed by quantifying surface area coverage using a light microscope (Leica DM RBE). All blood samples were processed within 1 h after being drawn.

Flow cytometry

The expression of GPIbα (CD42b), GPIbβ (CD41/CD61), GP IV (CD36), GPIa/IIa (CD49b/CD29) and GPVI was examined by flow cytometry. Briefly, citrated PRP was prepared as described above and 10 μl PRP was diluted with 40 μl PBS in the presence of a GPIbβ/IIa antagonist, tirofiban (5 μM; Aggrastat, Merck Sharp & Dome), to prevent platelet aggregation. The anti-GP Ibα monoclonal antibody G27C9 (22) and the anti-GP Ibβ monoclonal antibody 8G7A7 were made in our laboratory. The anti-GP IV antibody was purchased from Immunotech and the anti-GP IIa antibody from BD Biosciences. The anti-GP VI antibody HY101 was kindly donated by Dr Mark Kahn (University of Pennsylvania).

PCR amplification of the dystrophin isoforms

Blood was anticoagulated with EDTA and contaminating leukocytes from the platelet fraction were isolated using CD45-positive magnetic beads (Dynal beads, Invitrogen). Fibroblasts were trypsinized and centrifuged at 150g for 10 min. Total RNA was extracted from the platelets and fibroblasts using the TRizol reagent (Invitrogen) according to manufacturer’s recommendations. Approximately 1 μg RNA was used for the oligo (dT)-primed first-strand complementary DNA synthesis using M-MLV RT (Invitrogen). Purity of platelet RNA was confirmed by amplification of CD45, using CD45 specific primers. Forward primers to amplify dystrophin isoforms were constructed to recognize the specific isoform alone (Supplementary Material, Fig. S1) and sequences were analysed using BigDye terminator chemistry on an ABI310 (Perkin Elmer) sequencer. Details of the PCR conditions are available on request.

Statistical analysis

The significance of differences was determined by using the Mann–Whitney test. Two-sided P-values less than 0.05 were considered significant.
REFERENCES


SUPPLEMENTARY MATERIAL

Supplementary Material is available at HMG Online.

REFERENCES


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