Novel integrin-dependent platelet malfunction in siblings with leukocyte adhesion deficiency-III (LAD-III) caused by a point mutation in FERMT3

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Summary

Leukocyte adhesion deficiency-III (LAD-III) also called leukocyte adhesion deficiency-1/variant (LAD1v) is a rare congenital disease caused by defective integrin activation of leukocytes and platelets. Patients with LAD-III present with non-purulent infections and increased bleeding symptoms. We report on a novel integrin-dependent platelet dysfunction in two brothers with LAD-III syndrome caused by a homozygous mutation 1717C>T in the FERMT3 gene leading to a premature stop codon R573X in the focal adhesion protein kindlin-3. Stimulation of patients´ platelets with all used agonists resulted in a severely decreased binding of soluble fibrinogen indicating a defect in inside-out activation of the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa). Patients´ platelets did not respond to the $\alpha_2\beta_1$-integrin agonist aggretin-A at all. Our data on granula secretion indicate for the first time that the thrombin receptor PAR-4 but not PAR-1 may be important in integrin-triggered granule secretion in response to thrombin. In contrast, collagen mediated platelet granule secretion was not affected in LAD-III-patients. Thus, integrin-signalling may be not essential in collagen-induced granule secretion. The patients' peripheral blood mononuclear cells showed a severe loss of adhesion capacity to VCAM-1 and to endothelial cells compared to cells from healthy donors. Rap-1 activation after PMA stimulation could be observed in controls´ but not in patients´ cells. After haematogenesis stem cell transplantation (HSCT) the brothers showed no symptoms of bleeding or immunodeficiency and the integrin-dependent platelet and leukocyte functions normalised.

Keywords

Leukocyte adhesion deficiency, platelets, integrin, kindlin-3

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Introduction

Leukocyte adhesion deficiency-III (LAD-III), also described as leukocyte adhesion deficiency-1/variant (LAD1v), is associated with severe defects in leukocyte and platelet β_1-, β_2-, β_3-integrin activation despite of normal integrin expression (1–3). Platelets of LAD-III (LAD1v) patients fail to aggregate because of an impaired activation of the integrin β_3 (a subunit of the platelet receptor $\alpha_{IIb}\beta_3$, GPIIb/IIIa) leading to Glanzmann's thrombasthenia like bleeding symptoms (4). Leukocytosis in these patients is caused by a defect in leukocyte extravasation because of the impaired leukocyte integrin function (5). Patients with LAD-III suffer from recurrent bacterial infections (6).

Since Ca^{2+} and diacylglycerol-regulated guanine nucleotide exchange factor I (CalDAG-GEFI) knock-out mice show a combination of defects in leukocyte and platelet functions similar to that of LAD-III it had been hypothesised that *CalDAG-GEFI* is a candidate gene for LAD-III (7–10).

More recently *FERMT3*, encoding for kindlin-3, was identified as candidate gene for LAD-III (11–13). Kindlin-3, a member of an important family of focal adhesion proteins, the kindlins, contains a FERM domain located at the carboxyl terminus that bind to β-integrin cytoplasmic tails and cooperate with talin in integrin activation. Kindlin-3 is restricted to haematopoietic cells and is abundantly expressed in megakaryocytes and platelets (14, 15).

Here, we characterised extensively the integrin-dependent platelet function of two siblings with LAD-III before and after allogeneic stem cell transplantation (HSCT) and illustrate novel insights of integrin-dependent platelet granule secretion induced by thrombin and collagen. In addition, we analysed integrin-mediated leukocyte adhesion and Rap1 activation of leukocytes before and after HSCT. A homozygous nonsense mutation in the *FERMT3* gene, but no *CalDAG-GEFI* mutation was identified as cause for LAD-III in these two patients. This homozygous mutation in the *FERMT3* gene has been identified in only one other patient before (12).

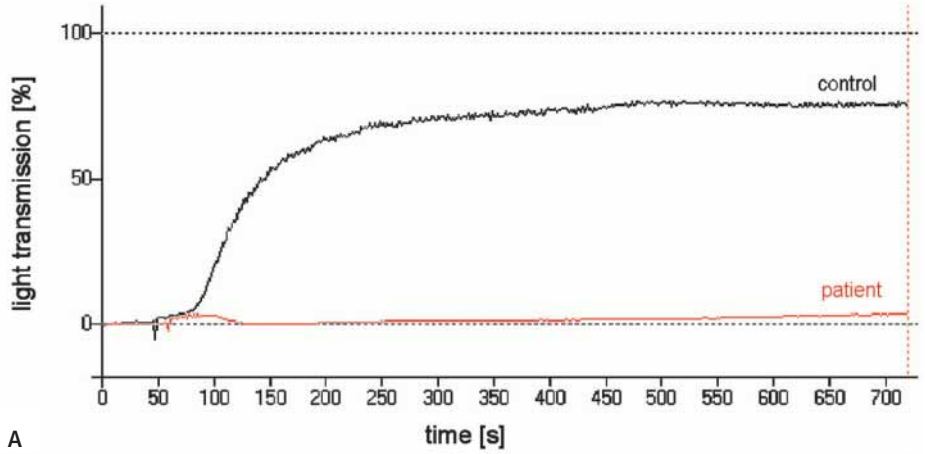
Patients, materials and methods

Patients

Patient 1

The older brother, born 1999, presented with recurrent spontaneous bleeding episodes of oral mucosa, epistaxis and recurrent bacterial infections since infancy. He received platelet-transfusions weekly and red blood cell transfusions every 4–6 weeks since two years of age. LAD-III was diagnosed at the age of 6.5 years; no severe or recurrent infections have been documented. At that time the patient presented with a splenomegaly, leukocytosis and hypochromic anemia, however, he showed no radiographic signs of osteosclerosis.

At the age of 6.8 years the patient underwent HSCT from an HLA-matched unrelated CMV-seropositive female donor. For pre-conditioning and GvHD prophylaxis the following drugs were used: Busulfex (4.4 mg/kg/day for 4 days), Fludarabin (40 mg/m²/day for 4 days), Cyclophosphamid (60 mg/kg/day for 2 days), ATG (Sangstat, Rabbit, 3 mg/kg/day for 3 days), Cyclosporin A (3 mg/kg/day) and Mycophenolatmofetil (1,200 mg/m²/day). The haematopoietic reconstitution was prompt, with granulocytes > 500/µl at day +23 and platelets > 20,000/µl at day +26; last platelet transfusion at day +18 and last erythrocyte transfusion at day +13. The complications mucositis °2–3, mild and transient elevation of liver enzymes without signs of VOD, acute GvHD °2, fever of unknown origin during aplasia, CMV reacti-



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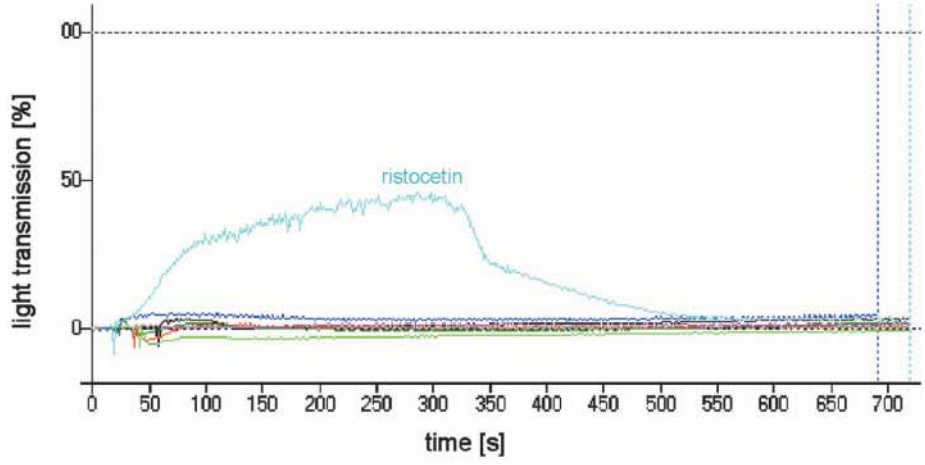

B

	stimulation	maximum aggregation
✓	collagen [2 µg/ml]	5,0 %
✓	collagen [10 µg/ml]	3,7 %
✓	ADP [2µmol/l]	no aggregation
✓	ADP [10µmol/l]	1,6 %
✓	epinephrine [8 µmol/l]	1,7 %
✓	arachidonic acid [0,5 mg/ml]	1,8 %
✓	ristocetin [1,2 mg/ml]	44,4 %

Figure 1: Platelet aggregation/agglutination of LAD-III patient before HSCT. A) Platelet aggregation after stimulation with collagen [2 µg/ml] in LAD-III patient and control. B) Platelet agglutination/aggregation after stimulation with ristocetin, collagen, ADP, epinephrine, and arachidonic acid. Data shown from patient 1 (n=1) are representative for both studied LAD-III patients.

vation 40 days after HSCT, bacterial pneumonia 50 days after HSCT, pneumonia and bacteriaemia (*Streptococcus pneumonia*) about six months after HSCT were successfully treated. Thirty-six months after HSCT the boy is alive and well with full donor chimerism documented by XY-FISH analysis (female donor) of peripheral blood leukocytes.

Patient 2

The younger brother, born 2005, presented with the same clinical symptoms. He was diagnosed with LAD-III at age of 2.75 years when the molecular diagnosis was made in his older brother. In addition, the younger brother showed radiographic evidence of os-

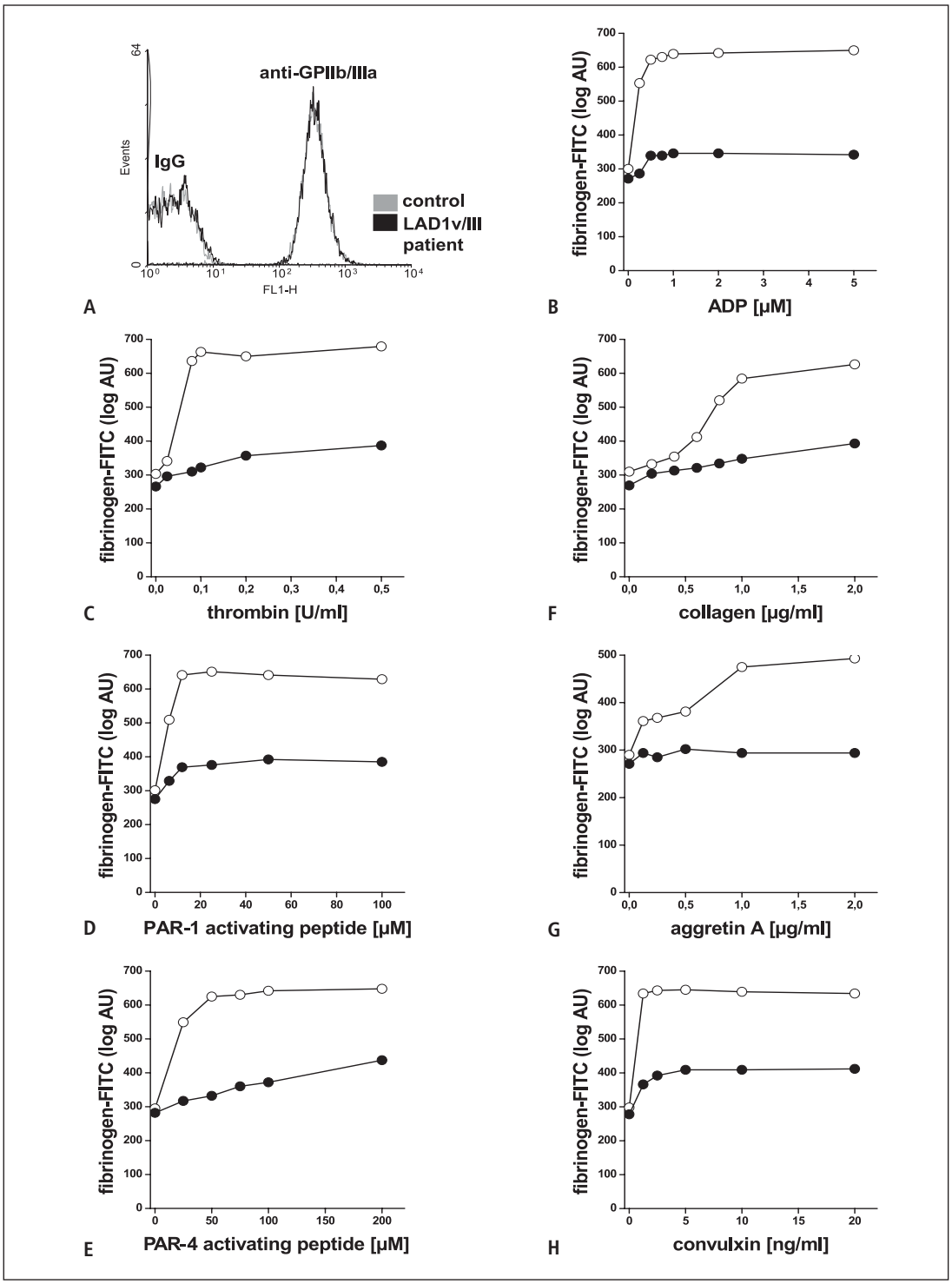

Figure 2: Analysis of LAD-III patient's platelet integrin GPIIb/IIIa before HSCT. Flow-cytometric analysis of platelet integrin GPIIb/IIIa surface expression (A) and agonist (ADP, thrombin, PAR-1 activating peptide, PAR-4 activating peptide, collagen, aggretin-A, convulxin)-induced binding of soluble fibrinogen to platelets (B-H). ❍ control, ● LAD-III patient. Data shown from patient 1 (n=1) are representative for both studied LAD-III patients.

teosclerosis resembling a mild form of osteopetrosis. At the age of 3.5 years the younger brother received HSCT from the same HLA-matched unrelated female CMV seropositive donor as his brother. The same protocol was used. The haematopoietic reconstitution was prompt, with granulocytes > 500/µl at day +22 and platelets > 20,000/µl at day +32; last platelet transfusion at day +27 and last erythrocyte transfusion at day +20. GvHD °I, CMV reactivation and bacterial pneumonia after HSCT were successfully treated. The patient now is 18 months after transplantation and suffers from mild chronic GvHD affecting the lungs which is treated with azathioprin and hydroxychloroquin. Both brothers are of Turkish descent.

Values for platelet and leukocyte function before and after HSCT obtained from both patients demonstrate in depth the native disordered haematopoietic system before HSCT and the donor hematopoietic system analysed 16 months and 12 months, respectively, after HSCT (▶Figs.1–6, ▶Table 1). Because data of both brothers were very similar before and after HSCT, the representative data from patient 1 are depicted.

Characterisation of platelet function in LAD-III patients

Isolation of platelets and platelet-poor plasma

Citrated-anticoagulated blood samples were obtained after informed consent from the controls, patient and the parents, respectively. For platelet function analysis platelet-rich plasma (PRP) and platelet-poor plasma (PPP) was prepared from citrated blood by centrifugation as described (16).

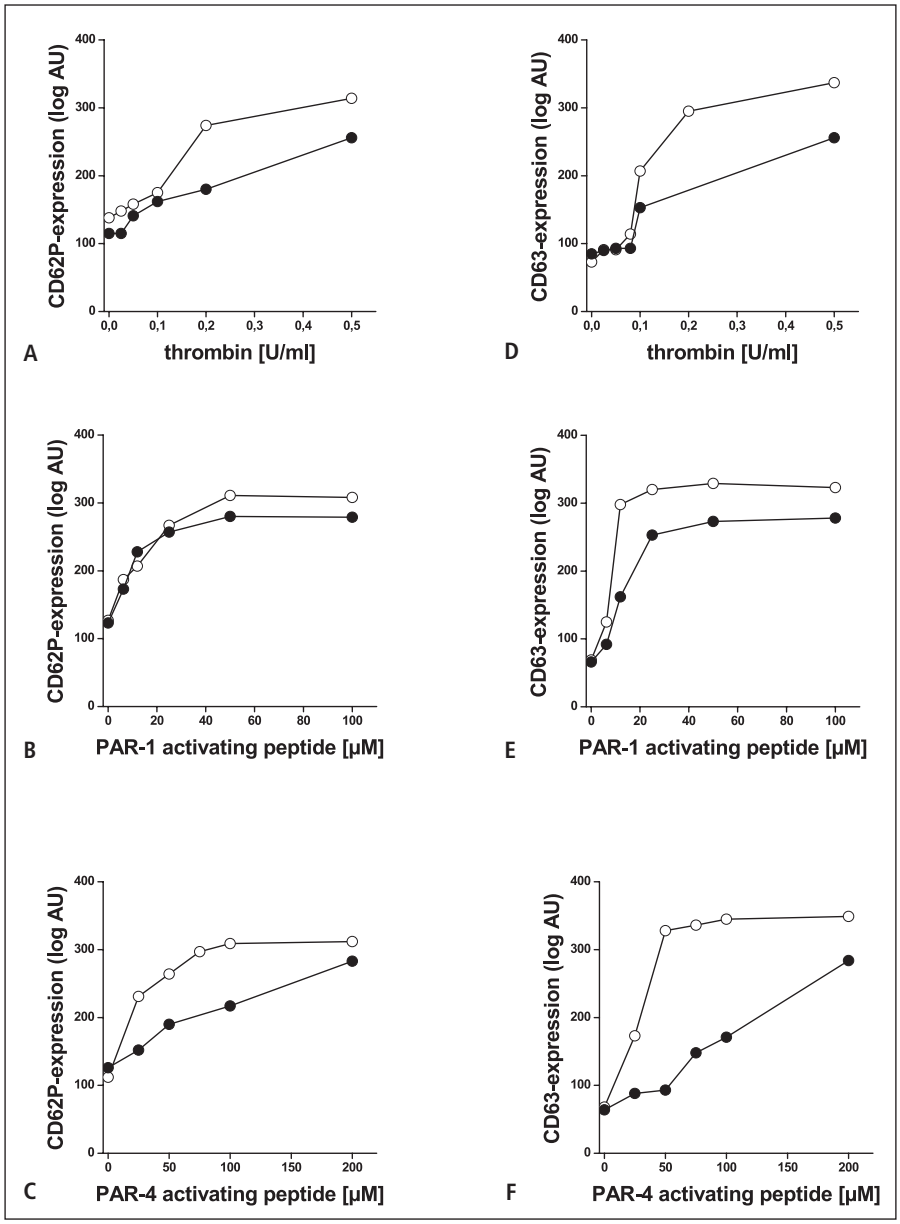

Figure 3: Thrombin-induced platelet granule secretion of LAD-III patient before HSCT. Thrombin-mediated expression of CD62P (A-C) and CD63 (D-F) on platelets detected by flow cytometry. ❍ control, ● LAD-III patient. Data are expressed as median of logarithmic fluorescence intensities. Data shown from patient 1 (n=1) are representative for both studied LAD-III patients.

Platelet aggregation assay

Platelet numbers in PRP were adjusted to a concentration of $25x10^7$ platelets/ml with PPP. Platelet aggregation was analysed on platelet aggregometer APACT 4 (Labor Fibrintimer, Ahrensburg, Germany) using following agonists: collagen (2.0 µg/ml, 4.0 µg/ml and 10.0 µg/ml, respectively; Nycomed, Zurich, Switzerland), adenosine diphosphate (ADP 2.0 µM, 4.0 µM, respectively; MP Biomedicals, Santa Ana, CA, USA), epinephrine (8 µM; Sanofi-aventis, Frankfurt, Germany), arachidonic acid (0.5 mg/ml; möLab, Langenfeld, Germany) and ristocetin (1.2 mg/ml; American Biochemical and Pharmaceutical LTd, London, UK).

Flow cytometry analysis of platelet surface GPIIb/IIIa and GPIb/V/IX ex vivo

Aliquots of diluted PRP ($5x10^7$ platelets/ml) were fixed as described (16). Fixed platelets were stained by fluorescein-labelled

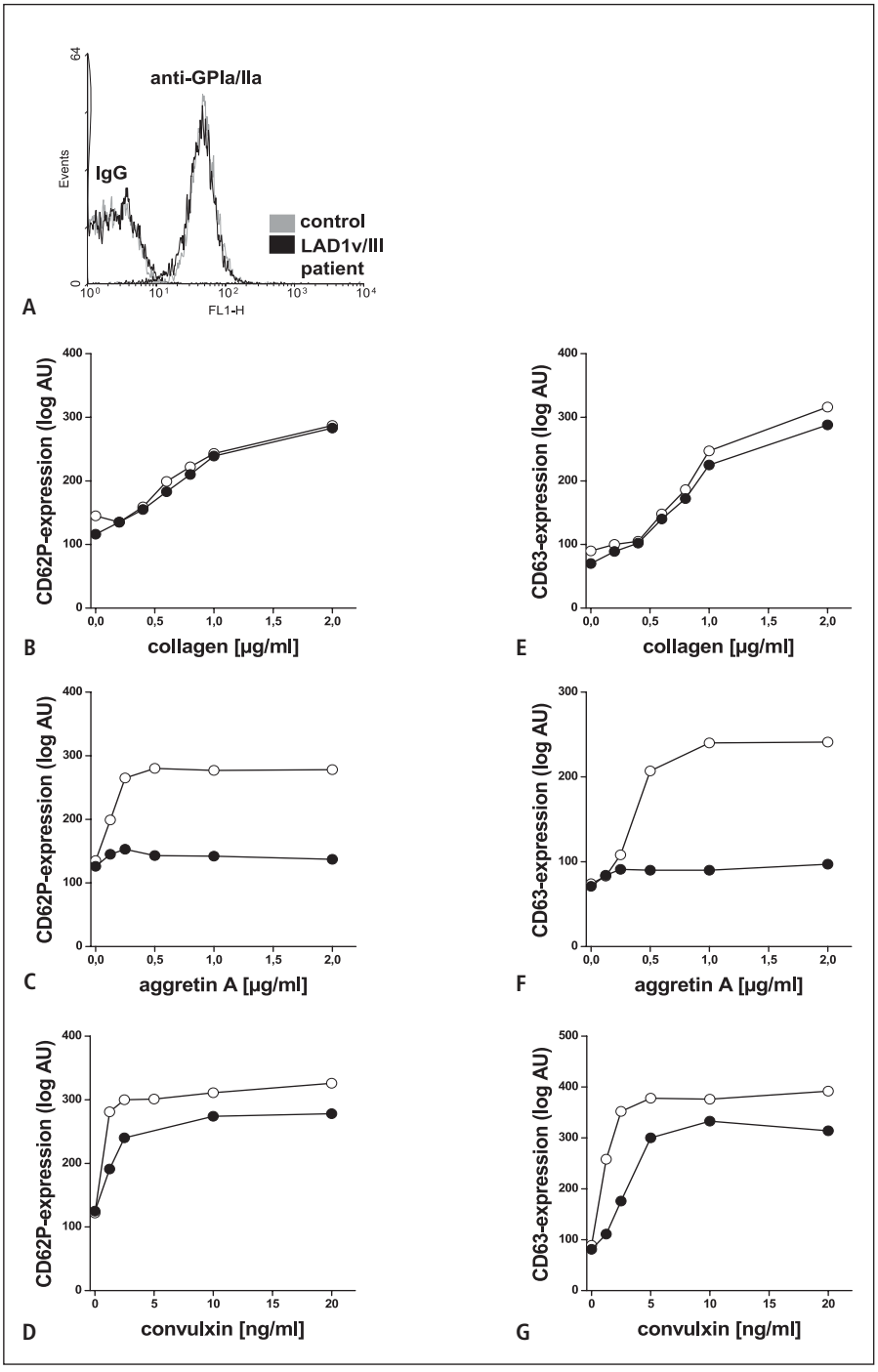

Figure 4: Collagen-induced platelet granule secretion of LAD-III patient before HSCT. Flow-cytometric analysis of platelet $\alpha_2\beta_1$-integrin (GPIa/IIa) surface expression (A) and collagen-mediated expression of CD62P (B-D) and CD63 (E-G) detected by flow cytometry. ❍ control, ● LAD-III patient. Data are expressed as median of logarithmic fluorescence intensities. Data shown from patient 1 (n=1) are representative for both studied LAD-III patients.

monoclonal antibody (mab) against CD41/CD61 (clone P2, Coulter, Immunotech, Marseilles, France) and analysed as described before (16). To analyse GPIb/V/IX expression a fluorescein-labelled mab against CD42b/CD42d/CD42a was used (clone SZ1, Coulter, Immunotech).

Flow cytometry analysis of fibrinogen binding to platelets

Diluted PRP ($5x10^7$ platelets/ml) was preincubated with fibrinogen-FITC as described (16) and stimulated with different concentrations of ADP (Sigma, Taufkirchen, Germany), soluble type I

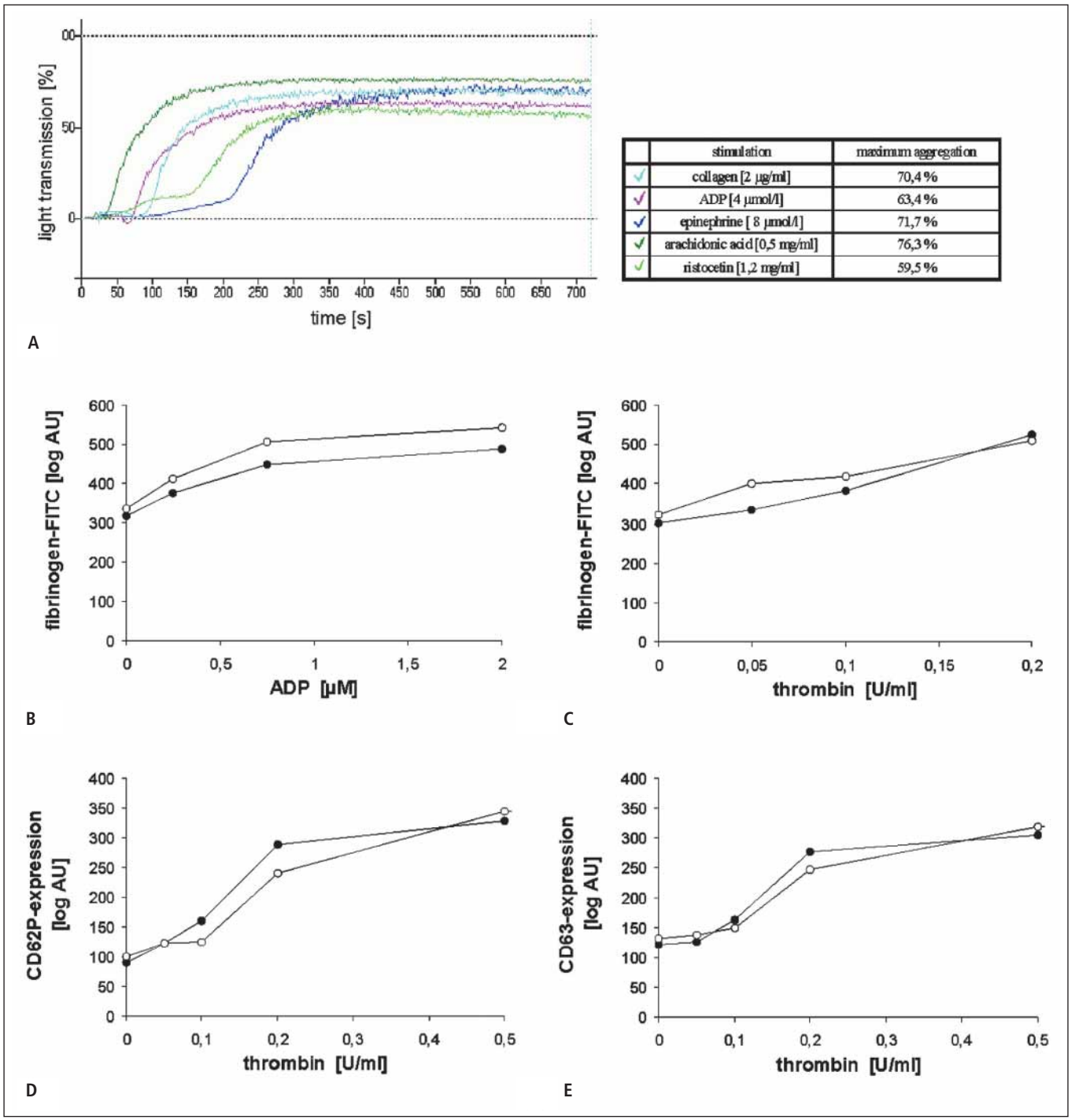

Figure 5: Platelet function of LAD-III patient after HSCT. A) Platelet agglutination and aggregation in LAD-III patient and control after stimulation with collagen, ADP, epinephrine, arachidonic acid and ristocetin. B, C) ADP- (B) and thrombin-induced (C) binding of soluble fibrinogen to platelets detected by flow-cytometric analysis. D, E) CD62P (D) and CD63 (E) expression on platelets after thrombin stimulation detected by flow cytometry. ❍ control, ● LAD-III patient. Data are expressed as median of logarithmic fluorescence intensities. Data shown from patient 1 (n=1) are representative for both studied LAD-III patients.

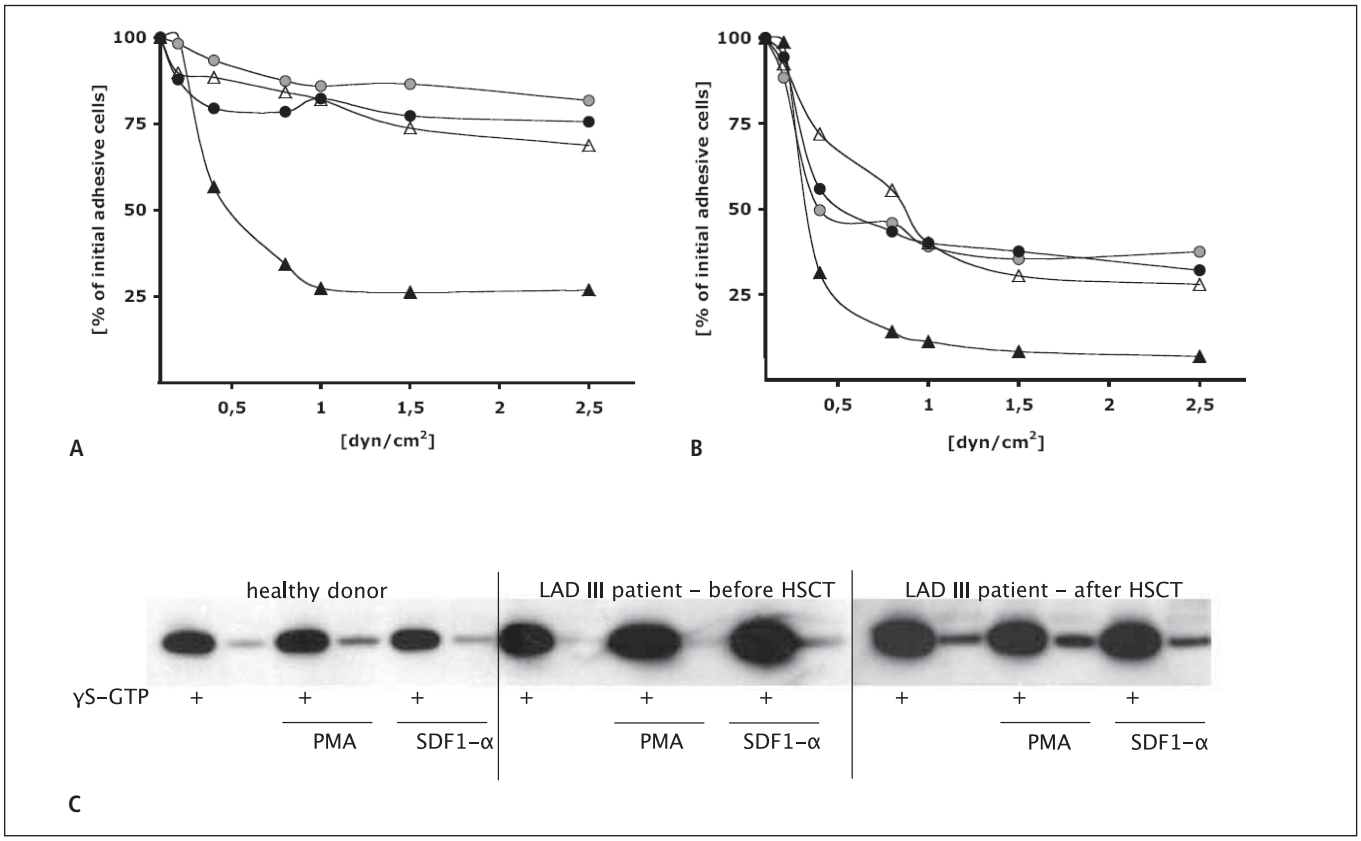

Figure 6: Leukocyte function of LAD-III patient before and after HSCT. Adherence capacity of PBMCs on VCAM-1 (A) and HUVEC (B) under different shear forces under laminar flow conditions. Values are normalised over initial adhesive cells at 0.1 dyn/cm^2. LAD-III cells have an impaired adhesive capacity compared to cells from healthy and transplanted donors on VCAM-1 and HUVEC. ● / ● healthy donors, ▲ LAD-III patient before HSCT, Δ LAD-III patient after HSCT. Data are expressed as mean of three independent experiments. C) Rap-1 activation in primary lymphocytes. Samples from a healthy donor and a LAD-III patient before HSCT and after HSCT were analysed in Rap-1 activation assay. γS-GTP was incubated with cells to detect total Rap-1. Stimulation with PMA [10 ng/ml], or SDF1-α [100 ng/ml]. LAD-III cells showed a reduced basal Rap-1 activity and failed to be stimulated by PMA. After HSCT Rap-1 activity is restored to normal levels. SDF1-α stimulation has no detectable effect on Rap-1 activity.

collagen (17), aggretin-A (kindly provided by KJ Clemetson, see Navdaev et al. [18]), convulxin (AXXORA, Lörrach, Germany, see Polgar et al. [19]), PAR-1 activating peptide SFLLRN (TRAP-6, Bachem, Weil am Rhein, Germany), PAR-4-agonist peptide Ala-Tyr-Pro-Gly-Lys-Phe (AYPGKF, Sigma) and alpha-thrombin (Sigma), for 3 minutes (min) at room temperature, respectively. Platelet activation by thrombin was performed in the presence of 1.25 mM of the peptide Gly-Pro-Arg-Pro to prevent fibrin-polymerisation. Platelets were fixed and analysed by flow cytometry as described (16).

Flow cytometry analysis of von Willebrand factor (VWF) binding to platelets

To measure VWF-binding, aliquots of diluted PRP (5x10^7 platelets/ml) were incubated with ristocetin (0.0 mg/ml; 0.2 mg/ml; 0.3 mg/ml; 0.5 mg/ml; 1.0 mg/ml) for 3 min at room temperature and fixed (16). Platelets were stained by anti-VWF-FITC (Serotec, Kidlington, UK) antibody and analysed as described before (16).

Flow cytometry analysis of platelet granule secretion

Diluted PRP (5x10^7/ml) was stimulated with different concentrations of ADP, soluble type I collagen, aggretin-A, convulxin, thrombin-receptor PAR-1 activating peptide SFLLRN (TRAP-6),

Table 1: Haematological parameters before and after HSCT.

	Before HSCT	After HSCT	Normal values
Haemoglobin [g/dl]	8.1	12.6	11.0 – 15.0
Haematocrit [%]	28	41	32 – 45
Red blood cell count [T/l]	3.7	5.06	3.9 – 5.1
Leukocytes [G/l]	23.9	11.4	5.0 – 14.0
Platelets [G/l]	279	364	100 – 436
MCV [fl]	74.7	80.0	77.0 – 89.0
MCH [pg]	22.0	25.0	25.0 – 31.0
MCHC [g/dl]	29.5	31.0	32.0 – 36.0

PAR-4-agonist peptide AYPGKF and alpha-thrombin, respectively. Platelet activation and fixation was performed as described above. Platelets were stained by monoclonal anti-CD62P antibody (CLBthromb/6-FITC, Coulter Immunotech) and anti-CD63 antibody (CLB-gran/12-FITC), respectively and analysed by flow cytometry.

Characterisation of leukocyte function in LAD-III patients

Flow chamber assay

Laminar flow chambers (Ibidi, Martinsried, Germany) were coated with 20 µg/ml human VCAM-1 (R&D Systems, Wiesbaden, Germany) for 30 min and blocked with 1% bovine serum albumin (BSA) prior to use. Alternatively chambers were pre-coated with 0.1% (wt/vol) gelatin (Sigma) and seeded with human umbilical vein ECs (HUVECs; Cambrex Bio Science, Verviers, Belgium) in EGM-2 medium (BulletKit, Cambrex Bio Science, Cambridge, UK) and grown till at least 90% confluency. Prior to use HUVECs were stimulated with 10 ng/ml TNFα (Tebu-Bio, Offenbach, Germany) for 4 hours. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation through Ficoll (Biochrom AG, Berlin, Germany), adjusted to 10^6 cells/ml in HBSS supplemented with 1% human plasma and 25 mM HEPES, and incubated for 30 min at 37°C, 5% CO_2. Using a perfusor pump connected to the inlet port via a 1/16-inch diameter tube, a uniform laminar flow was applied, setting the calculated shear forces. Prewarmed cells (5x10^6cells/ml) were allowed to adhere for 5 min. The flow rate was then increased stepwise to yield indicated shear forces and allowed to reach steady-state condition. Photographs were taken using a CCD camera (D-73431; Sony, Cologne, Germany) mounted on an inverted-stage microscope (Axiovert 135; Zeiss, Oberkochen, Germany) equipped with a 10 x objective (Zeiss). Numbers of adherent cells were counted with assistance of image software (National Institutes of Health, Bethesda, MD, USA).

Determination of Rap-1 activation

Epstein-Barr virus (EBV) transformation of peripheral blood lymphocytes from patients and a healthy donor was performed following a protocol reported by Steel et al. (20). After prestimulation of phosphate-buffered saline (PBS)-washed cells with PMA (10 ng/ml or 100 ng/ml for 5 min at 37°C; Sigma, see Kinashi et al. [8]) or SDF1-α [100 ng/ml] 10 seconds at 37°C (R&D Systems) stimulation was stopped by addition of cold PBS (4°C). Cells were spun down at 4°C and further treated according to manufacturers´ protocol (Pierce # 89872Y). In brief: cell lysates were divided into two parts, of which one was treated with GTPγS to activate all Rap-1 *in vitro* as a control, representing total Rap-1. In the other part, only active – GTP-bound-Rap-1 – was analysed. Active Rap-1 was precipitated with RalGDS-RBD and detected by Western analysis.

DNA-sequencing analyses

DNA was isolated from mononuclear cells prior to HSCT. PCR products were generated to amplify genomic DNA of both *CalDAG-GEFI* (complete gene, all exons according to NCBI Gene ID#10235) and *FERMT3* (all exons according to Kuijpers et al. [12], NCBI Gene ID#83706) genes by standard protocols. Oligonucleotide primers were designed to allow sequencing of all exons including 20 nucleotides of the splice junctions. Single PCR products were gel purified and subjected to sequencing. Primer sequences for *CalDAG-GEFI* are available upon request.

Results

Analyses of haematological and coagulation parameters

Before HSCT, hypochromic anaemia was observed in both children (haemoglobin [Hb] 8.1 g/dl, red blood cells 3.7 G/l, haematocrit 28%, MCV 75.2 fl, MCH 22.3 pg, MCHC 29.7 g/dl; 26% reticulocytes) (in patient 1). Leukocyte count was markedly increased (31.3 G/l with 63% neutrophil granulocytes, 25% lymphocytes, 6% monocytes, 2% eosinophils) (in patient 1), whereas the platelet count was normal. In spite of the normal platelet count (279 G/l), the bleeding time (Ivy) was severely prolonged (> 15 min; normal 2–6 min) indicating impaired primary haemostasis. Von Willebrand disorder was excluded because of normal values for von VWF antigen (71 %), VWF collagen binding capacity (95%) and VWF multimeric analysis. Therefore, extensive analyses of platelet function using platelet aggregometry and flow cytometry analyses were performed (in patient 1).

After HSCT normal values for red blood cell count (Hb 11.1 g/dl), leukocytes count (12.7 G/l), platelet count (356 G/l) and bleeding time (Ivy) were measured (4 min) (in patient 1).

Platelet function before HSCT

Platelet aggregrometry

Before HSCT no platelet aggregation after stimulation with collagen (2 µg/ml and 10 µg/ml, respectively) was detected (▶Fig. 1A, B). Additionally, no platelet aggregation was seen after stimulation with ADP (2 µM, 10 µM), epinephrine (8 µM) or arachidonic acid (0.5 mg/ml) (▶Fig. 1B). The maximal platelet agglutination after stimulation with ristocetin (1.2 mg/ml) was 44%.

Platelet surface expression of GPIIb/IIIa, GPIb/V/IX and binding of fibrinogen and of VWF

FACS analysis of platelets from both LAD-III patients showed no preactivation *in vivo* and normal surface expression patterns of the integrin GPIIb/IIIa (CD41/CD61) (▶Fig. 2A) *ex vivo*. Expression

patterns of GPIb/V/IX (CD42b/CD42d/CD42a) as well as ristocetin-induced VWF-binding measured by flow cytometry showed normal values (data not shown).

However, stimulation of patients´ platelets with ADP, thrombin, collagen, PAR-1 agonist (TRAP/thrombin-receptor PAR-1 activating peptide SFLLRN), PAR-4 agonist (peptide AYPGKF), collagen, aggretin-A ($\alpha_2\beta_1$-integrin agonist) and convulxin (GPVI-agonist) *in vitro* resulted in a severely decreased binding of soluble fibrinogen; e.g. up to less than 10% of control binding (▶Fig. 2B-H) indicating an activation defect of the platelet integrin GPIIb/IIIa receptor. Data are expressed as the median of fluorescence intensities in a logarithmic mode obtained from flow cytometry analysis.

Platelet-granule-secretion

Stimulation of patients' platelets with thrombin or PAR-4 activating peptide AYPGKF showed impaired α-granule and dense body/lysosome secretion, measured by surface expression of P-selectin (CD62P) and CD63, respectively. In contrast, granule secretion induced by the PAR-1 agonist did not differ from control platelets (▶Fig. 3A-F). In addition, collagen and convulxin (GPVI agonist)-induced granule secretion was either not or only very mildly impaired in patients' platelets. However, platelet stimulation with the $\alpha_2\beta_1$-integrin agonist aggretin-A did not result in any granule secretion response despite of normal $\alpha_2\beta_1$-integrin surface expression (▶Fig. 4).

Platelet function after HSCT

Platelet aggregometry

After HSCT platelet aggregation parameters turned to normal values after stimulation with the following agonists: collagen (2 µg/ml), ADP (4 µM), epinephrine (8 µM) and arachidonic acid (0.5 mg/ml) and ristocetin (1.2 mg/ml), respectively (▶Fig. 5A).

Platelet surface expression of GPIIb/IIIa, GPIb/V/IX and binding of fibrinogen and of VWF

After HSCT the surface expression patterns of integrin GPIIb/IIIa (CD41/CD61) and GPIb/V/IX (CD42b/CD42d/CD42a) of patients´ platelets *ex vivo* stayed within normal limits (data not shown). Fibrinogen binding of platelets after stimulation with ADP or thrombin restored to normal values (▶Fig. 5B, C). The VWF binding of platelets after stimulation with ristocetin was normal (data not shown).

Platelet-granule-secretion

After HSCT thrombin-induced CD62P- and CD63-surface expression on patients´ platelets was normalised (▶Fig. 5D, E).

Adhesion of peripheral blood mononuclear cells on vascular cell adhesion molecule (VCAM)-1 and human umbilical vein endothelial cells (HUVECs)

Peripheral blood mononuclear cells (PBMCs) of both patients were assayed before and after HSCT for their *in vitro* adhesion capacity in a defined shear flow system. On VCAM-1, a major endothelial adhesion receptor for leukocytes, a severe loss of adhesion capacity of LAD-III cells was observed compared to cells from healthy donors. This defect was restored by PBMCs after HSCT. One experiment was performed on VCAM-1 showing a severe loss of adhesion capacity of LAD-III cells compared to cells from healthy donors (▶Fig. 6A). The experiment performed on HUVECs had a lower basal adhesiveness of cells but paralleled the results obtained on VCAM-1 (▶Fig. 6B). Thus, a defect in shear stress resistant adhesion of PBMCs cells is observed in LAD-III.

Rap-1 activity of blood lymphocytes

Defective activation of the Rap-1 GTPase has been described in patients with LAD-III (10, 21). Therefore, we determined Rap-1 activation in primary and EBV-transformed blood lymphocytes using the RalGDS-RBD pull down assay. With PMA stimulation Rap-1 activation was observed with exception of LAD-III cells before HSCT, indicating a loss of function. This defect was reconstituted after HSCT (▶Fig. 6C). These results were shown twice in primary cells. However, these results could not be reproduced in EBV cell lines.

DNA-sequencing analyses

CalDAG-GEFI is a pivotal regulator of Rap-1 activation in platelets. Granulocytes and mice deficient for *RASGRP2* (gene which encodes CalDAG-GEFI) phenocopy defects which are observed in LAD-III (7). Therefore, we screened all 17 exons including the intron-exon junctions, but did not detect any mutation. The intronic position adjacent to the splice site at the beginning of exon 16 which was mutated in the LAD-III patients originally described by Pasvolsky et al. (22) was not mutated in the patients described here.

We next analysed kindlin-3 and sequenced the *FERMT3* gene. Both brothers had wild-type sequence at positions W229 and R509, but harbored the homozygous mutation 1717C>T leading to a premature stop codon R573X in kindlin-3 (▶Fig. 7). Thus, our data demonstrate that the patients do not have a fully functional kindlin-3 protein.

Discussion

We have identified two new siblings with LAD-III syndrome caused by a point mutation in *FERMT3*. Detailed platelet function

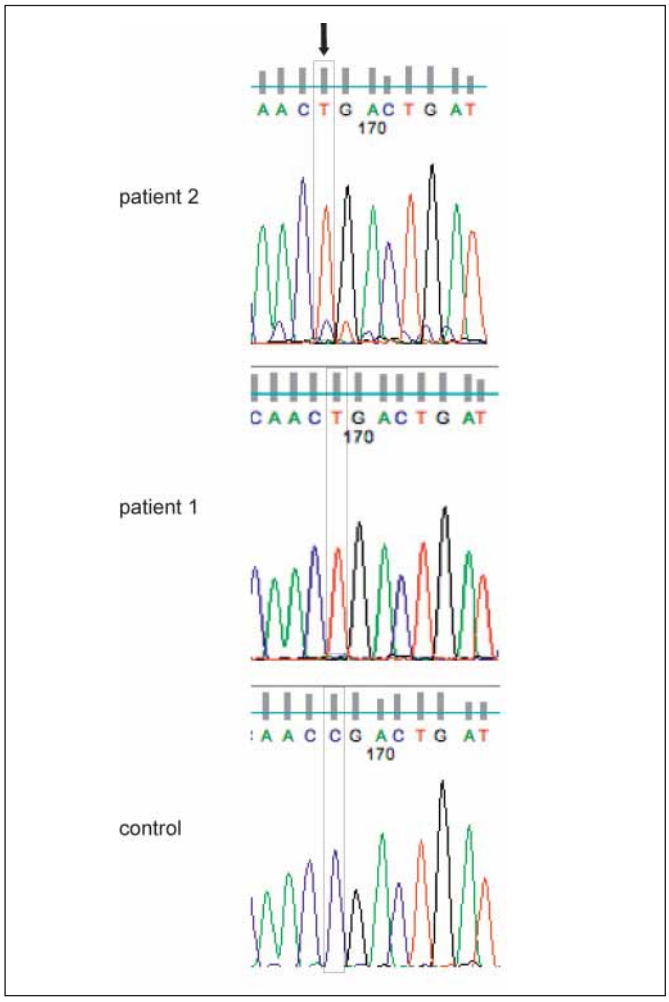

Figure 7: Sequencing analysis of FERMT3. Genomic DNA was PCR-amplified and subjected to sequencing. The homozygous mutation 1717C>T results in R573X stop codon.

analyses of these patients showed for the first time that in response to thrombin the thrombin receptor PAR-4 but not PAR-1 may be important in integrin-triggered granule secretion whereas integrin-signalling may be not essential in collagen-induced granule secretion. Allogeneic stem cell transplantation abrogated the patients' clinical symptoms as well as integrin-mediated functional defects in platelets and leukocytes.

Prior HSCT both patients presented with hypochromic anaemia, recurrent bacterial infections, leukocytosis, recurrent mucocutaneus bleeding episodes associated with severe prolonged bleeding time but normal platelet count since birth. A defect in primary haemostasis was suspected. Interestingly, standard platelet aggregometry tests showed Glanzmann's thrombasthenia-like characteristics, e.g. near complete missing response to collagen, ADP, epinephrine and arachidonic acid and normal or reduced response to ristocetin, indicating a quantitative or qualitative defect of the integrin $\alpha_{IIb}\beta_3$ (GPIIb/IIIa) (for review, see Jurk and Kehrel [23]). Von Willebrand syndrome was excluded. Markedly persistant leukocytosis suggested a leukocyte adhesion defect. The ability of the patients' blood mononuclear cells to adhere to the β1-integrin ligand VCAM-1 or to the macrovascular endothelial cells HUVECs, predominantly via β1- and β2-integrins, was impaired under shear stress conditions. Our results are consistent with recent studies in mice where neutrophils and mononuclear cells lacking kindlin-3 expression showed abolished firm adhesion and arrest on VCAM as well as on activated endothelial cells (24).

Because of the observed integrin-dependent platelet and leukocyte dysfunction a LAD-III syndrome was suspected which is caused by a defect in the activation of β_1-, β_2- and β_3-integrins on platelets and leukocytes despite of normal integrin expression (2, 12).

Rap-1, a small GTPase, which functions as a key regulator of inside-out integrin activation, had been described as abnormally regulated in LAD-III lymphocytes (10). Crittenden et al. (10) demonstrated that LAD-III cells had reduced Rap-1 activity, not even activatable with PMA, a potent stimulant of CalDAG-GEF1. Alon and Pasvolsky (21, 22) also have demonstrated an aberrant activity of Rap-1 in LAD-III and reduced Rap-1 activation in a single EBV-transformed B-cell line from one patient. Interestingly, a defect of Rap1 activation was also detected in the patients described here. However, the cause of this Rap1 activation defect could not be clarified so far.

Several intracellular signalling molecules are involved in the activation of leukocyte and platelet integrins. In CalDAG-GEFI-deficient platelets impaired β-integrin activation has been identified (10). Therefore, it had been postulated that CalDAG-GEFI might be a candidate gene for LAD-III. Kilic et al and Mory et al. described that they had identified impaired expression of CalDAG-GEFI in human haematopoietic cells as result of a homozygous splice junction mutation in exon 16 in patients with LAD-III (9, 11). Although CalDAG-GEFI is a potent activator of Rap-1 and our LAD-III patients showed impaired Rap-1 activation, no splice junction mutation was identified in the *CalDAG-GEFI* gene.

Recently, several groups described mutations in the kindlin-3 gene *FERMT3*, leading to LAD-III syndrome (6, 11–13, 25). The focal adhesion protein kindlin-3 contains a FERM domain which interacts with integrin β tails resulting in activation of the integrins. *FERMT3*$^{-/-}$ mice suffered from severe defect in platelet β_3- and β_1-integrin and leukocyte β_2-integrin activation (26). Mory et al. (11) and Kuijpers et al. (12) demonstrated that a homozygous stop codon in R513 (CGA>TGA) of kindlin-3 caused LAD-III in three patients and in seven other families. All these patients presented with the splice site mutation in exon 16 of *CalDAG-GEFI*. In contrast, Svensson et al. (6) and Malinin et al. (13) presented non-Turkish LAD-III patients with distinct stop codon mutations in the *FERMT-3* gene but without mutations in *CalDAG-GEFI* and normal Rap-1 activity in platelets and leukocytes. In addition, Kuijpers et al. (1) illustrated two other patients with LAD-III carried different stop codons in kindlin-3 (R573X and W229X, respectively) which caused LAD-III and interestingly, these two patients carry the wild-type *CalDAG-GEFI* gene, too. These observations lead to the hypothesis that the *CalDAG-GEFI* splice site mutation might co-segregate with the R513X mutation in kindlin-3.

We identified the nonsense homozygous mutation R573X in exon 14 of *FERMT3* gene in the two Turkish brothers (1717C>T, p.

R573X). This mutation causing LAD-III has been described only in one patient so far who originated from the South-Eastern region of Turkey (12). As these two brothers the Turkish patient did not carry a *CalDAG-GEFI* mutation.

Interestingly, *FERMT3*$^{-/-}$ mice developed severe anaemia (26). The two brothers also suffered from anaemia. If the anaemia is caused by the *FERMT3* mutation or is due to the recurrent bleeding symptoms remains unsolved. Remarkably, the younger brother was diagnosed with osteopetrosis by radiologic changes showing mild osteosclerosis as has been observed from kindlin-3-deficient mice, too (26). Radiologic signs of osteopetrosis disappeared within one year after transplantation. Malinin et al. (13) reported also about two patients with osteopetrosis and LAD-III caused by a kindlin-3 mutation.

The brothers´ expression of $\alpha_{IIb}\beta_3$ on platelets was normal as described for other LAD-III patients previously (12, 13). The brothers' activated platelets showed severely impaired binding of soluble fibrinogen, confirming the defect of $\alpha_{IIb}\beta_3$-integrin inside-out activation in kindlin-3-deficient human platelets (26) as well as in LAD-III platelets with defective Rap-1 activation (8, 22).

Platelet granule secretion induced by thrombin is triggered by $\alpha_{IIb}\beta_3$-mediated outside-in signalling subsequent to fibrinogen ligation (27). Therefore, we assessed thrombin- as well as the human thrombin receptors PAR-1 and PAR-4-mediated P-selectin (marker of α-granule secretion) and CD63 (marker of dense body and lysosome secretion)-surface expression on platelets from LAD-III patients by flow cytometry. Consistently with others we observed reduced thrombin-induced translocation of P-selectin on kindlin-3-deficient human platelets (13, 26). Additionally, thrombin-induced CD63 surface expression was significantly diminished in these patients, too. Furthermore, the LAD-III platelets showed significantly impaired P-selectin and CD63 surface expression in response to PAR-4 agonist peptide whereas PAR-1-agonist peptide-induced granule secretion was normal in these patients. Our data indicate for the first time that in response to thrombin the thrombin receptor PAR-4 rather than PAR-1 may be important in integrin-triggered platelet granule secretion. Although intracellular signals generated from both thrombin receptors activate $\alpha_{IIb}\beta_3$-integrin and mediate granule secretion in human platelets there is recent evidence that the signalling pathways downstream of the two PARs are different. PAR-1 as well as PAR-4 are coupled to $G\alpha_q$ and $G\alpha_{12/13}$ pathways (28). However, recent studies demonstrate that PAR-1 seems to be additionally coupled to $G\alpha_{i/0}$ pathways amplifying the increase in intra-platelet calcium concentration and granule secretion due to a PI3K-dependent extracellular calcium entry (29). Therefore, outside-in signalling via activated $\alpha_{IIb}\beta_3$-integrin seems to be important for entire platelet granule secretion induced by thrombin. Additionally, our results suggest that defective $\alpha_{IIb}\beta_3$-integrin activation and subsequent lack of outside-in signalling in LAD-III platelets may be bypassed and /or compensated in PAR-1- but not in PAR-4-mediated granule secretion through $\alpha_{IIb}\beta_3$-integrin-independent signalling pathways.

To test whether our LAD-III patients with normal platelet $\alpha_2\beta_1$-integrin expression show also defects in β_1-integrin-mediated platelet function we investigated platelet granule secretion induced by collagen, by the $\alpha_2\beta_1$-integrin agonist aggretin-A and by the GPVI-agonist convulxin. Patients´ platelet granule secretion induced by collagen and convulxin was normal whereas no response was observed of aggretin-A-treated LAD-III platelets. Although aggretin-A has been described to be not only a selective $\alpha_2\beta_1$-integrin agonist but also activates GPIb (18) we concluded that the missing granule secretion response in our LAD-III patients after aggretin-A treatment is due rather to a $\alpha_2\beta_1$-integrin defect than to a GPIb defect. Thus, $\alpha_2\beta_1$-integrin/GPIb-dependent signalling seems to be not essential in collagen-mediated platelet

What is known about this topic?

- Leukocyte adhesion deficiency-III (LAD-III), also called leukocyte adhesion deficiency-1/variant (LAD1v), is a rare congenital disease caused by defective activation of β_1-, β_2- and β_3-integrins of leukocytes and platelets despite of normal integrin expression.
- Patients with LAD-III suffer from recurrent bacterial infections and show a defect in leukocyte extravasation leading to leukocytosis due to the impaired leukocyte integrin function.
- Platelets of LAD-III patients fail to aggregate because of an impaired activation of the β_3-integrin subunit of the platelet receptor $\alpha_{IIb}\beta_3$, GPIIb/IIIa, leading to Glanzmann's thrombasthenia like bleeding symptoms.
- CalDAG-GEFI and FERMT3 (encoding the β-integrin adapter protein kindlin-3) have been described as candidate genes for LAD-III.

What does this paper add?

- We identified two young brothers with LAD-III syndrome who presented with recurrent mucocutaneus bleeding episodes since infancy, hypochromic anaemia, leukocytosis and splenomegaly. The younger brother showed radiographic evidence of osteosclerosis resembling a mild form of osteopetrosis.
- LAD-III platelets show a Glanzmann's thrombasthenia-like phenotype associated with impaired granule secretion in response to thrombin and PAR-4 activating peptide but not in response to PAR-1 activating peptide TRAP. Thus, the thrombin receptor PAR-4 but not PAR-1 may be important in integrin-triggered granule secretion induced by thrombin.
- LAD-III platelets show normal platelet granule secretion induced by collagen and by the GPVI-agonist convulxin whereas no response was observed by the $\alpha_2\beta_1$-integrin agonist aggretin-A. Therefore, integrin-signalling may be not essential in collagen-induced granule secretion.
- A homozygous nonsense mutation 1717C>T in the FERMT3 gene leading to a premature stop codon R573X was identified as cause for LAD-III in these two patients. This homozygous mutation in the FERMT3 gene has been identified in only one other LAD-III patient before.
- After haematogenesis stem cell transplantation the brothers showed no symptoms of bleeding or immunodeficiency and the integrin-dependent platelet and leukocyte functions normalised, which has been described in only very few infants with LAD-III before.

granule secretion confirming other studies that the $\alpha_2\beta_1$-integrin is primarily important for platelet adhesion to collagen whereas GPVI is a major receptor for collagen-induced platelet signal transduction (30). In addition, our results demonstrate that $\alpha_{IIb}\beta_3$-integrin-mediated outside-in signalling in response to collagen might not be essential for collagen-induced platelet granule secretion. Therefore, it is important to distinguish the differential role of integrin-signalling in thrombin- and collagen-mediated granule secretion for supporting a diagnosis of LAD-III syndrome.

In conclusion, LAD-III platelets show a Glanzmann's thrombasthenia-like phenotype associated with impaired granule secretion in response to thrombin whereas defective granule secretion induced by collagen is not mandatory.

Patients with LAD-I and patients with LAD-III, respectively, have been treated successfully with bone marrow transplantation (11, 13, 31). Our report shows that both patients showed no further symptoms of bleeding or immunodeficiency and that the activation of the platelet β_3 integrin normalised after HSCT. Additionally, the defective adhesion of patients' blood mononuclear cells to the β_1 integrin ligand VCAM-1 and to HUVECs under shear stress conditions was restored after HSCT. Thus, in addition to the correction of the platelet adhesion defect, normalisation has also been reached in lymphocytes after HSCT. After HSCT, Rap-1 activation was normal in the brothers' lymphocytes, too. Besides clinical and other laboratory parameters, a detailed analysis of platelet and leukocyte integrin function provided a good monitoring of LAD-III syndrome before and after HSCT.

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