Platelet Proteome Analysis Reveals Integrin-dependent Aggregation Defects in Patients with Myelodysplastic Syndromes*§

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Bleeding complications are a significant clinical problem in patients with myelodysplastic syndromes even at sufficient platelet counts (>50,000/µl). However, the underlying pathology of this hemorrhagic diathesis is still unknown. Here, we analyzed the platelet proteome of patients with myelodysplastic syndromes by quantitative two-dimensional difference gel electrophoresis followed by mass spectrometric protein identification. Proteins identified with lower concentrations, such as Talin-1, Vinculin, Myosin-9, Filmain-A, and Actin play critical roles in integrin αIIbβ3 signaling and thus platelet aggregation. Despite normal agonist receptor expression, calcium flux, and granule release upon activation, the activation capacity of integrin αIIbβ3 was diminished in myelodysplastic syndrome platelets. Förster resonance energy transfer analysis showed a reduced co-localization of Talin-1 to the integrin’s β3-subunit, which is required for receptor activation and fibrinogen binding. In addition, platelet spreading on immobilized fibrinogen was incomplete, and platelet aggregation assays confirmed a general defect in integrin-dependent platelet aggregation in patients with myelodysplastic syndromes. Our data provide novel aspects on the molecular pathology of impaired platelet function in myelodysplastic syndromes and suggest a mechanism of defective integrin αIIbβ3 signaling that may contribute to the hemorrhagic diathesis observed in these patients. Molecular & Cellular Proteomics 12: 10.1074/mcp.M112.023168, 1272–1280, 2013.

Myelodysplastic syndromes (MDS)¹ are among the most frequent hematological malignancies (1). Each of the hematopoietic lineages may be affected by dysplasia and/or cytopenia, and patients suffering from this clonal bone marrow disease are affected by anemia and infectious as well as hemorrhagic complications (1, 2). Thrombocytopenia itself is prevalent in up to 65% of patients with MDS at the time of diagnosis (3). Recently, a detailed study of the clinical features of 2900 patients with MDS reported that even among patients with platelet counts of >50,000/µl, 19% had signs of bleeding at the time of diagnosis (4). Taken together, these data suggest a platelet count-independent functional platelet defect in MDS, which is supported by sporadic reports indicating insufficient platelet aggregation (3, 5–8). The existence of such a functional platelet defect may very well contribute to the high rate of hemorrhagic complications observed in patients with MDS.

Platelet aggregation following a vascular injury or other stimuli is a highly organized process to ensure optimal efficacy. Exposure to collagen, von Willebrand factor, or soluble platelet agonists such as ADP, thrombin, or thromboxane A2 at the site of injury attracts platelets and initiates platelet adhesion. This activates and recruits additional platelets from the blood to finally cover the injury (9, 10). The fibrinogen receptor (integrin αIIbβ3) plays an essential role in this process (10–13). On resting platelets, integrin αIIbβ3 resides in a low affinity state and depends on intrinsic signals for activation and function (14). The final step in this activation process is the co-localization of Talin-1 from the cytoplasm to the β-subunit of the integrin (12, 13, 15, 16). Once activated, αIIbβ3 is linked to the actin cytoskeleton by several linker proteins, which is required for platelet aggregation and spreading (12, 17, 18). Hence, a series of key proteins like Talin-1, Kindlin-3, Vinculin, Actin, and Myosin-9 need to be expressed in platelets for sufficient integrin αIIbβ3 function and hemostasis. Despite some evidence that a functional platelet defect may exist in MDS, no data are yet available to explain the hemorrhagic diathesis in those patients who present with sufficient platelet counts. In this study, we performed a comprehensive analysis validating peptide, CD, cluster of differentiation; MFI, mean fluorescence intensity.

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¹ The abbreviations used are: MDS, myelodysplastic syndrome; PE, phycoerythrin; APC, allophycocyanin; TRAP, thrombin receptor activating peptide, CD, cluster of differentiation; MFI, mean fluorescence intensity.
of the platelet proteome of MDS patients and MDS platelet aggregation. We show that the critical integrin αMβ2 is functionally impaired and that a series of proteins essential for its proper function have a reduced expression in MDS.

**EXPERIMENTAL PROCEDURES**

**Patient Characteristics**—The 66 MDS patients included in this study had a median age of 66 years and a median platelet count of 100,000/μl and did not receive platelet transfusion or any medication possibly interfering with platelet function. Please note that not all patient samples were used in each experiment, and for detailed patient characteristics and application to the single experiments, see supplemental Table S1. Informed consent was obtained according to a protocol approved by the ethics committee of the Heinrich-Heine-University, Düsseldorf, Germany.

**Platelet Preparation**—Platelets were prepared according to a standard operating procedure of the Heinrich-Heine-University Clinic. Citrate-anticoagulated peripheral blood was centrifuged for 30 min at 150 × g to obtain platelet-rich plasma. Platelets were isolated from platelet-rich plasma by a 10-min centrifugation at 300 × g, washed, and stored in modified Tyrode’s/Hepes buffer (12). The purity of the samples for residual leukocytes and erythrocytes was checked by flow cytometry.

**Two-dimensional Difference Gel Electrophoresis**—Platelets of seven MDS patients and seven normal donors were lysed (7 mM urea, 2 mM thiourea, 25 mM Tris, 4% CHAPS, protease, and phosphatase inhibitors), sonicated, and centrifuged (60 min, 35,000 × g, 4 °C). The protein concentration of the supernatants was determined by protein assay according to the manufacturer’s instructions (Bio-Rad). Fluorescence labeling of the single lysates with cyanine dyes was done as recommended by the manufacturer (GE Healthcare). To eliminate dye-specific differences, 1 aliquot of each sample was labeled with Cy3 and one with Cy5 (dye-swap). An internal standard for all 14 dye-specific differences, 1 aliquot of each sample was labeled with Cy2, one MDS patient labeled with Cy3, and one healthy donor labeled with Cy5 or vice versa) were combined per gel and applied to Immobiline™ DryStrips (GE Healthcare) with cup loading. Isoelectric focusing was performed on a MultiPhor II (GE Healthcare) as described previously (19). The strips were then loaded onto linear 12.5% polyacrylamide gels, and second dimension separation was performed using a Laemmli buffer system on an EttanDalt 12 system (GE Healthcare) as described previously (19).

**Gel Imaging and Analysis**—Gels were scanned using a Typhoon 9400 (GE Healthcare), and protein spot abundances and statistics were determined by ProteomeWeaver 4.0 (Bio-Rad). Gel images were grouped for comparison. Images of the 14 internal standard gels were combined in group 1, and group 2 contained the 14 images of all normal donor samples. Each of the seven patients comprised an individual group containing the two dye-swap gel images. All gel images were matched automatically and normalized using the internal standard (labeled with Cy2), one MDS patient labeled with Cy3, and one healthy donor labeled with Cy5 or vice versa) were combined per gel and applied to Immobiline™ DryStrips (GE Healthcare) with cup loading. Isoelectric focusing was performed on a MultiPhor II (GE Healthcare) as described previously (19). The strips were then loaded onto linear 12.5% polyacrylamide gels, and second dimension separation was performed using a Laemmli buffer system on an EttanDalt 12 system (GE Healthcare) as described previously (19).

**Protein Identification and Pathway Analysis**—Protein spots of interest were excised from the gels using a Gelpix spot picker (Genetix). Gel pieces were washed and proteins trypsin-digested, and the resulting peptides were eluted as depicted before (19) and applied to a MALDI Prespotted AnchorChip target (Bruker Daltonics). Samples were analyzed in an Ultraflex-ToF/ToF mass spectrometer, and acquired mass spectra were annotated using Compass 1.3 software (both from Bruker Daltonics). All spectra were re-calibrated internally using the mass-list generated by Bruker Daltonics (based on 400 identified spectra to assign laboratory-specific peaks that should be excluded, see supplemental Data S1). Protein identification was performed using Biotools 3.2 (Bruker Daltonics) by searching SwissProt (SwissProt_57.12.fasta, 513877 sequences) and NCBIpr (NCBIpr_20090324.fasta, 8097822 sequences) databases with Mascot 2.2.04 (Matrix Science) using the following search parameters: enzyme “trypsin”, species “human,” fixed modifications “carbamidomethyl,” optional modifications “methionine oxidation,” missed cleavages “1,” and mass tolerance “50 ppm.” Calculated pl and molecular mass data were obtained by Mascot. For peptides matching different isoforms or multiple members of a protein family, we used the following reporting criteria. The experimental pl and molecular mass taken from the two-dimensional gels were compared with the theoretical data of the different isoforms/protein members. If no conflicts in molecular mass or pl were found, the isoform/protein member with the highest mass score was reported. Proteins were considered as identified with a Mascot score of >56 (p < 0.05) on at least two physically different gels. Pathway and signaling network analysis was performed by Ingenuity Pathway Analysis tools (Ingenuity Systems).

**Western Blot Analysis**—The pooled platelet lysates of the seven MDS patients and seven normal donors were separated by SDS-PAGE, blotted onto a PVDF membrane, and incubated with appropriate antibodies followed by nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (Roche Applied Science) development. Antibodies used were mouse anti-human-talin-1 (LSBio; 1:2500), rabbit anti-human-MYH9 (Sigma-Aldrich; 1:500), mouse anti-human-vinculin, and mouse anti-human-filamin-A (both Abcam; 1:2500). Secondary antibodies were AP-conjugated donkey anti-mouse and donkey anti-rabbit (both Abcam; 1:2500). Rabbit anti-human-β-tubulin (Abcam; 1:2500) was used as loading control. Bands were quantified by pixel intensity using ImageJ software (rsweb.nih.gov).

** Förster Resonance Energy Transfer (FRET)**—Washed platelets from five MDS patients and normal donors were incubated with 1 unit/ml thrombin and simultaneously stained for 20 min at RT in the dark with anti-CD41 (Acris Antibodies) and anti-CD49b (BD Biosciences) conjugated to phycoerythrin (PE) and allophycocyanin (APC) using Lync Rapid Conjugation kits (AbD Serotec). FRET was measured using a Cytomics FC500 flow cytometer (Beckman Coulter) equipped with 488- and 635-nm lasers. If the APC-labeled talin-1 probe was in close physical proximity (<10 nm) to the 488-nm excited PE-labeled integrin, it absorbed the energy emitted at 575 nm and emitted light at 675 nm without the use of the 635-nm laser. The increase in the MFI at 675 nm was recorded to quantify co-localization of talin-1 and the integrin. All samples were checked for sufficient staining prior to FRET analysis.

**Whole Blood Flow Cytometry**—Within 30 min after blood sampling, 10 μl of citrate-anticoagulated whole blood from 11 MDS patients and normal donors was diluted in PBS, incubated with agonists, simultaneously stained with antibodies for 20 min at RT in the dark, and subsequently analyzed using a FACScalibur (BD Biosciences). Agonists used were 5 × 10^{-6} M thrombin receptor-activating peptide (TRAP), 10^{-5} M phorbol 12-myristate 13-acetate, and 10^{-3} M MnCl2. Antibodies used were anti-CD41-FITC (clone P2, Acris Antibodies), goat anti-human GPV1 (Santa Cruz Biotechnology), rabbit anti-human P2Y1, and P2Y12 (Alomone Labs), and PAC-1-FITC, CD42b-PE, CD49b-PE, CD61-PE, CD62P-APC, and isotype controls (all from BD Biosciences). Unconjugated antibodies were stained with AF488-conjugated goat anti-rabbit or rabbit anti-goat secondary antibodies (Invitrogen). Cytosolic free calcium concentration ([Ca^{2+}]) of the platelets of 10 MDS patients and normal donors was measured upon

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activation with 50 mM ADP or 1 unit/ml thrombin using whole blood diluted 1:10 in calcium-free PBS and incubated with 10 μM Fluo-4 AM (Invitrogen) for 20 min at 37°C in the dark.

Aggregometry—Aggregometry of 58 MDS patients and 10 normal donors was performed according to a standard operation procedure at the Department of Clinical Chemistry and Laboratory Diagnostics, Heinrich-Heine-University Clinic. Within 2 h after blood collection, platelet-rich plasma was isolated and stimulated with 20 μM ADP, 0.5 mg/ml arachidonic acid, 10 μg/ml collagen, or 1.5 mg/ml ristocetin. Light transmission was recorded on a platelet aggregation profiler PAP8 (mÔ® Lab) over 20 min.

Platelet Spreading Experiments—Glass-bottom dishes (MatTek) were coated with 1 mg/ml human fibrinogen and blocked with 1% BSA in PBS. BSA-coated dishes were used as controls. Washed platelets of four MDS patients and normal donors were seeded at a concentration of 0.5 × 10⁶/ml, activated with 1 unit/ml thrombin, and analyzed by differential interference contrast microscopy over 30 min.

Statistical Analysis—Frequency tables (z-test) and mean comparison (t test) were used for statistical examination. P values are given in the text and stars are used throughout the figures to indicate the level of significance (*p<0.05, **p<0.01, ***p<0.001). No corrections for multiple hypothesis testing were performed to allow for maximum detection of potentially differentially expressed proteins. Established methods for correction lowered the number of false-positive discoveries at the expense of increasing false-negative discoveries. χ² automatic interaction detection was used for modeling the dependence of the aggregation defect on multiple clinical parameters (all SPSS 19, IBM).

RESULTS

Platelet Proteome Analysis Reveals Low Levels of Proteins Critical for Integrin Receptor Signaling and Platelet Function—To gain insights into the molecular pathology of platelet dysfunctions in MDS, we conducted a comprehensive analysis of the entire MDS platelet proteome. We performed two-dimensional difference gel electrophoresis for a quantitative comparison of platelet lysates obtained from seven MDS patients and seven normal donors. We detected a total number of 1649 protein spots of which 120 spots (7%) significantly differed between MDS and normal donor platelets regarding their concentration (Fig. 1). Among the differentially expressed protein spots, 101 spots (84%) had a lower expression in MDS platelets. We identified 35 of the differentially expressed protein spots (29%) representing 16 distinct proteins (Table I). Whereas nine protein species were identified from one spot each, seven protein species could be identified from more than one spot indicating different post-translational modification statuses of these proteins. The fold changes of these proteins discovered in more than one spot had similar values in the same direction. Ten of the 16 proteins (Talin-1, Kindlin-3, Vinculin, Filamin-A, Actin, Myosin-9, Myosin regulatory light chain 2 [MRLC2], Integrin-linked protein kinase [ILK], Cysteine and glycine-rich protein 1 [CSRP1], and Pleckstrin), all with lower levels in MDS, are involved in integrin signaling and function (see supplemental Data S1 for mass spectra and peptide coverage). The decreased expression of Talin-1, Myosin-9, Filamin-A, and Vinculin was also confirmed by Western blot showing a 23% decrease of Talin-1 amount in MDS platelets compared with healthy donor platelets as well as 42% of Vinculin, 17% of Filamin-A, and 63% of Myosin-9, respectively, although β-tubulin levels were not altered (Fig. 2). Subsequent ingenuity network and pathway analysis of the 16 identified proteins with altered expression in MDS revealed integrin signaling and cytoskeletal reorganization, both critical for platelet function, to be the most affected pathways (Fig. 3, A and B).
Normal Surface Receptor Expression and Primary Activation in MDS Platelets—The aberrant platelet protein profile in MDS indicated an abnormal integrin signaling as a potential cause of defective platelet function in MDS. We therefore investigated the expression profiles of several integrins and other platelet surface receptors (GPIIa, GPIIb, GPIb, GPVI, VLA-α2, P2Y1, and P2Y12) and found no differences in the MFI of MDS and normal platelets (n = 10, Fig. 4A). Furthermore, we examined the very first step in platelet activation following agonist stimulation as we looked at the transient rise in $\left[\text{Ca}^{2+}\right]_i$ due to discharge from internal stores and found no differences between MDS and healthy donors (n = 10, Fig. 4B). As the calcium mobilization is critical for granule movement and secretion, we further analyzed granule release upon platelet activation using P-selectin. Our flow cytometric analysis also revealed no difference in the activation-dependent transport of P-selectin to the platelet

### Table I

| No. | Accession no. | Title of protein | Spot intensity healthy | Spot intensity MDS | FC   | p value       |
|-----|---------------|------------------|------------------------|--------------------|------|---------------|
| 1a  | FLNA_HUMAN    | Filamin-A        | 0.1254                 | 0.0515             | -2.43| 0.000009      |
| 1b  | FLNA_HUMAN    | Filamin-A        | 0.2053                 | 0.1004             | -2.04| 0.006578      |
| 1c  | FLNA_HUMAN    | Filamin-A        | 0.1310                 | 0.0599             | -2.19| 0.003582      |
| 2a  | MYH9_HUMAN    | Myosin-9         | 0.0675                 | 0.0257             | -2.63| 0.020416      |
| 2b  | MYH9_HUMAN    | Myosin-9         | 0.6042                 | 0.3622             | -1.67| 0.004491      |
| 2c  | MYH9_HUMAN    | Myosin-9         | 0.5107                 | 0.2604             | -1.96| 0.019720      |
| 2d  | MYH9_HUMAN    | Myosin-9         | 0.1421                 | 0.0598             | -2.38| 0.013282      |
| 2e  | MYH9_HUMAN    | Myosin-9         | 0.1304                 | 0.0572             | -2.28| 0.020173      |
| 2f  | MYH9_HUMAN    | Myosin-9         | 0.1026                 | 0.0548             | -1.87| 0.025893      |
| 2g  | MYH9_HUMAN    | Myosin-9         | 0.1688                 | 0.0805             | -2.10| 0.011641      |
| 2h  | MYH9_HUMAN    | Myosin-9         | 0.2767                 | 0.1297             | -2.13| 0.003862      |
| 3a  | TLN1_HUMAN    | Talin-1          | 0.3525                 | 0.1741             | -2.02| 0.013270      |
| 3b  | TLN1_HUMAN    | Talin-1          | 0.3283                 | 0.1223             | -2.69| 0.019528      |
| 3c  | TLN1_HUMAN    | Talin-1          | 0.4103                 | 0.2079             | -1.97| 0.000318      |
| 3d  | TLN1_HUMAN    | Talin-1          | 2.7395                 | 1.3022             | -2.10| 0.000014      |
| 3e  | TLN1_HUMAN    | Talin-1          | 3.0228                 | 1.4448             | -2.09| 0.000095      |
| 4a  | VINC_HUMAN    | Vinculin         | 0.1102                 | 0.0364             | -3.03| 0.000000      |
| 4b  | VINC_HUMAN    | Vinculin         | 0.4126                 | 0.1434             | -2.88| 0.000427      |
| 4c  | VINC_HUMAN    | Vinculin         | 0.4146                 | 0.1396             | -2.97| 0.000025      |
| 4d  | VINC_HUMAN    | Vinculin         | 0.2806                 | 0.0987             | -2.64| 0.01262       |
| 5   | FIBP_HUMAN    | Fibroblast growth factor-binding protein | 0.0684 | 0.0272 | -2.51 | 0.039802 |
| 6   | URP2_HUMAN    | Kindlin-3        | 0.0664                 | 0.0321             | -2.07| 0.002287      |
| 7a  | ACTG_HUMAN    | Actin, cytoplasmic 2 | 0.0874 | 0.0403 | -2.17 | 0.003818 |
| 7b  | ACTB_HUMAN    | Actin, cytoplasmic 1 | 0.1352 | 0.0473 | -2.86 | 0.044908 |
| 8   | CLIC1_HUMAN   | Chloride intracellular channel protein 1 | 0.4149 | 0.0681 | -6.09 | 0.005478 |
| 9   | HSPB1_HUMAN   | Heat shock protein β1 | 0.0285 | 0.0714 | 2.51  | 0.002229 |
| 10a | MLRM_HUMAN    | Myosin regulatory light chain 2 | 0.8381 | 0.4336 | -1.93 | 0.000007 |
| 10b | MLRN_HUMAN    | Myosin regulatory light chain 2 | 0.6212 | 0.2070 | -3.00 | 0.000001 |
| 11  | ILK_HUMAN     | Integrin-linked protein kinase | 0.8299 | 0.4089 | -2.03 | 0.000340 |
| 12  | FIBA_HUMAN    | Fibrinogen α chain precursor | 0.1414 | 0.0549 | -2.58 | 0.005281 |
| 13  | AMPM1_HUMAN   | Methionine aminopeptidase 1 | 0.2179 | 0.0866 | -2.52 | 0.007359 |
| 14a | PLEK_HUMAN    | Pleckstrin       | 0.6820                 | 0.2551             | -2.67| 0.003865      |
| 14b | PLEK_HUMAN    | Pleckstrin       | 0.6858                 | 0.2313             | -3.01| 0.001464      |
| 15  | CSRP1_HUMAN   | Cysteine and glycine-rich protein 1 | 0.9050 | 0.4459 | -2.03 | 0.000235 |
| 16  | HBB_HUMAN     | Hemoglobin subunit β | 0.8877 | 2.3011 | 2.59  | 0.011719 |

The serial number listed here is identical to the respective number in Fig. 1, showing the position of the identified proteins. FC indicates the fold change between the mean spot intensities of MDS and healthy platelet samples.

### Fig. 2

Western blot of platelet lysates from MDS patients and healthy donors. The pooled platelet lysates from seven MDS patients and seven healthy donors show the decreased levels of Talin-1, Vinculin, Filamin-A, and Myosin-9 in MDS platelets.
membrane in MDS platelets compared with their healthy counterparts (n = 11, Fig. 4C) indicating a normal primary activation of the MDS platelets.

**MDS Platelets Show Insufficient Integrin αIIbβ3 Activation (Inside-out Signaling)**—In the subsequent activation process, intact bidirectional signaling of integrin αIIbβ3 was a prerequisite for platelet activation and aggregation. To properly activate this receptor, Talin-1 and Kindlin-3 have to relocate from the cytoplasm to the cell membrane and bind to distinct domains of the integrin β3. By utilizing FRET, we found that the energy transfer between Talin-1 and integrin αIIbβ3 upon activation with thrombin was markedly reduced in MDS platelets compared with normal ones (n = 5, p < 0.05), which indicates insufficient activation capacities of integrin αIIbβ3 (Fig. 5). To analyze the capability of this receptor to shift from its inactive low affinity to an active high affinity conformation, we assessed PAC-1 binding to this receptor by flow cytometry. PAC-1 exclusively recognizes the fibrinogen-binding site of integrin αIIbβ3 that is only accessible in its activated high affinity state (20). Following stimulation with TRAP as well as with agonist receptor-independent phorbol 12-myristate 13-acetate, PAC-1 binding was significantly reduced in MDS platelets (n = 8, p < 0.05 and 0.01, Fig. 6), demonstrating that in MDS platelets a significantly lower percentage of integrin αIIbβ3 was able to shift from its inactive to its active state. To rule out nonspecific nonsignaling-related defects of integrin αIIbβ3, such as a structural defect at the fibrinogen-binding site itself, we examined its PAC-1 binding capability following activation through Mn2+, which is independent from intracellular signaling events. Here, we detected no differences between MDS and normal platelets (n = 3, Fig. 6) demonstrating principal functionality of the receptor itself.

**Impaired Integrin αIIbβ3 Activation Leads to Inadequate Platelet Spreading and Aggregation**—To further address po-
Integrin-dependent Platelet Defects in MDS

Platelets labeled with anti-integrin \( \alpha_{IIb}\beta_3 \) conjugated to PE (upper panel) and in combination with anti-talin-1 conjugated to APC (lower panel) showing the reduced emission at 675 nm in MDS. B, box plot showing the significantly reduced MFI at 675 nm after excitation with 488 nm in MDS platelets compared with normal donors (n = 5, p < 0.05).

Fig. 5. FRET analysis of talin-1 co-localization to the integrin \( \beta_3 \). A, bi-parametric analysis of emissions at 575 nm (PE, abscissa) and 675 nm (APC, ordinate) after excitation at 488 nm of MDS and healthy platelets labeled with anti-integrin \( \alpha_{IIb}\beta_3 \) conjugated to PE (upper panel) and in combination with anti-talin-1 conjugated to APC (lower panel) showing the reduced emission at 675 nm in MDS.

Potential functional defects in MDS platelets, we analyzed outside-in signaling of the affected integrin \( \alpha_{IIb}\beta_3 \). Washed platelets of MDS patients and normal donors were exposed to immobilized fibrinogen. Following stimulation with thrombin, MDS platelets showed a spreading defect as they did mainly form filopodia but only few lamellipodia. Consequently, they failed to spread for up to 30 min, confirming the impaired outside-in signaling of integrin \( \alpha_{IIb}\beta_3 \) (Fig. 7A), which is in line with the reduced concentrations of Talin-1, Kindlin-3, Vinculin, Filamin-A, Actin, Myosin-9, and Myosin regulatory light chain 2 (MLC2). Although MDS and normal platelets adhered similarly to the immobilized fibrinogen (integrin \( \alpha_{IIb}\beta_3 \)-activation-independent), only 21% of the MDS platelets were fully spread after 30 min compared with 95% of healthy platelets (n = 4, p < 0.001, Fig. 7B). The impaired bidirectional signaling of integrin \( \alpha_{IIb}\beta_3 \) suggested a general defect in integrin-dependent platelet aggregation in MDS. However, shape change is not an essential prerequisite for aggregation, because adrenaline causes aggregation without shape change, and cytochalasin B inhibits shape change but not aggregation (21, 22). Therefore, we tested the ability of 58 MDS patients’ platelets to aggregate in response to different agonists (Fig. 8A). In accordance with the observed defective integrin \( \alpha_{IIb}\beta_3 \) receptor activation in MDS, we found an insufficient aggregation in response to all four agonists (collagen p < 0.001, arachidonic acid p < 0.01, ADP p < 0.01, and ristocetin p < 0.05). Statistical \( \chi^2 \) automatic interaction detection modeling of the dependence of the aggregation defect on multiple clinical parameters revealed that platelet count had the highest influence with 77% of the patients with platelets below 157,000/μL showing an aggregation defect compared with 21% of patients with higher platelet counts (p < 0.001, Fig. 8B). Other parameters that influenced aggregation capacity were the World Health Organization subtype and International Prognostic Scoring System (IPSS). Whereas patients with del(5q) showed a lower incidence of the aggregation defect compared with the other subtypes (22% versus 61%; p < 0.05), patients with chronic myelomonocytic leukemia (CMML) had a higher incidence (78% versus 51%, p < 0.05). Increasing IPSS correlated with increased aggregation defects (p < 0.05), with all high risk patients having the defect.

**DISCUSSION**

Platelet count-independent hemorrhages are a significant clinical problem in patients with MDS (3, 4). The presence of a functional platelet defect may aggravate bleeding complications in patients with low platelet counts and in general increase the complication rate during invasive procedures in MDS patients. So far, it has not been elucidated if abnormal hemostasis in MDS is systematic and if it follows a common platelet-intrinsic pathology.

Once platelets have been shed from megakaryocytes, they already contain most of the proteins required for their function. Therefore, quantitative platelet proteome analysis is an ideal tool to uncover platelet-intrinsic defects that may contribute to functional platelet defects. Our analysis of the MDS and healthy platelet proteomes revealed that the majority of proteins with different concentrations showed lower levels in MDS. This suggested that functional platelet defects in MDS may result from inadequate levels of proteins critical for platelet function. This hypothesis was further supported by our pathway/network analysis that showed that the majority of these lower concentrated proteins are essential for integrin signaling. Proper function of integrin \( \alpha_{IIb}\beta_3 \) is critical for platelet aggregation and hemostasis (13, 23). Among the proteins with lower levels in MDS were TAlin-1 and Kindlin-3, both binding to the \( \beta_3 \)-subunit of integrin \( \alpha_{IIb}\beta_3 \), which is referred to as the common final step necessary for integrin activation.
The proteins of the kindlin family and Talin-1 also establish major links between the β₃-subunit and the actin filaments of the cytoskeleton, which is necessary for initiation of subsequent platelet spreading (26, 27). Both of these signaling pathways are absolutely required for platelet function, and consistently, lack of Talin-1 or Kindlin-3 in platelets has been shown to abrogate integrin β₃ activation with loss of platelet spreading and aggregation in vivo (12, 13, 24).

The reduced expression of these two proteins and their central role in integrin activation pointed toward abnormal integrin α₅β₃ function in MDS. While surface receptor expression and early activation (calcium flux and granule release) did not differ between MDS and normal platelets, the integrin inside-out signaling upon activation was markedly reduced in MDS. Consequently, platelet spreading on immobilized fibrinogen was almost abolished in patients with MDS. Besides activation of integrin α₅β₃, the ability to spread on immobilized fibrinogen requires the presence of Vinculin and cytoskeletal proteins such as Filamin-A, Actin, and Myosin-9, which were also expressed lower in MDS platelets. It has been shown that loss of Vinculin decreases cytoskeletal mechanics and spreading capacities (31, 32), and Filamin-A is responsible for the organization of F-actin into bundles and networks and the linkage of transmembrane receptors to the cytoskeleton (33, 34). The reduced expression of the major cytoskeletal proteins Actin and Myosin-9 also aggravates the poor spreading performance (35). This may explain why the spreading defect observed in MDS platelets was more severe than suggested solely by the incomplete integrin α₅β₃ activation. Inside-out and thus Talin-1- or Kindlin-3-independent integrin α₅β₃ functions such as adhesion to fibrinogen (36) or receptor activation following Mn²⁺ stimulation (37) remain intact in MDS platelets, pointing toward a specific integrin α₅β₃ activation deficit in MDS originating from the reduced expression of essential signaling proteins such as Talin-1, Kindlin-3, Vinculin, Filamin-A, and Actin. With respect to their functional phenotype, MDS platelets mimic the platelet phenotype of conditional Talin-1 or Kindlin-3 knockout mice (12, 13, 24).

From a functional perspective, the impaired bidirectional integrin α₅β₃ signaling in MDS suggests a general defect in integrin-dependent platelet aggregation. In line with this assumption, we found considerably decreased aggregation in response to the integrin-dependent agonists arachidonic acid and collagen when compared with normal donor platelets. We also found a diminished aggregation in response to ADP, although not as severe as against the other two. This could be
due to ADP’s two P2Y receptors, of which only one is dependent on Chloride intracellular channel protein 1 (Clic1) that we found 3-fold down-regulated in MDS platelets. The absence of Clic1 was recently found to induce a lower ADP-stimulated platelet aggregation in mice that is dependent on the P2Y12 receptor but not the P2Y1 receptor (38). We also detected a reduced response to ristocetin, which is independent of integrin \( \alpha_{IIb}\beta_3 \) function as it initiates platelet agglutination rather than aggregation (39). The reduced response to ristocetin observed in MDS patients might be explained by the lower levels of the cytoskeletal protein Myosin-9 as patients with inherited disorders involving the corresponding \( MYH9 \) gene locus also present with a similarly reduced platelet aggregation capacity in response to ristocetin (40), as well as giant platelets (18, 41). The Myosin-9 deficiency in MDS platelets may have conceivably contributed to the increased mean platelet volume (>11 fl), a common phenomenon in MDS referred to as “giant” or “balloon-like” platelets (17), observed in the majority of MDS patients examined here. We observed the described effects consistently in patients with platelet counts ranging between 19,000 and 734,000/μl, whereas the aggregation defect positively correlated with platelet count, World Health Organization subtype, IPSS, and signs of bleeding. Although in our statistical analysis a lower platelet count (<157,000/μl) had the highest influence on the aggregation capacity, the average platelet count (89,000/μl) did not suggest aggregation defects in these patients thus indicating the existence of a functional defect.

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