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CD34+ marrow progenitors from MDS patients with high levels of intramedullary apoptosis have reduced expression of α4β1 and α5β1 integrins

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Excessive intramedullary apoptosis is central in the pathogenesis of myelodysplastic syndromes (MDS). Growth-inhibiting cytokines, the Fas/FasLigand pathway, and autoreactive cytotoxic T-lymphocytes have been identified to be important proapoptotic factors in MDS. In normal hematopoiesis, α4β1 and α5β1 integrin-mediated interactions between progenitors and fibronectin are critical for progenitor cell survival. In this study, we have used flow cytometry to quantify the expression levels of members of the β1 integrin family on CD34+ marrow progenitors in 27 untreated patients with MDS, three with s-AML, and 25 control subjects. In MDS, we observed that nonapoptotic progenitors significantly downregulate cell surface expression levels of α4 and β1 integrin chains compared with healthy controls. Downregulation of α4, β1, and also α5 was present in MDS patients with ≥25% apoptotic progenitors, irrespective of their French, American, British subcategory. Reduced cell surface expression levels of α4, α5, and β1 did also correlate with decreased in vitro adhesiveness to fibronectin fragments. Therefore, our observations suggest that downregulation of α4β1 and α5β1 integrins on CD34+ progenitors could be a newly identified proapoptotic mechanism in MDS.

Introduction

Hematopoiesis occurs in close contact with a protective and permissive microenvironment consisting of a variety of cellular elements, extracellular matrix components, and membrane-bound and soluble cytokines. Contact-mediated interactions between hematopoietic progenitor cells (HPC) and stromal ligands play an important role in the orderly regulation of the hematopoietic process. Hematopoietic precursors interact with the extracellular matrix through a variety of cell surface adhesion receptors including selectins, the immunoglobulin-like superfamily, cell surface proteoglycans, cadherins, and integrins.[1–4] Integrins are heterodimeric transmembrane cell surface glycoproteins consisting of a noncovalently linked α and β chain. There are at least 18 α and eight β subunits, giving rise to more than two dozen distinct integrins in mammalian cells.[2,5,6] The β1 family of integrins is attributed a predominant role in the retention of hematopoietic progenitors in the bone with fibronectin, laminin, and collagen as their principal ligands.[2,7] In addition to providing an anatomical linkage, integrins also play a critical role in communication between cells and their microenvironment,

and in intercellular crosstalk. Several studies have proven that survival, proliferation and differentiation of HPC is dependent on α4β1 (or VLA-4) and α5β1 (or VLA-5) integrin-mediated interactions between progenitors and fibronectin.[8–11] A 75 kDa proteolytic fragment in the center of the fibronectin molecule contains the peptide RGD and interacts with most cells via the α5β1 integrin receptor. Adhesion to fibronectin can also occur in an RGD-independent manner via the 33/66 kDa COOH-terminal 'heparin-binding domain' containing the minimal recognition site for the α4β1 integrin receptor.[2] Stimulation of α4β1 integrin receptors by adhesion to the COOH-terminal heparin-binding domain of fibronectin,[9] or by direct monoclonal antibody crosslinking,[12] inhibits proliferation of normal clonogenic and immature hematopoietic progenitors by blocking transition from the G1 to S phase of the cell cycle. Interactions between α5β1[10] or α4β1[11] and fibronectin are also important in rescuing cells, including HPC, from apoptosis. As a consequence, the expression levels and/or functional status of β1 integrins, and more precisely α4β1 and α5β1, are important regulators of the survival and proliferation of normal HPC. The interactions between α4β1 and α5β1 receptors on leukemic cells and their stromal ligands have also been intensively studied both in acute and chronic leukemia.[13–17] For example, the interaction of α4β1 with fibronectin has been shown to promote the survival of AML blasts, and may therefore be of crucial importance in the persistence of minimal residual disease.[13]

Given their importance in normal and leukemic hematopoiesis, we were interested in the expression and function of α4β1 and α5β1 on HPC in myelodysplastic syndromes (MDS): a bone marrow failure disorder with a high propensity for leukemic transformation. Although peripheral blood cytopenia is a common feature in MDS, the bone marrow is frequently found to be hypercellular. Increased intramedullary apoptosis associated with progenitor hyperproliferation is the commonly accepted explanation for this paradox.[18,19] Apoptosis in MDS is generally accepted to be multifactorial in origin.[20] Cumulated genomic damage in HPC,[21] an imbalance between growth- and apoptosis-promoting cytokines like TNF-α,[22] activation of the Fas/FasL pathway,[23] and increased numbers of autoreactive cytotoxic T-lymphocytes[24] all can promote MDS apoptosis to a variable extent. If quantitative and/or functional abnormalities in the integrins of the β1 family, like α4β1 and α5β1, are involved in apoptosis induction and/or hyperproliferation of MDS progenitor cells, is to the best of our knowledge currently unknown.

In this study, we have measured the expression levels of the α4β1 and α5β1 integrin cell surface receptors on MDS-derived CD34+ marrow progenitors and studied their relationship with levels of intramedullary apoptosis and disease subtype.

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Materials and methods

Patient characteristics (Table 1)

Fresh bone marrow aspirates were consecutively obtained from 27 unselected patients with MDS and three with secondary AML. Patients were either in chronic follow-up or newly referred to our center. Mean patient age was 67 years (range 30–85 years). According to the French, American, British (FAB) morphological guidelines for MDS classification, 10 patients had RA, five RARS, five RAEB, and seven RAEB-t. A cytogenetic abnormality was observed in 16/27 patients. None of the patients had received anti-MDS therapy other than supportive care for at least 4 weeks prior to the bone marrow examination. In all, 25 healthy volunteer bone marrow donors served as controls.

Progenitor isolation

Bone marrow aspirations and sample handlings were performed in accordance with the guidelines of the local ethical committee of the Catholic University Leuven. Bone marrow samples were aspirated from the posterior iliac crest and collected in syringes prefilled with phosphate-buffered saline (PBS) and preservative-free heparin. All samples were processed immediately. Mononuclear cells were recovered after centrifugation for 30 min at 1400 rpm on a Lymphoprep gradient (1.077 g/ml; Nycomed Pharma AS, Oslo, Norway). Subsequently, mononuclear cells were washed twice in PBS supplemented with 0.3% bovine serum albumin (Sigma Chemical Co, St Louis, MO, USA) and

kept on ice until CD34 selection. CD34 enrichment was carried out by a double passage over MiniMACS CD34 isolation columns (Miltenyi Biotec, Bergish Gladbach, Germany) according to the manufacturer's instructions.
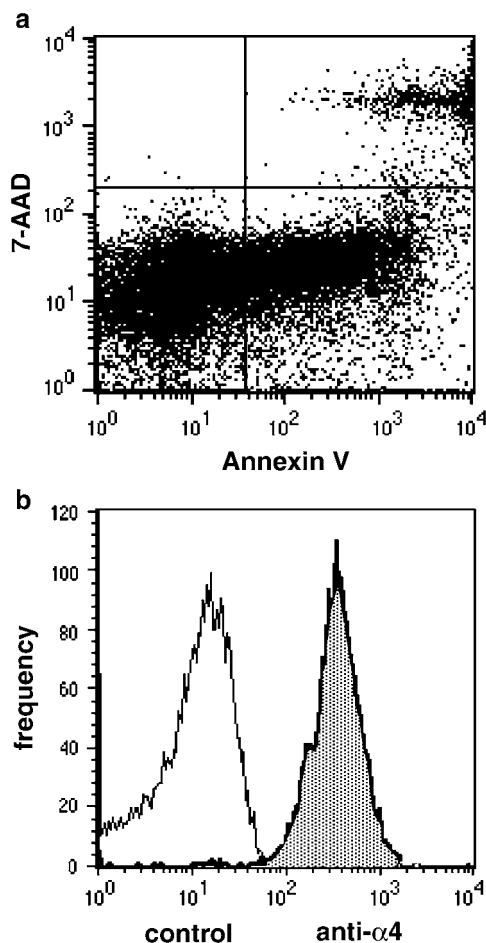
Flow cytometry

CD34$^+$ cells were resuspended in PBS and split equally into snap cap tubes at a final concentration of 50 000 cells/100 μl. Cells were incubated for 30 min at 4°C with phycoerythrin (PE)-conjugated monoclonal antibodies against the following adhesion receptor chains: β1 or CD29 (clone MAR4), α2 or CD49b (clone 12F1-H6), α4 or CD49d (clone 9F10), α5 or CD49e (clone IIA), and α6 or CD49f (clone GOH3). Cells stained with PE-conjugated isotype-specific antibodies were used as negative controls. All antibodies were purchased from Pharmingen (Becton Dickinson). After incubation, cells were washed with 1 ml cold HEPES binding buffer (10 mM HEPES, (pH = 7.4), 140 mM NaCl, 5 mM CaCl$_2$), and pelleted cells were resuspended in 100 μl of HEPES buffer. Subsequently, 5 μl of fluorescein isothiocyanate (FITC)-conjugated annexin V (Clontech Laboratories, Palo Alto, CA, USA) and 1 μl of 7-amino-actinomycin D (7-AAD at 157 mM) (Calbiochem, La Jolla, CA, USA) were added, followed by incubation for 10 min at room temperature in the dark. Subsequently, cells were kept on ice and flow cytometric analysis was carried out within 1 h. Three-color analysis was performed on a FACScan flow cytometer (Becton Dickinson, San Diego, CA, USA) equipped with a 488 nm argon-ion laser and a standard filter combination provided by the manufacturer. Electronic compensation was

Table 1 Patient characteristics

Patient No.	Sex	Age (years)	FAB subtype	Hgb (g/dl)	WBC (× 10³/μl)	Plt (10³/μl)	% BM blasts	Karyotype	% apoptosis
1	M	63	RARS	7.9	3.4	231	<5	Normal	38.1
2	M	68	RAEB	9.3	0.9	26	13	Normal	7.1
3	F	58	RARS	8.7	4.1	310	<5	Normal	68.1
4	F	65	sAML	8.9	1.9	79	36	−7	10.3
5	F	83	RA	8.3	2.1	156	<5	del(5q)	56.1
6	M	79	RAEB	6.7	3.0	9	5	Normal	32.3
7	M	85	RAEBt	9.0	8.3	14	29	+8	5.9
8	F	73	RA	7.3	3.9	64	<5	del(5q)	21.1
9	F	43	RAEBt	9.2	2.9	59	11[a]	Normal	6.4
10	M	72	RA	4.5	2.7	310	4	del(5q)	16.3
11	M	65	RA	8.3	3.9	47	<5	Normal	15.7
12	F	52	RAEBt	8.0	6.9	119	29	Normal	13.5
13	M	52	RA	7.6	4.4	86	<5	Normal	16.6
14	M	59	RAEB	13.2	2.5	118	6	−7	10.1
15	M	60	RAEBt	11.9	1.5	195	8[a]	Normal	70.9
16	M	68	RARS	9.5	2.3	36	<5	del(7q)	20.7
17	M	71	RA	8.8	4.3	350	<5	Normal	39.1
18	M	68	RARS	7.5	3.2	158	<5	Normal	64.3
19	F	71	RAEBt	8.3	1.0	28	9[a]	−7	12.7
20	M	74	sAML	8.6	0.8	43	56	No mitoses	4.7
21	M	75	sAML	8.4	2.0	40	94	−7	7.7
22	M	64	RAEB	8.8	3.7	49	14	+8	5.3
23	M	81	RAEB	10.9	1.7	113	14	45,X,−Y	5.2
24	F	30	RA	8.9	2.1	47	<5	del(5q)	52.1
25	M	55	RA	15.7	10.7	12	<5	del(20q)	8.1
26	M	81	RA	6.0	2.8	214	<5	Normal	45.9
27	M	73	RA	7.5	1.6	122	<5	−5	58.1
28	M	85	RARS	8.0	7.1	229	<5	Normal	49.5
29	M	72	RAEBt	9.1	15.2	43	16[a]	−7	36.5
30	M	75	RAEBt	8.9	12.3	51	25	+8	1.5

[a]Presence of Auer rods.

used to remove residual spectral overlap, and analysis of the acquired data was performed with CELLquest software (Becton Dickinson). A minimum of 20 000 events were collected in each sample. In every patient and control subject, forward scatter *vs* side scatter and CD34-PE expression were used to gate the target population, and annexin V and 7-AAD expression to discriminate viable (annexin V[neg]/7-AAD[neg]), apoptotic (annexin V[pos]/7-AAD[neg]), and dead (annexin V^{pos}/7-AAD^{pos}) cells (Figure 1a). Expression of adhesion receptors was measured on the viable annexin V^{neg}/7-AAD^{neg} CD34^{+} population. For each antibody tested, we have used the distribution histogram to determine the geometric mean of the fluorescence intensity (MFI) (Figure 1b).[25] The degree of positivity for each tested surface adhesion receptor was expressed as a numerical ratio calculated by dividing the MFI of the positively stained cells by that of cells stained with the isotype-matched control antibody.

Figure 1 Quantification of adhesion receptor expression on viable CD34^{+} progenitors. (a) Flow cytometric analysis of purified CD34^{+} progenitors. Cells were stained with FITC-conjugated annexin V and 7-AAD. Annexin V and 7-AAD staining allowed to discriminate viable (annexin V[neg]/7-AAD[neg]), apoptotic (annexin V[pos]/7-AAD[neg]), and dead (annexin V[pos]/7-AAD^{pos}) progenitor cells. (b) Measurement of expression levels of adhesion receptors. For each antibody tested, we have used the distribution histogram to determine the geometric MFI. The degree of positivity for each tested surface adhesion receptor was expressed as a numerical ratio calculated by dividing the MFI of the positively stained cells by that of cells stained with the isotype-matched control antibody. Results are shown for an individual patient.

Adhesion assays

For functional adhesion tests, we have used fibronectin fragments as adhesive ligands. Previously published methodology was slightly adapted in order to allow more accurate quantification of adherent cells. In summary, 24-well flat-bottomed tissue culture plates were coated overnight at 37°C with HBF (human fibronectin α-chymotryptic 40 K fragment, concentration 25 μg/ml, Gibco BRL, Life Technologies Ltd, Paisley, Scotland) containing the α4β1 high-affinity region CS-1 (the minimal recognition site for the α4β1 integrin), and with the peptide RGD (fibronectin-like engineered protein polymer, concentration 25 μg/ml, Sigma, St Louis, USA) containing the α5β1-binding domain. After overnight incubation, plates were washed twice with PBS to remove unbound protein, and remaining nonspecific protein binding sites were blocked for 1 h at 37°C with 5% BSA in PBS. Before plating the cells, excess BSA was removed by washing with PBS. Control wells were coated overnight with 5% BSA. After coating and washing, 50 000 CD34^{+} cells resuspended in 1 ml of PBS were allowed to adhere for 1 h at 37°C. Each experiment was performed in duplicate. Nonadherent cells were removed by three consecutive gentle washes with PBS. After the final wash, 1 ml of prewarmed trypsin-EDTA (Gibco BRL) was added to each well to detach the adherent cells. After 10 min at 37°C, 1 ml of ice-cold fetal bovine serum (StemCell Technologies, Vancouver, BC, Canada) was added to neutralize trypsin activity. The adherent cells were collected and microscopic inspection ensured that all adherent cells were recovered from the wells. Subsequently, adherent and nonadherent cells were washed separately, pelleted by centrifugation, and resuspended in 200 μl HEPES buffer. Adherent and nonadherent cells were counted by flow cytometry using TruCount® Absolute Count Tubes (Becton Dickinson). Briefly, cells were added to the TruCount® tubes and stained with 10 μl CD34 PE, 5 μl annexin V FITC, and 1 μl of 7-AAD, followed by incubation for 15 min at room temperature in the dark. The absolute number of adherent cells was obtained with the following formula: ((number of events in cell containing region/number of events in absolute count bead region) × total number of beads per test). The percentage of adherent cells could be subsequently calculated.

Statistical analysis

For statistical calculations, we have used GraphPad PRISM version 3.02. Values of adhesion receptors are expressed as the ratio of the MFI±s.e.m. Comparison between the expression levels of adhesion receptors was carried out using the Mann–Whitney test. Correlations were calculated with the Spearman rank test. Statistical significance in other experiments was calculated using a two-tailed Student's *t*-test. In all statistical calculations, a *P*-value of <0.05 was considered significant.

Results

Levels of apoptosis in MDS patients and controls

As shown in Figure 2a, levels of apoptosis in purified CD34^{+} progenitors were significantly higher in MDS patients (*n*=30) (mean=26.7%; range=1.5–70.9%) compared to control subjects (*n*=25) (mean=9.7%; range=2.5–20.7%) (*P*=0.005). Patients with RA, RARS (early phase MDS, *n*=15) displayed significantly higher levels of apoptosis (mean=38%;
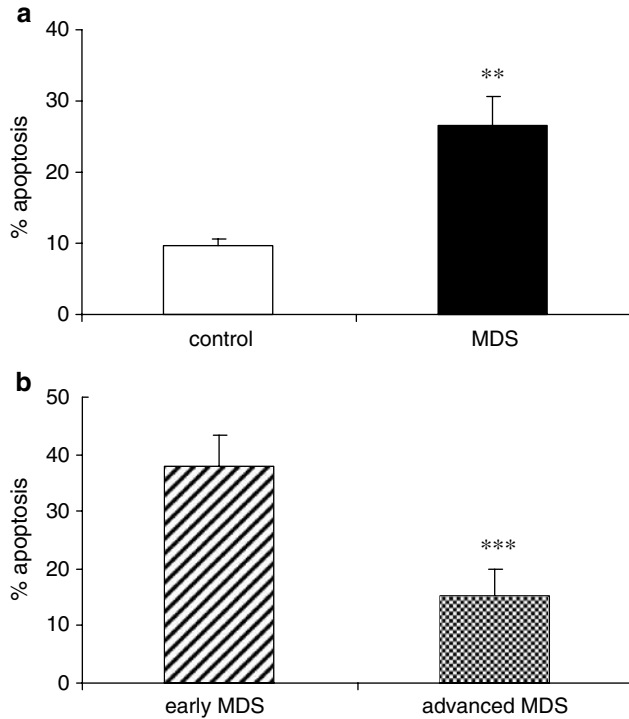
Figure 2   Apoptosis in CD34$^+$ progenitors from MDS patients and control subjects. (a) Apoptosis in control subjects ($n=25$) and MDS patients ($n=30$). (b) Apoptosis in early (RA, RARS) ($n=15$) and advanced (RAEB, RAEB-t, sAML) MDS ($n=15$). Shown are the mean values ($\pm$s.e.m.) of annexin V^{pos}/7-AAD^{neg} CD34$^+$ fractions **$P=0.005$, ***$P=0.001$.

range $=8.1–68.1\%$) compared with RAEB, RAEB-t, and sAML patients (advanced MDS, $n=15$) (mean $=15.4\%$; range $=1.5\%–70.9\%$) ($P=0.001$) (Figure 2b). Using the apoptosis levels in the control population as reference values, we have defined two MDS subgroups for the purpose of this study. Patients with $<25\%$ of their CD34$^+$ population being apoptotic were considered to have 'low apoptosis MDS' (LA-MDS; $n=18$) (mean $=10.5\%$; range $=1.5–21.1\%$) and had apoptosis levels in the range of the control population, whereas patients with $\geq$ 25% apoptotic progenitors are referred to as 'high apoptosis' MDS (HA-MDS; $n=12$) (mean $=51\%$; range $=32.3–70.9\%$). In the early phase MDS subgroup, 9/15 patients fell within the HA-MDS subcategory, whereas this was limited to only 3/15 patients with more advanced MDS subtypes.

Expression levels of adhesion receptors on normal and MDS CD34$^+$ progenitors

In order to avoid any possible influence of the apoptotic process itself on cell membrane adhesion receptors, we have only reported expression levels of adhesion receptors after gating on the viable annexin V^{neg}/7-AAD^{neg} CD34$^+$ progenitor fractions. As explained in the Materials and methods section, levels of adhesion receptors were expressed using a ratio between the MFI of the antibody directed against the adhesion receptor and an isotype-matched control antibody. Comparing these ratios between all MDS patients and control subjects, we observed a significant decreased expression of β1 or CD29 (4.69 [1.08–12.96] vs 5.69 [2.08–14.62]) ($P=0.05$), and of α4 or CD49d
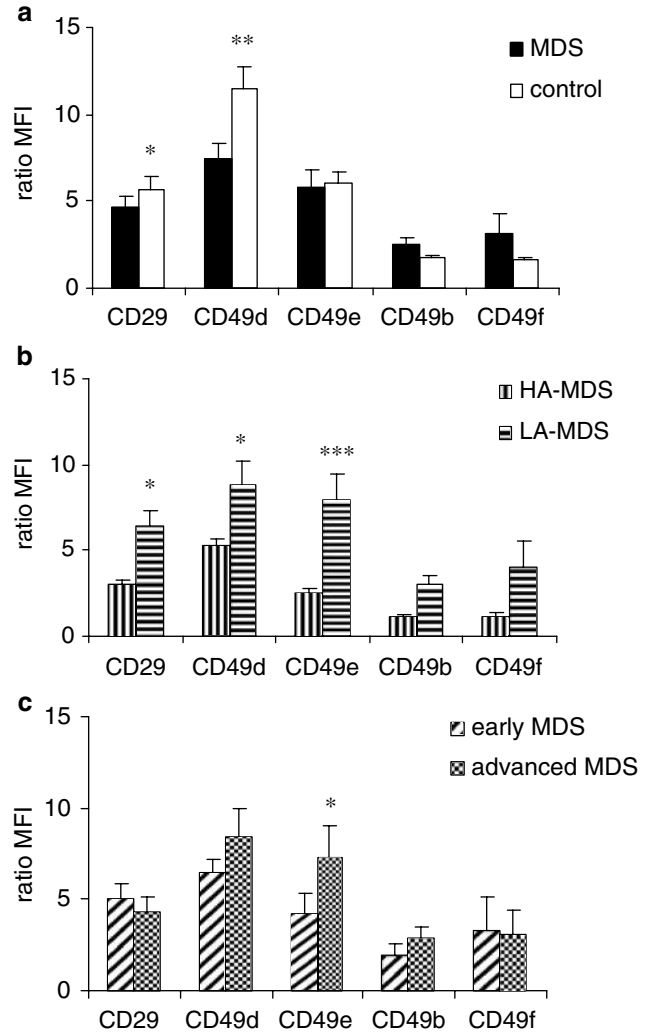
Figure 3   Adhesion receptor expression on CD34$^+$ progenitors from control subjects and MDS patients. Highly purified CD34$^+$ cells were incubated with PE-conjugated monoclonal antibodies against CD29 (or β1), CD49d (or α4), CD49e (or α5), CD49b (or α2), and CD49f (or α6). Cells incubated with PE-conjugated isotype-specific antibodies were used as negative controls. Analyses were carried out on a FACScan flow cytometer. A minimum of 20 000 events was analyzed for each sample, and gating was performed on the annexin V^{neg}/7-AAD^{neg} CD34$^+$ population. We have used the distribution histogram to determine the geometric MFI. The degree of positivity for each tested surface adhesion receptor was expressed as a ratio calculated by dividing the MFI of the positively stained cells by that of cells stained with the isotype-matched control antibody. Results are expressed as mean values$\pm$s.e.m. (a) MDS patients ($n=30$) vs control subjects ($n=25$). *$P=0.05$, **$P=0.001$. (b) 'high-apoptosis' MDS (HA-MDS) ($n=12$) vs 'low-apoptosis' MDS (LA-MDS) ($n=18$). *$P=0.02$, ***$P=0.0004$. (c) early MDS ($n=15$) vs advanced MDS ($n=15$). *$P=0.04$. All other P-values between data sets were not significant.

(7.43 [2.71–27.97] vs 11.45 [4.58–31.81]) ($P=0.001$). Expression levels of α5 or CD49e were comparable between MDS and controls (5.75 [1.48–24.07] vs 5.99 [2.50–15.12]), whereas MDS-derived progenitors had an upregulation of α2 or CD49b (2.49 [0.44–6.14] vs 1.73 [0.99–2.52]), and of α6 or CD49f (3.17 [0.57–16.33] vs 1.61 [0.79–2.56]), but these differences were not significant (Figure 3a).
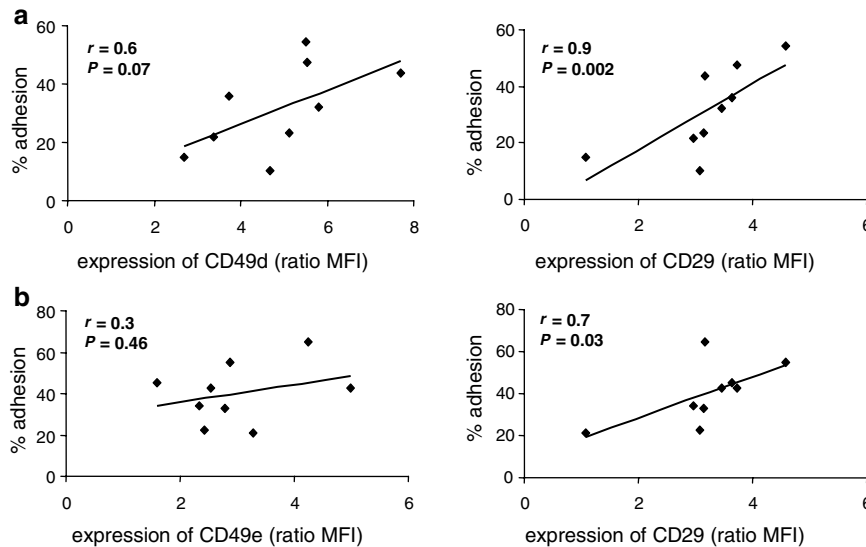
Figure 4 Relationship between expression levels of CD29, CD49d, CD49e and adhesion on HBF (a) and RGD (b) fibronectin fragments. Functional adhesion tests were performed in nine MDS patients by plating 50 000 CD34$^+$ cells in 24-well flat-bottomed tissue culture plates overnight coated with purified fibronectin fragments HBF (ligand for α4β1) and RGD (ligand for α5β1), with BSA as control. After adhesion for 1 h at 37°C, nonadherent and adherent cells were recovered, washed, and enumerated by flow cytometry using TruCount® Absolute Count Tubes (Becton Dickinson). Subsequently, the percentage of adherent cells could be calculated. r-values are the Spearman correlation coefficients. Regression lines were fitted by Graphpad PRISM version 3.02.

When we compared adhesion receptor profiles between HA-MDS and LA-MDS patients, we found in the HA-MDS subgroup a downregulation of all adhesion receptors tested. This downregulation reached statistical significance for CD29 (3.05 [1.08–3.76] vs 6.47 [2.62–12.96]) ($P = 0.02$), CD49d (5.30 [3.74–7.88] vs 8.85 [2.71–27.97]) ($P = 0.02$), and for CD49e (2.53 [1.60–4.98] vs 7.89 [1.48–24.07]) ($P = 0.0004$). Downregulation of CD49b (1.18 [0.87–1.64] vs 2.99 [0.44–6.14]) and CD49f (1.15 [0.57–1.59] vs 4.01 [0.8–16.33]) was also observed, but not in a statistically significant way ($P = 0.1$ for both) (Figure 3b). An inverse correlation was found between the percentage of apoptosis and the expression levels of CD49d ($r = -0.4$).

When patients were grouped according to their FAB subtype, MDS-derived progenitors from patients with early phase MDS (RA, RARS) had a significant downregulation of CD49e (4.24 [1.48–17.79] vs 7.26 [1.83–24.07]) compared with more advanced MDS (RAEB, RAEB-t, sAML) ($P = 0.04$). For CD29 (5.04 [1.80–12.57] vs 4.33 [1.08–12.96]) ($P = 0.4$), CD49d (6.47 [3.39–12.18] vs 8.39 [2.71–27.97]) ($P = 0.2$), CD49b (1.99 [0.97–5.19] vs 2.88 [0.44–6.14]) ($P = 0.6$), and CD49f (3.25 [0.57–16.33] vs 3.10 [0.80–13.31]) ($P = 0.3$), there were no significant differences between early and more advanced MDS subtypes (Figure 3c). When a comparison was made between LA-MDS patients in the early ($n = 6$) and advanced MDS subgroups ($n = 12$), expression levels of CD29 (7.5 [1.80–12.57] vs 4.79 [1.08–12.96]) ($P = 0.3$), CD49d (8.91 [3.39–12.18] vs 8.82 [2.71–27.97]) ($P = 0.4$),and CD49e (6.81 [2.33–17.79] vs 8.43 [3.27–24.07]) ($P = 0.5$) on CD34$^+$ HPC, were within the same range. In contrast, early phase MDS patients with HA-MDS ($n = 9$) had lower expression levels of CD29 (3.40 [1.90–4.58]), CD49d (4.85 [3.74–5.81]), and CD49e (2.53 [1.60–4.98]) compared with the LA-MDS subpopulation within the same FAB subgroups ($n = 6$, cfr. supra). Although P-alues were found to be < 0.05, we believe these sample numbers are too small to perform mathematically correct statistical comparisons.

α4β1 and α5β1-mediated adhesion of CD34$^+$ MDS progenitors on fibronectin fragments

In the next set of experiments, we have studied the relationship between the expression levels of the adhesion receptors CD29, CD49d, and CD49e on CD34$^+$ MDS progenitors and their in vitro adhesive capacity to their natural ligands: the fibronectin fragments HBF and RGD. In nine patients, we obtained sufficient progenitors to perform functional adhesion tests. CD34$^+$ progenitors were plated on purified HBF (ligand for α4β1) and RGD (ligand for α5β1), with BSA as control. The percentage of adhesion was quantified as described in the Materials and methods section, and correlated with the measured baseline expression levels of CD29, CD49d, and CD49e. The mean percentage of adhesion to HBF was 34% (range: 11–65%), and 42% (range: 21–65%) for RGD. The mean percentage of adhesion to BSA varied between 8 and 10% (mean = 9%) and was significantly lower compared to HBF and RGD peptide ($P = 0.001$ for both). We observed a positive correlation between the expression levels of CD49d and CD29 on MDS CD34$^+$ progenitors and their individual adhesive capacity to HBF ($r = 0.6$ and 0.9, respectively). Similar observations were made between expression levels of CD49e, CD29, and adhesion on RGD ($r = 0.3$, and $r = 0.7$, respectively) (Figure 4). These data suggest that decreased expression of CD29, CD49d, and CD49e is associated with a reduced in vitro adhesive capacity to fibronectin fragments.

Discussion

Excessive intramedullary programmed cell death or apoptosis is central in the pathogenesis of MDS. Although it can affect all FAB subtypes, literature evidence points towards higher levels of intramedullary apoptosis in patients with a low propensity for leukemic transformation (RA, RARS).[26] Our findings are in

support of these published observations: in comparison with the RA and RARS subgroups (early phase MDS), apoptosis levels of progenitor cells from patients with RAEB, RAEB-t, and sAML (advanced MDS) were significantly lower and in the range of what was observed in a control population. Currently, it is still a matter of debate if apoptosis in MDS is fundamentally related to the molecular pathogenesis of the disease or if it is merely a 'logical' consequence of progressive damage to genes and proteins essential for cell proliferation and survival. Additionally, the currently known proapoptotic triggers are not always identifiable in every individual MDS patient, so the challenge to unravel new proapoptotic factors in MDS still remains. The importance of α4β1- and α5β1-mediated interactions between progenitor cells and the extracellular matrix in the proliferation, differentiation, and survival of normal and leukemic progenitors has inspired us to study β1 integrin expression levels on MDS-derived progenitors. By comparing a randomly selected group of 30 untreated MDS patients with healthy control subjects, we have observed a significantly decreased expression of the β1 and α4 integrin chains on the cell surface of marrow-derived CD34+ MDS progenitors. On contrary, α2 and α6 were found to be upregulated, albeit not significantly. In other words, these observations give evidence for selective downregulation of α4β1 integrins on CD34+ progenitors in MDS patients. Given the elevated progenitor apoptosis levels in the overall MDS study population, and the established antiapoptotic role of α4β1, we were intrigued if our observation of downregulation of α4β1 could be related to the apoptotic process itself. Therefore, we have performed a reanalysis by grouping patients based on their levels of progenitor apoptosis. In the patient subgroup with apoptosis levels higher than what could be observed in a randomly selected healthy control population (what we called 'high apoptosis' MDS), we observed a significant downregulation of α4, β1, and also of α5 integrin chains in comparison with MDS patients having apoptosis levels within the range of the control population (so-called 'low apoptosis' MDS). Similar significancies were found when comparing high apoptosis patients and control subjects, but not between low apoptosis patients and controls (results not shown). In contrast, when patients were grouped according to their FAB subtypes into early phase MDS (RA, RARS) and advanced MDS (RAEB, RAEBt, and sAML), we found only a significant downregulation of α5 in the early phase MDS subgroup. However, it has to be considered that within this early phase MDS subgroup, 40% of the patients belonged to the low apoptosis subcategory, and their expression of α4, β1, and α5 was fully in the range of what could be observed in the majority of advanced MDS patients and the controls. In contrast, the 60% of early phase patients with high apoptosis levels had an overt downregulation of α4, β1, and α5 in comparison with their low apoptosis counterparts. In other words, our results suggest that the downregulation of α4, β1, and α5 is more related to the levels of progenitor cell apoptosis and not primarily to the FAB subtype.

Our observations raise the question as to what mechanisms could underlie the downregulation of adhesion receptors, and more precisely of α4β1 and α5β1, on progenitor cells in MDS.

It is well known that apoptosis and cell death cause cell membrane disintegration and protein cleavage.[27] Therefore, in order to avoid any interference from the apoptotic process itself on our results, we have performed all flow cytometric analyses after gating on viable, nonapoptotic, progenitors only. The annexin V assay that we used to discriminate viable and apoptotic cells measures the translocation of phosphatidylserine from the inner leaflet of the plasma membrane to the outer surface. This is a characteristic early change in apoptotic cells occurring prior to the loss of cell membrane integrity and nucleosomal DNA fragmentation.[28] Therefore, it seems very unlikely that the adhesion receptor downregulation on MDS progenitors that we observed would be a consequence of an already advanced apoptotic process. In contrast, our observations of reduced α4β1 and α5β1 expression levels would rather support the concept that this downregulation is not a consequence of the apoptotic process itself, but eventually a newly identified proapoptotic trigger in MDS. Cell death caused by decreased adhesion to stromal ligands is called anoikis[29] and is primarily controlled by the ECM in a α4β1 and α5β1 integrin-specific manner.[10,11] In our functional adhesion studies, we observed that decreased expression levels of α4, α5, and β1 integrin chains did correlate with reduced in vitro adhesiveness to fibronectin fragments, their principal natural ligands. These in vitro findings might support the concept that α4β1 and α5β1 downregulation on hematopoietic progenitors from MDS patients could exert its proapoptotic effect by decreased in vivo adhesiveness to the extracellular matrix component fibronectin.

Progenitor hyperproliferation, frequently observed concomitantly with apoptosis in MDS,[30] could also be related to decreased α4β1 or α5β1 expression, but was beyond the scope of our present study.

Since it is very unlikely that the apoptotic process itself is responsible for α4β1 and α5β1 downregulation, the possible underlying mechanisms still need to be elucidated. Intrinsic structural abnormalities in cell membrane proteins or exogenous microenvironmental factors like impaired cytokine production could be responsible for adhesion receptor downregulation. Altered cell surface receptor expression has already been sporadically reported in MDS.[31] Additionally, a recent report has shown that patients with less advanced (RA/RARS) and more advanced MDS subtypes (RAEB and RAEBt) can be discriminated using cell surface immunophenotyping.[32]

On the other hand, it is well known that several cytokines can influence the number and/or the ligand binding affinity of members of the β1 integrin family. Both in normal and in AML progenitor cells, cytokines like IL-3, GM-CSF, and SCF can upregulate the functional state of α4β1 and α5β1 integrins, resulting in increased adhesion to fibronectin.[14,33] Growth-inhibiting cytokines like TNF-α and IFN-γ are known to promote cell death in MDS by several mechanisms.[34,35] Although they can also modulate the expression of adhesion receptors,[36] it is to be elucidated if they also contribute to altered α4β1 and α5β1 expression levels in MDS.

Finally, despite the predominant clonal nature of hematopoiesis in MDS, the possibility remains that the CD34+ fractions of MDS patients with early phase disease (RA, RARS) could still contain minor nonclonal fractions.[37,38] This is a drawback of many MDS studies when separation of clonal and nonclonal progenitors is not possible. Nevertheless, we believe that minor nonclonal fractions could only result in neglectable variations of the highly significant differences in α4β1 and α5β1 expression levels we observed in this study.

In summary, we have shown that CD34+ progenitor cells from MDS patients with high levels of intramedullary apoptosis have a significant downregulation of the α4β1 and α5β1 integrin receptors. Given the importance of these particular integrin receptors in cell survival, this downregulation could be a newly identified proapoptotic factor in MDS. Reduced adhesion to the extracellular matrix component fibronectin might be the principal mechanism by which α4β1 and α5β1 downregulation reduces progenitor cell survival.

References

1 Eaves CJ, Cashman JD, Sutherland HJ, Otsuka T, Humphries RK, Hogge DE *et al.* Molecular analysis of primitive hematopoietic cell proliferation control mechanisms. *Ann NY Acad Sci* 1991; **628**: 298–306.

2 Verfaillie C, Hurley R, Bhatia R, McCarthy JB. Role of bone marrow matrix in normal and abnormal hematopoiesis. *Crit Rev Oncol Hematol* 1994; **16**: 201–224.

3 Verfaillie CM. Adhesion receptors as regulators of the hematopoietic process. *Blood* 1998; **92**: 2609–2612.

4 Chan YH, Watt SM. Adhesion receptors on haematopoietic progenitor cells. *Br J Haematol* 2001; **112**: 541–557.

5 Clark EA, Brugge JS. Integrins and signal transduction pathways: the road taken. *Science* 1995; **268**: 233–239.

6 Parise LV, Lee JW, Juliano RL. New aspects of integrin signaling in cancer. *Semin Cancer Biol* 2000; **10**: 407–414.

7 Williams DA, Rios M, Stephens C, Patel VP. Fibronectin and VLA-4 in haematopoietic stem cell–microenvironment interactions. *Nature* 1991; **352**: 438–441.

8 Verfaillie CM, McCarthy JB, McGlave PB. Differentiation of primitive human multipotent hematopoietic progenitors into single lineage clonogenic progenitors is accompanied by alteration in their interaction with fibronectin. *J Exp Med* 1991; **174**: 693–703.

9 Hurley RW, McCarthy JB, Verfaillie CM. Direct adhesion to bone marrow stroma via fibronectin receptors inhibits hematopoietic progenitor proliferation. *J Clin Invest* 1995; **96**: 511–519.

10 Zhang Z, Vuori K, Reed JC, Ruoslahti E. The α5β1 integrin supports survival of cells on fibronectin and up-regulates Bcl-2 expression. *Proc Natl Acad Sci USA* 1995; **92**: 6161–6165.

11 Wang MW, Consoli U, Lane CM, Durett A, Lauppe MJ, Champlin R *et al.* Rescue from apoptosis in early (CD34-selected) versus late (non-CD34-selected) human hematopoietic cells by very late antigen 4- and vascular cell adhesion molecule (VCAM)1-dependent adhesion to bone marrow stromal cells. *Cell Growth Differ* 1998; **9**: 105–112.

12 Hurley RW, McCarthy JB, Wayner EA, Verfaillie CM. Monoclonal antibody crosslinking of the α4 or β1 integrin inhibits committed clonogenic hematopoietic progenitor proliferation. *Exp Hematol* 1997; **25**: 321–328.

13 Matsunaga T, Takemoto N, Sato T, Takimoto R, Tanaka I, Fujimi A *et al.* Interaction between leukemic-cell VLA-4 and stromal fibronectin is a decisive factor for minimal residual disease of acute myelogenous leukaemia. *Nat Med* 2003; **9**: 1158–1165.

14 Bendall LJ, Makrynikola V, Hutchinson A, Bianchi AC, Bradstock KF, Gottlieb DJ. Stem cell factor enhances the adhesion of AML cells to fibronectin and augments fibronectin-mediated anti-apoptotic and proliferative signals. *Leukemia* 1998; **12**: 1375–1382.

15 Mudry RE, Fortney JE, York T, Hall BM, Gibson LF. Stromal cells regulate survival of B-lineage leukemic cells during chemotherapy. *Blood* 2000; **96**: 1926–1932.

16 Bhatia R, Munthe HA, Verfaillie CM. Role of abnormal integrin-cytoskeletal interaction in impaired β1 integrin function in chronic myelogenous leukemia hematopoietic progenitor cells. *Exp Haematol* 1999; **27**: 1384–1396.

17 De la Fuente MT, Casanova B, Garcia-Gila M, Silva A, Garcia-Pardo A. Fibronectin interaction with alpha4beta1 integrin prevents apoptosis in B cell chronic lymphocytic leukemia: correlation with Bcl-2 and Bax. *Leukemia* 1999; **13**: 266–274.

18 Yoshida Y. Hypothesis: apoptosis may be the mechanism responsible for the premature intramedullary cell death in the myelodysplastic syndrome. *Leukemia* 1993; **7**: 144–146.

19 Raza A, Gezer S, Mundle S, Gao XZ, Alvi S, Borok R *et al.* Apoptosis in bone marrow biopsy samples involving stromal and hematopoietic cells in 50 patients with myelodysplastic syndromes. *Blood* 1995; **86**: 268–276.

20 Delforge M. Understanding the pathogenesis of myelodysplastic syndromes. *Hematol J* 2003; **4**: 303–309.

21 Willman CL. Molecular genetic features of myelodysplastic syndromes (MDS). *Leukemia* 1998; **12**: S2–S6.

22 Kitagawa M, Saito I, Kuwata T, Yoshida S, Yamaguchi S *et al.* Overexpression of tumor necrosis factor (TNF)- α and interferon (IFN)-γ by bone marrow cells from patients with myelodysplastic syndromes. *Leukemia* 1997; **11**: 2049–2054.

23 Bouscary D, De Vos J, Guesnu M, Jondeau K, Viguier F, Melle J *et al.* Fas/Apo-1(CD95) expression and apoptosis in patients with myelodysplastic syndromes. *Leukemia* 1997; **11**: 839–845.

24 Sloand EM, Kim S, Fuhrer M, Risitano AM, Nakamura R, Maciejewski JP *et al.* Fas-mediated apoptosis is important in regulating cell replication and death in trisomy 8 hematopoietic cells but not in cells with other cytogenetic abnormalities. *Blood* 2002; **100**: 4427–4432.

25 Watson JV. A brief history of numbers and statistics with cytometric applications. *Cytometry* 2001; **46**: 1–22.

26 Parker JE, Mufti GJ, Rasool F, Mijovic A, Devereux S, Pagliuca A. The role of apoptosis, proliferation, and the Bcl-2-related proteins in the myelodysplastic syndromes and acute myeloid leukemia secondary to MDS. *Blood* 2000; **96**: 3932–3938.

27 Greenberg PL. Apoptosis and its role in the myelodysplastic syndromes: implications for disease natural history and treatment. *Leukemia Res* 1998; **22**: 1123–1136.

28 Merchant SH, Gonchoroff NJ, Hutchison RE. Apoptotic index by Annexin V flow cytometry: adjunct to morphologic and cytogenetic diagnosis of myelodysplastic syndromes. *Cytometry* 2001; **46**: 28–32.

29 Frish SM, Francis H. Disruption of epithelial cell-matrix interactions induces apoptosis. *J Cell Biol* 1994; **124**: 619–626.

30 Raza A, Alvi S, Borok RZ, Span L, Parcharidou A, Alston D *et al.* Excessive proliferation matched by excessive apoptosis in myelodysplastic syndromes: the cause-effect relationship. *Leukemia Lymphoma* 1997; **27**: 111–118.

31 Mazzone A, Porta C, Fossati G, Gritti D, Mazzucchelli I, Ricevuti G. Granulocyte dysplasia and dysfunction, and CD11/CD18 defects in myelodysplastic syndromes. *Leukemia Lymphoma* 1996; **23**: 267–275.

32 Maynadié M, Picard F, Husson B, Chatelain B, Cornet Y, Le Roux G *et al.* Groupe d'Etude Immunologique des Leucemies (GEIL). Immunophenotypic clustering of myelodysplastic syndromes. *Blood* 2002; **100**: 2349–23565.

33 Lévesque JP, Leavesley DI, Niutta S, Vadas M, Simmons PJ. Cytokines increase human hematopoietic cell adhesiveness by activation of very late antigen (VLA)-4 and VLA-5 integrins. *J Exp Med* 1995; **181**: 1805–1815.

34 Shetty V, Mundle S, Alvi S, Showel M, Broady-Robinson L, Dar S *et al.* Measurement of apoptosis, proliferation and three cytokines in 46 patients with myelodysplastic syndromes. *Leuk Res* 1996; **20**: 891–900.

35 Allampallam K, Shetty V, Hussaini S, Mazzoran L, Zorat F, Huang R *et al.* Measurement of mRNA expression for a variety of cytokines and its receptors in bone marrows of patients with myelodysplastic syndromes. *Anticancer Res* 1999; **19**: 5323–5328.

36 Defilippi P, Truffa G, Stefanuto G, Altruda F, Silengo L, Tarone G. Tumor necrosis factor α and interferon γ modulate the expression of the vitronectin receptor (integrin β3) in human endothelial cells. *J Biol Chem* 1991; **266**: 7638–7645.

37 Asano H, Ohashi H, Ichihara M, Kinoshita T, Murate T, Kobayashi M *et al.* Evidence for nonclonal hematopoietic progenitor cell populations in bone marrow of patients with myelodysplastic syndromes. *Blood* 1994; **4**: 588–594.

38 Delforge M, Demuynck H, Verhoef G, Vandenberghe P, Zachee P, Maertens J *et al.* Patients with high-risk myelodysplastic syndrome can have polyclonal or clonal haemopoiesis in complete haematological remission. *Br J Haematol* 1998; **102**: 486–494.