The Role of Platelet/Endothelial Cell Adhesion Molecule–1 (CD31) and CD38 Antigens in Marrow Microenvironmental Retention of Acute Myelogenous Leukemia Cells

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Abstract

In acute myelogenous leukemia (AML), leukemic cell-microenvironment interactions within various niches (stromal/osteoblastic or sinusoidal endothelial cell niches) have a role in leukemia cell survival and drug resistance. The AML leukemic cells express platelet/endothelial cell adhesion molecule–1 (CD31) and CD38, two adhesion molecules that could interact with microenvironmental elements, i.e., CD31 on the surface of marrow endothelial cells (CD31/CD31 and CD31/CD38 interactions) and hyaluronate (CD38/hyaluronate interactions). We report a physical association of these two antigens on the plasma membrane of myeloid leukemic cells. In this context, in vitro experiments done using interaction-blocking anti-CD31 and anti-CD38 monoclonal antibodies (CLB-HEC75 and OKT10, respectively) indicate that an excess of CD31 on the cell membrane of leukemic cells (CD31/CD38 MFI ratio >1) promotes a homotypic interaction with marrow endothelial cells, resulting in higher transendothelial migration. Conversely, an excess of CD38 (CD31/CD38 MFI ratio <1) allows leukemic cells to be entrapped within the bone marrow microenvironment through hyaluronate adhesion. The results obtained in vitro using fluorescence resonance energy transfer, co-capping, and co-immunoprecipitation experiments, and hyaluronate adhesion and transendothelial migration assays, are supported by immunophenotypic characterization of marrow leukemic cells from 78 AML patients on which CD38 expression levels were found to be positively correlated with those of CD31. Importantly, the excess of CD31 in those samples was associated with a higher peripheral WBC count. These findings indicate that bone marrow retention of AML cells depends on CD31 and CD38 coexpression levels. [Cancer Res 2007;67(18):8624–32]

Introduction

Acute myelogenous leukemia (AML) is characterized by uncontrolled proliferation within the bone marrow of malignant myeloid progenitors and the egress of these abnormal cells into the circulation. A major problem in the treatment of AML remains the recurrence of the pathology following chemotherapy, which is due to resistant leukemic cells localized in the bone marrow. It has been shown that cell-cell or cell-matrix adhesion processes are involved in chemoresistance (1). The retention of leukemic cells in the bone marrow compartment is controlled by interactions between the cells and elements of the marrow microenvironment.

In vivo, marrow endothelial cells play an important role in the trafficking of hematopoietic cells by acting as gatekeepers separating the stroma from the sinusoidal lumen. The marrow vascular niche consists of a network of thin-walled and fenestrated sinusoidal vessels. Venous sinusoids are the site of large-scale traffic of cells between the extravascular hematopoietic compartment and the blood stream. In comparison with typical basement membranes in other tissues, the marrow sinusoidal basement membrane has a discontinuous nature, lacking network organization. Moreover, this membrane presents an exceptionally large quantity of chondroitin sulfate proteoglycan (CSPG; ref. 2). This structural organization promotes the interactions between the hematopoietic cells and the marrow endothelium.

The CD31 and CD38 cell surface antigens are expressed in all AML subtypes, but their expression varies considerably from one patient to another. Human platelet/endothelial cell adhesion molecule–1 belongs to the immunoglobulin gene superfamily (3). It is expressed on endothelial cells and AML cells and has been identified as having a role in transendothelial migration via homophilic interaction (4). Human CD38 is a transmembrane glycoprotein present on AML cells whose extracellular domain contains an enzymatic site that can generate cyclic ADP ribose (cADPR) and ADPR from nicotine adenine dinucleotide (NAD+) and nicotinic acid adenine dinucleotide phosphate (NAADP) from NAADP+ (5). Moreover, three hyaluronate-binding sites have been reported in CD38, two of which existing in the extracellular domain of this antigen (6). CD31 and CD38 are known to be ligands of CD31 in homophilic and heterophilic interactions (7). Because CD31 is expressed on the entire endothelial cell surface and CSPG is able to complex with hyaluronate (8, 9), we postulated that the balanced control between extramedullary dissemination and marrow retention of leukemic cells may depend on the cell surface expression of CD31 and CD38 on leukemic cells via CD31/CD31 interactions with the vascular wall and CD38/hyaluronate interactions with the extracellular matrix.

In the present study, we report that CD31 and CD38 are important for leukemic cell trafficking in AML. We show a physical association of these two antigens on the plasma membrane of leukemic cells. In this context, an excess of CD31 (CD31/CD38 ratio >1) promotes a homotypic interaction with marrow endothelial cells, resulting in higher transendothelial migration. Conversely, an excess of CD38 (CD31/CD38 ratio <1) allows leukemic cells to be...
entrained within the marrow microenvironment through adhesion to hyaluronate. The strategy adopted for this study included the analysis of the distribution of CD38 and CD31 on the cell surface, the effects of CD38 overexpression on the *in vitro* adhesion to hyaluronate, and the effects of anti-CD31 and anti-CD38 blocking monoclonal antibodies (mAb) on the *in vitro* adhesion to marrow endothelium and transendothelial migration of leukemic cells. The results obtained *in vitro* were supported by the immunophenotypic characterization of marrow leukemic cells of 78 consecutive AML patients on which we found that CD38 and CD31 expression levels were positively correlated, and that an excess of CD31 (CD31/CD38 ratio >1) was associated with a higher peripheral WBC count.

**Materials and Methods**

**Human cells and reagents.** Marrow cells of 78 adult patients (Table 1) presenting *de novo* AML were analyzed from samples collected after obtaining informed consent from each patient and approval by the ethics committee of the University Hospital of Tours. HL-60 and U937 (ECACC) were cultured in RPMI 1640 with 20 μMOL/L L-glutamine supplemented with 10% FCS, 100 units/mL penicillin G, and 100 μg/mL streptomycin (Invitrogen). The TrHBMEC cell line (10) was cultured in α-MEM with ribonucleosides with 5% FCS, 100 units/mL penicillin G, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B (Bristol-Myers Squibb) in gelatin-coated flasks.

**Phenotype analysis by flow cytometry.** The cells were incubated with the following mAbs from BD Biosciences: APC-conjugated anti-CD45 immunoglobulin G1 (IgG1; HI30), PE-conjugated anti-CD34 IgG1 (8G12), PE-conjugated anti-CXCR4 (12G5), FITC-conjugated anti-CD31 IgG1 (WM59), FITC-conjugated anti-CD38 IgG1 (HIT2), PE-conjugated anti-CD44 IgG2a (G44-26), FITC-conjugated anti-CD44 IgG1 (IM7), and FITC- or PE-conjugated isotypic controls. CD168 (HMMR) expression was evaluated using purified goat polyclonal antibody and goat IgG as negative control, followed by FITC-rabbit anti-goat IgG (all from Santa Cruz Biotechnology).

**Fluorescence resonance energy transfer and co-capping experiments.** FRET experiments were carried out on a FACSCalibur flow cytometer as previously described (11). Purified anti-CD38 mAb (T16, Beckman Coulter) was conjugated with Cy3 dye using FluoroLink MAb Cy3 Labeling kit (Amersham Biosciences). Leukemic cells were incubated at 4°C for 1 h with the anti-CD38 Cy3-conjugated mAb alone or in combination with FITC-conjugated anti-CD31 FITC- or anti-CD44 mAbs (WM59 and HP2/1, respectively). For capping experiments, the cells were incubated with the purified mouse anti-human CD31 mAb (CLB-HEC75) or CD49D (HP2/1) for 30 min on ice, washed, and reacted with TRITC-labeled goat anti-mouse Ig (GaM; Sigma) on ice. Samples were then moved to 37°C for 30 min to induce capping, after which ice-cold PBS plus 0.5% bovine serum albumin (BSA) containing 0.1% azide was added. The cells were maintained on ice for counterstaining with biotinylated mAbs against CD38 (AT13/5, Serotec) and further incubated with FITC-conjugated streptavidin (Sigma). Cells settled on poly-L-lysine–coated coverslips (Sigma) were observed at room temperature with a DMR microscope (Leica Microsystems).

**Co-immunoprecipitation and immunoblotting.** Cells were lysed in 10 mmol/L HEPES (pH 7.9), 10 mmol/L KCl, 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, and 10% [vol/vol] NP40 containing protease inhibitors. Western blot analyses of CD38 and CD44 and CD168 were done as described (12). Immunoprecipitation was done with 2 μg anti-CD31 goat polyclonal Ab or goat IgG as negative control. Immune complexes were captured with protein A/G PLUS-Agarose (Santa Cruz Biotechnology) and subjected to SDS-PAGE and immunoblotting with anti-CD31 goat polyclonal Ab. All antibodies were purchased from Santa Cruz Biotechnology. The reactions were developed with peroxidase-conjugated secondary antibodies and the ECL Plus kit (Amersham Biosciences).

**Quantitative reverse transcription-PCR.** Total RNA of HL-60 and U-937 leukemic cell lines was isolated using TRizol (Invitrogen). The relative quantification of CD38, CD44, and HMRR gene expression was done by real-time PCR on an ABI Prism 7900HT Sequence Detection System. cDNA was generated using random primers and M-MLV RT (Invitrogen). Q-PCR reactions were carried out in a total volume of 10 μL on 25 to 50 ng of CDNA using TaqMan Universal Master Mix (Applied Biosystems) and Universal ProbeLibrary assays designed with the ProbeFinder software (Roche Applied Science). The primer sequences and Universal Probe numbers used are available upon request. ABI Prism 7900HT SDS was programmed to an initial step of 2 min at 50°C and 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 1 min at 60°C. All reactions were run in triplicate, and the average values were used for quantification. The human *LOC100085788* (18S rRNA) predeveloped TaqMan assay (Applied Biosystems) was used as the endogenous control to normalize the expression values of CD38, CD44, and HMRR (*ACT = Ctarget - Cendogenous control*). Relative fold differences were determined using the ΔΔCT method.

**Hyaluronate adhesion assays.** Culture dishes with grid (35 mm; Sarstedt) were coated with hyaluronate (Sigma) at 10 μg/mL in PBS overnight at 4°C. The cells were incubated with anti-CD44 blocking mAb (BU75, Ancell) or anti-CD38 blocking mAb (OKT10, Prof. F. Malavasi, Laboratory of Immunogenetics, Turin, Italy) and plated into the dishes. After incubation for 2 h at 37°C, adherent leukemic cells were counted in 10 separate microscope fields at 100× magnification (DMIL; Leica Microsystems). Hyaluronate-specific adhesion was calculated as follows: (number of cells adhering to hyaluronate-coated dish) – (number of cells adhering to PBS-coated dish).

**Adhesion to TrHBMEC and transendothelial migration assays.** The experiments were done using anti-CD38 (OKT10) and anti-CD31 (CLB-HEC75, Monosan) blocking mAbs. Before adhesion/migration assays, leukemic cells were preincubated for 1 h at room temperature with blocking mAbs. The percentage of adhesion/migration in the presence of blocking mAbs was compared with the percentage obtained with the isotopic control. Adhesion of HL-60 and U937 cells to the TrHBMEC cell line was studied using a conventional adhesion assay using 51Cr-labeled cells (13), where the cells were labeled with 51CrO4Na2 (100 μCi/5 × 106 cells; Life Science Products) and were added to the endothelial cell monolayer. For the transmigration experiments, TrHBMEC cells were seeded on 5-μm microporous membranes (Transwell, Corning Incorporated) coated with fibronectin (Sigma). Confluenzy was verified by light microscopy after fixation in 100% methanol and May-Grünwald-Giemsa staining. Leukemic cells were added to the upper chamber in 0.1 mL of assay medium, and 0.6 mL of assay medium with 200 ng/mL of stromal derived factor-1 (SDF-1; R&D Systems) was added to the lower compartment. The membrane expression of CXCR4 on leukemic cells was checked by indirect immunofluorescence staining using mouse anti-human CXCR4 IgG2a (12G5, BD Biosciences). Transendothelial migration was allowed to proceed at 37°C in 5% CO2 for 24 h. The migrated fraction was counted in the bottom well of the migration chamber using a Malassez chamber. The cells were then stained with FITC-conjugated anti-CD146 (S-Endo1, Biocytex) and APC-conjugated anti-CD45 (HI30, BD Biosciences), and the percentages of leukemic cells (CD146/CD45*) were determined.

**Scanning electron microscopy.** Adhering TrHBMEC cells were fixed with a solution of 4% paraformaldehyde and 1% glutaraldehyde for at least 1 h at room temperature, and then preparations were stored at 4°C. The preparations were postfixed for 30 min with 1% osmium tetroxide in phosphate buffer. Preparations were dehydrated in progressive concentrations of ethanol (50–100%), dried by the critical point method (CO2), and mounted on stubs. Then, a thin layer of platinum was deposited with a Magnetron sputtering device. Preparations were studied under an FEG Gemini 982 scanning electron microscope (Zeiss).

**Statistical analysis.** Statistical analyses were done with Statview software, version 4.5 (Abacus Concept Inc.) using the Mann-Whitney *U* test for nonzero proportions, Wilcoxon test, χ2 contingency test, and *z* test for nonzero correlation, as appropriate. The level of significance was set at 0.05.

**Results**

CD31 and CD38 are closely associated on myeloblastic leukemic cells. We investigated a lateral association between CD38 and its ligand CD31 on HL-60 and marrow AML cells using...
fluorescence resonance energy transfer (FRET), co-capping and co-immunoprecipitation strategies. FITC-conjugated anti-CD31 induced a 98.3 ± 21.7% increase in geometric mean fluorescence intensity (MFI) of Cy-3 on HL-60 cells. Conversely, the MFI of Cy3 was not clearly affected using FITC-conjugated anti-CD49d (+15.0 ± 3.0%). The association being significant and specific, as shown by the lack of energy transfer with anti-CD49d mAb, these results suggest a physical association of CD31 and CD38 on leukemic cells (Fig. 1A). Because a very stringent way of demonstrating protein association in living cells is to impair the cell surface distribution of one protein by capping it with a mAb and investigating whether a second protein moves with it or co-caps, we conducted co-capping experiments with HL-60 and marrow AML cells. Capping of CD31 was first induced with CLB-HEC75 in cell suspensions at 37°C. The cells were then fixed and labeled with anti-CD38 mAbs. CD31 and CD38 displayed very similar patterns of labeling following capping. To determine the specificity of this assay, we induced capping of CD49d with clone HP2/1 and investigated whether it also capped CD38. As illustrated in Fig. 1B, CD38 was not co-capped with CD49d, showing that the colocalization observed for CD31 and CD38 was specific. Consistent with these findings, CD38 co-immunoprecipitated with CD31 in HL-60 cells, whereas no CD38 could be detected when immunoprecipitating with control goat IgG (Fig. 1C).

**CD38 promotes the adhesion of AML cells to hyaluronate.**

Leukemic cells were treated with all-trans-retinoic acid (ATRA; or left untreated) to induce CD38 overexpression and were then plated onto a hyaluronate-coated dish. As verified by quantitative reverse transcription-PCR (RT-PCR), Western blot, and flow cytometry, ATRA effectively induced overexpression of CD38 in all experiments (Fig. 2A). Because CD44 and CD168 are also able to interact with hyaluronate on leukemic cells (14, 15), we verified that ATRA exposure did not increase their basal level of mRNA and protein expression (Fig. 2A). Because CD44 can also promote adhesion to hyaluronate, all in vitro adhesion assays were done in the presence of anti-CD44 blocking mAB to minimize its role. As expected, these antibodies inhibited the adhesion to hyaluronate in all experiments (data not shown). HL-60 and U937 cells exposed to ATRA exhibited a 9.2 ± 2.7- and 2.1 ± 0.1-fold increase in CD38 expression (S/N of MFI), and a 44.7 ± 10.9% and 38.5 ± 5.4% increase in adhesion to hyaluronate (n = 5, P < 0.001), respectively. Five experiments were also done with marrow leukemic cells from AML patients with more than 80% marrow blastosis (Table 1). The leukemic cells exposed to ATRA exhibited an average of 26.3 ± 11.4% increase in CD38 expression (S/N of MFI) and 40.3 ± 10.0% increase in adhesion to hyaluronate (Fig. 2A). These results are further supported by the fact that anti-CD38 (OKT10) and anti-CD44 mAbs were found to inhibit, with the same order of magnitude, the adhesion of untreated HL-60 cells to hyaluronate (52.9% and 34.1%, respectively; data not shown). Together, these results suggest that CD38 participates in the in vivo adhesion of marrow AML cells to the extracellular matrix.

**CD38 is more effective than CD38 in promoting adhesion of leukemic cells to marrow endothelium.**

The roles of CD31 and CD38 antigens in the adhesion of human leukemic cells to the marrow endothelium were studied using anti-CD31 (CLB-HEC75) and anti-CD38 (OKT10) blocking mAbs in assays with HL-60 and U937 cells plated on the confluent monolayer of a human marrow endothelial cell line (TrHBMEC). As presented in Fig. 2B, adhesion of HL-60 and U937 cells at 4°C was inhibited by CLB-HEC75 (by

<table>
<thead>
<tr>
<th>FAB subtype</th>
<th>Patients</th>
<th>Age (y)</th>
<th>Marrow blasts (%)</th>
<th>WBC count (/mm³)</th>
<th>CD31 (S/N MFI)</th>
<th>CD38 (S/N MFI)</th>
<th>Ratio CD31/CD38</th>
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<td></td>
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<tr>
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<td>2.2 (1.8–5.7)</td>
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<tr>
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<tr>
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<td>2.7 (1.1–27.7)</td>
<td>6.1 (1.7–32.1)</td>
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<tr>
<td>M3</td>
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<td>90 (72–98)</td>
<td>10.4 (1.6–66.9)</td>
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<tr>
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<td>75 (23–97)</td>
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<tr>
<td>M5</td>
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<td>85 (35–97)</td>
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<td>5.0 (1.7–33.7)</td>
<td>5.0 (1.5–11.6)</td>
<td>1.04 (0.22–2.91)</td>
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<tr>
<td>M7</td>
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<td>2.6</td>
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<td>Total</td>
<td>78</td>
<td>46 (17–78)</td>
<td>80 (17–98)</td>
<td>14.1 (1.1–154.0)</td>
<td>3.7 (1.1–33.7)</td>
<td>4.7 (1.0–35.6)</td>
<td>0.81 (0.09–2.91)</td>
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Patients analyzed by in vitro assays

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<th>Patients</th>
<th>Age (y)</th>
<th>Marrow blasts (%)</th>
<th>WBC count (/mm³)</th>
<th>CD31 (S/N MFI)</th>
<th>CD38 (S/N MFI)</th>
<th>Ratio CD31/CD38</th>
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<tr>
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<td>31.9</td>
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<tr>
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<td>98</td>
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<tr>
<td>M2</td>
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<td>51.6</td>
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NOTE: Data provided are median (range).
Abbreviations: FAB, French American British classification; S/N MFI, signal/noise ratio of mean fluorescence intensities.
The ability of CXCR4+ leukemic cells to migrate from the previously reported French-American-British (FAB) subgroups (17). The antibody-captured proteins were analyzed by Western blotting with anti-CD38 antibody. C. co-capping experiment with HL-60 cells and marrow AML blast cells. The cells were capped with purified anti-CD31 mAb or anti-CD49d mAb as control and TRITC-conjugated GaM for 30 min at 37°C. The experiment was blocked by the addition of cold PBS, and counterstaining was done with biotin-conjugated anti-CD38 antibody-captured proteins were analyzed by Western blotting with anti-CD31 goat polyclonal Ab or goat IgG. The antibody-captured proteins were analyzed by Western blotting with anti-CD38 antibody.

CD31 is more effective than CD38 in promoting transendothelial migration of leukemic cells through marrow endothelium. To further investigate the roles of CD31 and CD38 in the transendothelial migration of leukemic cells, we used blocking mAbs in a Transwell system, where SDF-1 was used as a chemoattractant for CXCR4+ leukemic cells (Fig. 3A). Transendothelial cells were cultured until confluency on the 5-μm microporous membrane (Fig. 3B). Confluency of the endothelial cells was checked by microscopy analysis after May-Grünwald-Giemsa staining (Fig. 3C). The CXCR4 expression was not different from the previously reported French-American-British (FAB) subgroups (17). The ability of CXCR4+ leukemic cells to migrate in response to SDF-1 was also verified after staining with PKH26 fluorescent dye (Fig. 3A). The levels of transendothelial migration displayed by HL-60 cells and leukemic cells of AML patients were 3.5 ± 0.4% and 10.2 ± 3.9%, respectively (data not shown). As presented in Fig. 4A, anti-CD31 mAb blocked transmigration of HL-60 cells across the TrHBMEC monolayer by 49.7 ± 6.3%, and inhibition was lower (29.2 ± 4.1%) using anti-CD38 mAb (n = 5; P < 0.0001). The difference was not due to selective transmigration of smaller cells because their forward scatter was not different. Moreover, we verified the absence of CD45+ CD146+ cells in the lower chamber of the Transwell system in all experiments (data not shown). Transendothelial migration experiments were also done using marrow leukemic cells of seven AML patients, and the effects of blocking mAb were analyzed in terms of antigen density on the cell surface (Fig. 4B). In all cases, CXCR4 was expressed on these cells (data not shown). The inhibition of migration by anti-CD31 blocking mAb ranged from 2.3% to 66.3% of controls and was correlated with CD31 expression level (R = 0.9236; P < 0.0001) as shown in Fig. 4C, indicating that migration blocking was stronger when the expression of CD31 on AML leukemic cells was higher. In contrast, migration in the presence of anti-CD38 blocking mAb was about 80% that of the control level (range from 70.6% to 89.6%) without correlation with CD38 expression level (Fig. 4C), suggesting a minor role in the transendothelial migration process.

Study of CD31 and CD38 distribution in 78 AML patients. To evaluate the influence of the CD31/CD38 ratio on extramedullary
malignant cell dissemination in vivo, we studied the phenotypes of marrow leukemic cells and the peripheral WBC counts of 78 consecutive adult patients with newly diagnosed de novo AML. Patient characteristics, peripheral WBC counts, and the expression of CD31 and CD38 antigens for each FAB subtype are presented in Table 1. Expression levels of CD38 and CXCR4 were not different in terms of FAB subtypes, whereas CD31 expression was higher on the M4/M5 FAB subtypes (P < 0.0001). The peripheral WBC count was statistically influenced by the CD31/CD38 ratio. As presented in Fig. 5A, an excess of CD31 antigen was associated with an increased WBC count in peripheral blood when comparing patients with ratio >1 to those with ratio <1 (P < 0.0001). Interestingly, peripheral WBC count was correlated with the CD31/CD38 ratio (R = 0.5286; P = 0.0129), whereas such a correlation was not found when considering CD38 or CD31 expression on marrow leukemic cells separately. In comparison with the group of patients with ratio <1, the group with ratio >1 was characterized by higher expression of CD31 (S/N MFI = 7.9 ± 1.4 versus 4.0 ± 0.5, P = 0.0081) and lower expression of CD38 (S/N MFI = 5.0 ± 0.7 versus 8.6 ± 1.1, P = 0.0058). Moreover, although the CD31/CD38 ratio was higher in AML (myelo)monocytic subtypes when comparing FAB M4/M5 to FAB M0/M1/M2/M3 subtypes (1.21 ± 0.08 and 0.72 ± 0.08, respectively; P < 0.0001), the median peripheral WBC count in the subgroup of patients with a CD31/CD38 ratio >1 was higher when considering each FAB subtype separately (Fig. 5B). These findings are in accordance with the results of the in vitro functional analyses and are supported by a correlation between the expression levels of membrane CD31 and CD38 on marrow leukemic cells (R = 0.6621; P < 0.0001). Thus, as shown in Fig. 5C, marrow leukemic cells expressed CD38 and CD31 simultaneously, suggesting a heterotypic model of CD38/CD31 interaction in accordance with the colocalization of these two antigens shown in vitro.

**Discussion**

In this study, we focused on the role of CD31 and CD38 cell surface antigens in controlling this process via the CD38/ extracellular matrix and CD31/marrow endothelial cell interactions.

CD38 has been mostly studied in immune cells (reviewed in ref. 18), on which CD38 ligation delivers activation/antiapoptotic signals and induces cytokine production. Many groups have shown that its short cytoplasmic tail is not required for CD38-mediated signals and induces cytokine production. Many groups have shown that its short cytoplasmic tail is not required for CD38-mediated signal transduction. CD38 is localized on lipid microdomains (rafts) within the plasma membrane (19), and its intrinsic ineptitude to

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**Figure 2.** Effects of ATRA-induced CD38 overexpression on the adhesion of leukemic cells to hyaluronate and bone marrow endothelial cells. The cells were exposed to 1 μmol/L ATRA for 24 h at 37°C or left unexposed. A, adhesion to hyaluronate of leukemic cells exposed to ATRA or left unexposed. Columns, percentages of adhesion after ATRA exposure compared with adhesion without ATRA. The experiments were done on both HL-60 and U937 cell lines (mean ± SEM of five independent experiments) and marrow leukemic cells of five AML patients whose characteristics are described in Table 1. The effects of ATRA on the expression of CD38, CD44, and CD168 in HL-60 and U937 cells were evaluated by quantitative PCR, Western blot, and flow cytometry. In quantitative PCR experiments (lower right), mRNA levels were determined by calculating the ∆CT values where the levels of mRNA for CD38, CD44 (all isoforms), and HMR (CD168) are normalized according to the endogenous control gene LOC100008588 (18S rRNA; ∆CT = C_target − C_endogenous control). The graphs show the basal level of mRNA expression (∆CT) and the relative fold difference after ATRA exposure. The Western blot analysis was done with 50 μg lysate protein, which were analyzed with anti-CD38, anti-CD44, and anti-CD168 antibodies and reprobed with α-tubulin (lower line) to verify equal loading. The flow-cytometric expression of CD38, CD44, and CD168 antigens before (blue lines) and after (red lines) ATRA exposure was determined by comparison with negative controls (black lines). B, adhesion assays done with HL-60 and U-937 cells labeled with 51Cr. Cells were exposed (black column) or left unexposed (gray column) to 1 μmol/L ATRA and then incubated with anti-CD31 or anti-CD38 blocking mAbs for 1 h at room temperature. Adhesion in the presence of blocking mAbs was compared with adhesion in the presence of nonrelevant mAbs (IgG1). Columns, mean results; bars, SEM. Representative histograms of phenotypic profiles of CD31 expression on HL60 and U937 cells by flow cytometry before (gray histogram) and after (black line) ATRA exposure.
transduce signals is overcome by working in physical and functional association with specialized signaling molecules such as TCR/CD3, BCR, and CD16 on the cell surface of lymphocytes (20–22) and with CD83, CD11b, and CCR7 on mature monocyte-derived dendritic cells (23). The ability of CD38 to contribute to molecular complexes on the cell surface of myeloid leukemic cells has not been reported to date. CD31 is a ligand of CD38 (7), and we showed here using FRET and co-capping experiments that CD31 and CD38 are closely associated on AML cells. Interestingly, this colocalization was not been found on chronic lymphocytic leukemia (CLL) B cells (24). The fact that it is known that CD38 and CD31 are polymorphic in the white cell population may explain this difference (25, 26). Moreover, the specific CD38 molecular complex on these lymphoid cells could physically hamper their lateral association with CD31 on the cell surface. Our experimental results are supported by a correlation between CD38 and CD31 expression on marrow leukemic cells from this cohort of 78 de novo AML patients. This simultaneous expression has also been reported on CLL B cells and marrow neoplastic plasma cells (27–29). The small number of CD31 or CD38 molecules not engaged in the CD31/CD38 membrane complex on leukemic cells could therefore interact more easily with the microenvironment and promote either extracellular matrix anchorage or transendothelial migration.

Hyaluronate is a key component of the extracellular matrix and is abundant at the periphery of the marrow microvasculature (30), whose basal membrane specifically presents a huge quantity of CSPG (2) known to complex with hyaluronate (9). Nishina et al. (6) reported two hyaluronate-binding sites in the extracellular domain of CD38. We verified the functionality of these [B(X7)B] motifs by demonstrating that the ATRA-induced up-regulation in CD38 expression concomitantly increased cell adhesion to hyaluronate. A high number of CD38 molecules on the cells may therefore promote mechanical cell anchorage to the extracellular matrix in vivo. In certain cases, hyaluronate binding can modify the AML cell biology, such as the CD44/hyaluronate interaction known to induce differentiation (31). In contrast to CD44, the cytoplasmic tail of CD38 is very short and probably unable to transduce signals after hyaluronate binding. In accordance with this hypothesis, Todisco et al. (32) reported that hyaluronate is not able to induce the marked inhibition of the leukemic myelopoiesis observed after CD38 ligation by anti-CD38 mAb in the presence of stromal feeder layers mimicking the in vivo marrow microenvironment.

CD31 plays a role in transendothelial migration of various CD31+ cell types such as CD34+ hematopoietic cells (33, 34), lymphocytes (7, 16), and monocytes (4). Because CD31 is distributed over the entire endothelial cell surface (8), it could interact with CD38 or...
CD31 expressed on marrow AML cells. We developed a model of leukemic cell trafficking using the TrHBMEC cell line that expresses CD31 and retains the morphology, phenotype, and function of primary marrow endothelial cells (10). By analogy to studies on mature lymphocytes, CD38 and CD31 expressed on AML leukemic cells (17) could be important for the adhesion of leukemic cells to marrow microvascular endothelial cells and for the transendothelial migration process (7, 16). The role of the CD38/CD31 interaction has been clearly shown for lymphocytes in the adhesion to endothelium process (7), and a similar role for AML cells has recently been suggested (35). Nevertheless, the CD38/CD31 interaction is selectin-like, weaker than that of CD31/CD31 (16). In accordance with this, our results show that anti-CD38 blocking mAb induces a decrease in adhesion of leukemic cells to the endothelium only in experiments done at 4°C to minimize integrin-mediated binding (16). Moreover, the strong up-regulation of CD38 after ATRA exposure does not affect the effects of anti-CD38 blocking mAb used at saturating concentrations. The low levels of migration displayed by leukemic cells in the transendothelial migration experiments are in agreement with the findings of other groups who also used marrow endothelial cells (33, 34, 36). The results indicated a major role of the CD31/CD31 interaction and a minor role of the CD38/CD31 interaction in promoting leukemic cell transmigration. In contrast to anti-CD38 blocking mAb, which induces a slight and invariable inhibition of migration, anti-CD31 mAb reduces this process all the more strongly when the antigen is highly expressed on AML cells.

All these elements support the concept that the densities of CD31 and CD38 antigens on the cell surface of leukemic cells are critical to promoting either their marrow retention via the CD38/hyaluronate interaction or their extramedullary dissemination via the CD31/CD31(endothelial cell) interaction. Evaluation of peripheral leukocytosis and the densities of these two antigens on the marrow leukemic cells of 78 de novo AML patients supports this concept. In accordance with the results reported by Brouwer et al. (17), the expression of CD31 was higher in the M4/M5 FAB subgroup, and peripheral WBC counts showed no correlation with the levels of expression of this antigen. However, when considering the concomitant expression of CD31 and CD38 using the CD31/CD38 ratio, we show that an excess of CD31 (ratio > 1) is associated with a higher peripheral WBC count. Hyaluronate is abundant in lymph nodes (14), which participate in its catabolism (reviewed in ref. 37). Interestingly Jaksic et al. (27) applied the concept of the CD31/CD38 ratio to study the distribution of CLL B cells. They reported that a higher CD38 expression was associated with lymphoid compartments, particularly lymph nodes, and that these results suggested a functional interaction between CD38 and hyaluronate.

Figure 4. Effects of anti-CD31 and anti-CD38 blocking mAbs on the transendothelial migration of myeloblastic cells through bone marrow endothelial cells. A, transendothelial migration of myeloblastic cells preincubated with blocking mAbs. Migration in the presence of blocking mAbs was compared with that in the presence of nonrelevant mAbs (100%). Red columns, migration with anti-CD38 mAbs; blue columns, migration with anti-CD31 mAbs for HL-60 cells (mean ± SEM of five independent experiments) and marrow leukemic cells of seven AML patients whose characteristics are reported in Table 1. B, expression of CD31 (blue) and CD38 (red) on the marrow leukemic cells of the seven AML patients investigated by flow cytometry. C, correlation between the signal/noise (S/N) ratio of MFI of CD31 (blue) or CD38 (red) and percentages of in vitro migration of marrow leukemic cells from the seven AML patients.
CD38 and CD31 could interact with other structures found in the marrow microenvironment and possibly involved in the cell migration process. CD31 is able to recognize αvβ3 integrin on the endothelial cell surface, but these molecules interact on the same cell surface in a cis manner (38) and do not act as counter-receptors in mediating leukocyte transmigration (39). Moreover, it has been reported that CD38 and cADPR are required for chemotaxis of human monocytes and dendritic cells in response to SDF-1, the ligand of CXCR4 (40, 41). Because the SDF-1/CXCR4 axis plays a key role in the control of leukemic cell dissemination (42–45), these elements suggest that a functional interaction between CD38 and CXCR4 might contribute to leukemic cell anchorage in the marrow microenvironment.

In conclusion, the results presented here lead us to propose a model in which CD31 and CD38 are closely associated on AML cells and where excess of CD31 promotes egress of the cells from the marrow compartment, whereas an excess of CD38 enhances their anchorage to the marrow microenvironment (Fig. 5D). Most investigators are aware that cell-microenvironment interactions within various niches (stromal/osteoblastic or sinusoidal endothelial cell niches) have a role in leukemia stem cell survival and drug resistance (1, 46, 47). In this sense, recent therapeutic strategies (CXCR4 antagonists) propose to release AML cells from their adhesive sites to render them more chemosensitive (48). To this end, our results predict the potential benefits of new therapeutic approaches targeted at CD38 and CD31 to prevent the acquisition of a chemoresistant phenotype.

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Figure 5. Relative expression of CD31 and CD38 antigens on marrow leukemic cells of 78 AML patients and peripheral WBC count. A and B, comparison between the CD31/CD38 ratio (S/N of MFI) and peripheral WBC count without discrimination of FAB subtypes (A) and in each FAB subtype (B). The results are represented using box plots in which the bottom of each box is the 25th percentile and the top is the 75th percentile. Medians are drawn inside the boxes. The lower and upper adjacents show the 10th and 90th percentiles, respectively. ○, patients below the 10th and above the 90th percentiles. C, correlation between CD31 and CD38 expression on marrow leukemic cells of AML patients. Data are expressed as S/N of MFIs obtained by flow cytometry. D, schematic diagrams of a proposed model to explain the concomitant roles of CD31 and CD38 in the marrow retention of AML leukemic cells. CD31 and CD38 are closely associated on the cell surface. An excess of CD31 promotes their egress to the circulation through homophilic CD31 interaction with marrow endothelial cells. Conversely, an excess of CD38 promotes cell attachment to hyaluronate, whereas the interaction between CD38 and CD31 on marrow endothelial cells is too weak to counteract matrix anchorage, resulting in weaker egress of leukemic cells to the peripheral blood.
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