Expression of the 67-kDa Laminin Receptor in Acute Myeloid Leukemia Cells Mediates Adhesion to Laminin and Is Frequently Associated with Monocytic Differentiation[1]

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ABSTRACT

Lodgement, proliferation, and migration of leukemic cells within bone marrow (BM) microenvironment involves adhesion of these cells to the BM extracellular matrix molecules fibronectin and laminin. The 67-kDa laminin receptor (67LR) is a nonintegrin protein with high affinity for laminin, which plays a critical role in basement membrane invasion and metastasis of cancer cells. By Western blotting, we documented that 67LR was strongly expressed in myelomonocytic THP1 and histiocytic U937 cells and was weakly expressed in promyelocytic HL-60 cells. In HL-60 cells, 67LR expression almost disappeared after retinoic-induced granulocytic differentiation, whereas it strongly increased after phorbol ester-induced monocytic differentiation. We did not detect 67LR expression in normal BM hematopoietic cells, in precursor-B acute lymphoblastic leukemia, in chronic lymphocytic leukemia, or in chronic myeloid leukemia in chronic phase. By contrast, we detected enhanced 67LR expression in 40% of 53 *de novo* acute myeloid leukemias (AMLs), which frequently exhibited monocytic or myelomonocytic morphology and expressed CD14 and CD11a ($P < 0.05$). Using a colorimetric assay, we found that the expression pattern of this receptor corresponded to a higher adhesion to laminin; the adhesion was specific because *in vitro* addition to laminin-coated wells of recombinant 37-kDa laminin receptor precursor (37LRP), which is the cytoplasmic precursor containing both laminin-binding domains of cell surface 67LR, significantly reduced laminin binding of AML cells. The expression of 67LR on AML cell surface did not correlate with other differentiation and integrin antigens such as CD7, CD13, CD33, CD34, CD11b, CD11c, CD49d, CD49e, CD45RA, and CD45R0. In contrast with 67LR behavior in solid tumors, no statistically significant difference was found between 67LR expression and any hematological characteristic of the disease at diagnosis, nor between 67LR expression and outcome of the disease as measured by complete remission rate, disease-free survival, or overall survival. In conclusion, our results indicate that 67LR expression mediates specific adhesion to laminin and that the detection of this molecule may be a valuable addition to other lineage-associated antigens in identifying monocytic-oriented AML.

INTRODUCTION

Different molecules such as integrins, members of the immunoglobulin superfamily, and selectins are involved in maintaining early hematopoietic progenitors in close proximity to growth factor production sites and mediate direct transmembrane signaling, thus, promoting survival, proliferation, and maturation required for normal hematopoiesis (1). Disregulated expression of these molecules has been implicated in the acquisition of the malignant phenotype by hematopoietic cells (2). The BM[3] extracellular matrix proteins fibronectin and laminin have been found to preferentially mediate adhesion of AML cells to stroma, which may lead to their survival in the marrow during periods of apparent CR and to subsequent proliferation under the influence of stromal-derived myeloid growth factors (3–5). The major glycoprotein of the basement membrane is laminin, which is involved in the attachment, spreading, migration, and differentiation of normal and neoplastic cells (6). Interactions between cancer cells and laminin is a prerequisite for basement membrane invasion and metastasis (7, 8); in addition, this glycoprotein regulates interactions between malignant cells and the immune system (9). Several cell surface laminin-binding proteins have been described, including integrin and nonintegrin LRs (10). The 67LR is a nonintegrin

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[3] The abbreviations used are: BM, bone marrow; AML, acute myeloid leukemia; LR, laminin receptor; LRP, LR precursor; ATRA, all-*trans*-retinoic acid; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; CLL, chronic lymphocytic leukemia; CML, chronic myeloid leukemia; CR, complete remission; MNC, mononuclear cell; OS, overall survival; DFS, disease-free survival; MGG, May-Grunwald-Giemsa; NSE, non-specific esterase; ALL, acute lymphoblastic leukemia; FAB, French-American-British; 67LR, 67-kDa LR; 37LRP, 37-kDa LRP; MoAb, monoclonal antibody; r37LRP, recombinant 37LRP.

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protein with a high affinity for laminin (8). Its expression on the cell surface derives from posttranslational modifications of a 37LRP (11, 12). Two laminin-1-binding domains have been localized on 37LRP (13, 14): peptide G (residues 161–180) and peptide 11 (residues 205–229). Increased expression of 67LR has been reported in a variety of human carcinomas (colon, breast, stomach, liver, lung, and ovary) and directly correlates with a higher proliferation rate of malignant cells and tendency to metastasize (15). In addition, 67LR is detectable on the surface of CD30+ cells of anaplastic large cell lymphomas and in small subsets of high-grade B-cell non-Hodgkin's or Hodgkin's lymphomas (16); high levels of 67LR have been reported in normal human peripheral T cells and in T-lymphoma cell lines (17). Little information is available on the expression of 67LR in human AML. To gain insight into the role of 67LR in AML, we investigated the expression of 67LR in fresh leukemia BM AML cells and during granulocytic and monocytic differentiation of the promyelocytic cell line HL-60. We also investigated possible correlations with adhesive properties, clinical and hematological characteristics at presentation, and disease outcome.

MATERIALS AND METHODS

Cell Lines and Culture Conditions. HL-60 promyelocytic cells, THP-1 myelomonocytic cells, and U937 histiocytic cells were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in RPMI 1640 (Life Technologies, Inc., Grand Island, NY) supplemented with 10% heat-inactivated FCS (Life Technologies, Inc.), 2 mM L-glutamin, 100 units/ml penicillin, and 0.1 mg/ml streptomycin at 37°C in 5% CO_2.

Differentiation of HL-60 Cells. To induce differentiation, cells were seeded into 35-mm Petri dishes (Becton Dickinson, Lincoln Park, NJ); granulocytic or macrophage differentiation was induced with 1 μM ATRA (Hoffman-La Roche, Switzerland) and with 20 nM TPA (Sigma Chemical Co., St. Louis, MO), respectively. Cell differentiation profiles were analyzed after 4 days by morphology, NSE activity, and surface antigen expression. Cell morphology was assessed on cytocentrifuge preparations stained with MGG. NSE activity was demonstrated according to the method of Koski *et al.* (18), with a slight modification: the substrate was α naphthyl acetate (Sigma Chemical Co.) instead of naphthyl butyrate. At least 300 cells were evaluated for morphology and cytochemical analysis. Monocytic differentiation was further detected by flow cytometry using the monocyte-associated antigens OKM1 (CD11b; Ortho Diagnostic Systems, Inc., Raritan, NJ) and HLA-DR (DAKO, Golstrup, Denmark).

Patient Specimen Collection. BM samples were obtained, after informed consent, during diagnostic procedures from 5 normal BM donors and from 75 patients with acute or chronic leukemia (57 AMLs, 5 ALLs, 5 CLLs, and 8 CMLs). Diagnosis was based on MGG-stained BM smears, cytochemistry, and immunophenotyping (19). No patient had a history of prior therapy with anticancer drugs or a preceding diagnosis of myelodysplastic syndrome. AML cases were classified as follows: M0, 2 cases; M1, 15 cases; M2, 15 cases; M3, 4 cases; M4, 15 cases; and M5, 7 cases. ALL and CLL cases were all B-lineage committed. Seven CML patients were in chronic phase, and one patient was studied during a blastic crisis. AML patients [28 males and 29 females; median age 53 years (range 13–71)] were treated by intensive induction regimens differentiated according to age: twelve patients >60 years of age received a combination of idarubicin, etoposide, and cytarabine (20); twelve patients <60 years of age received daunorubicin combined with cytarabine (21). The other 30 patients <60 years of age were treated with daunorubicin or idarubicin plus etoposide and cytarabine (22). Three patients with acute promyelocytic leukemia received an induction course with idarubicin for 6 days either alone or associated with cytarabine (23). CR was defined by BM blast with blasts <5%. All patients who achieved CR received a postinduction therapy according to the respective protocols (20–23).

Separation of Leukemic Blasts. BM-MNCs were isolated by Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) centrifugation. Samples with <80% blasts ($n = 7$) were depleted from contaminating T cells using antibodies and magnetic beads, as described previously (24), resulting in a final blast purity of >95% as determined by morphology on cytospin preparations. Briefly, BM cells were incubated on ice with CD3 MoAb (50 μl for 2×10^6 cells) for 30 min, washed with PBS, and then incubated with antimouse IgG-conjugated magnetic beads (Dynal, Oslo, Norway) on ice for 10 min before separation using a magnetic separator. The degree of residual contamination was also evaluated using flow cytometry after staining cell aliquots with CD3 MoAb; BM cell fractions usually contained <2% $CD3^+$ cells.

Flow Cytometry. Immunophenotyping classification was performed by a large panel of MoAbs: HLA class II/anti-HLA-DR, CD2/OKT11, CD3/OKT3, CD4/OKT4, CD5/Leu1, CD8/OKT8, CD7/Leu9, CD10/OKB CALLA, CD11a/LFA-1, CD11b/Mac-1, CD11c/p150,95, CD13/LeuM7, CD14/LeuM3, CD15/Lewis^x, CD19/Leu12, CD20, CD24, CD25, CD33/LeuM9, CD34/IOM34, CD45RO/UCHL1, CD45RA/2H4, CD49d/VLA-4 and CD49e/VLA-5. MoAbs were purchased from Ortho Diagnostic Systems, Inc., Becton Dickinson (Mountain View, CA), Immunotech (Marseille, France), Coulter (Miami, FL), and DAKO. Antimyeloperoxidase (MP07; DAKO) was used in selected cases. Methods for direct and indirect immunofluorescence were reported previously (25). Controls were performed with isotype-matched IgG1, IgG2, and IgM MoAbs. FAB classification was integrated by cytochemistry and by the presence of an immunophenotypic profile of blast cells consistent with FAB subgrouping, according to consensus international guidelines (26).

Production of Anti-67LR Antibodies. Briefly, cDNA coding for the 67LR−cytosolic precursor 37LRP was cloned into the pTrcHis B expression vector (Invitrogen, San Diego, CA) and expressed in TOP-10 bacteria (Invitrogen) as a fusion protein containing at its amino terminus a His-tag. The r37LRP, containing both laminin-binding domains of the mature cell surface 67LR (11–14), was purified by nickel affinity cromatography according to manufacturer's specifications (Invitrogen), dialyzed in 50 mM Tris (pH 7.5), 0.1% Triton X-100, and adjusted to a concentration of 1 mg/ml. In adhesion inhibition assays, r37LRP was further diluted 1:10 and used at a final concentration of 100 μg/ml. An antiserum was produced by

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rabbit immunization with SDS-PAGE electroeluted r37LRP, as described previously (27, 28). Anti-r37LRP antibody was affinity purified by r37LRP-conjugated Affigel 10 resin as previously described (27). The specificity of affinity-purified anti-r37LRP polyclonal antibody was evaluated by Western blot on purified r37LRP. On Western blots of cancer cell lysates, anti-r37LRP antibody reacted with both 37LRP and 67LR (27, 28).

Western Blot. BM-MNCs were washed in PBS and lysed in 50 mM Tris (pH 7.5) containing 0.5% Triton X-100, 0.01%SDS for 10 min at 4°C. After a brief centrifugation, the supernatant was collected and the protein content was evaluated by a colorimetric assay (micro-BCA; Pierce Chemical Co., Rockford, IL), according to manufacturer's specifications. Protein (50 μg) from each cell lysate, together with molecular weight markers (Amersham, Little Chalfont, United Kingdom), were fractionated by 10% SDS-PAGE. Equal protein loading was assessed by Comassie-blue staining of SDS-PAGE gels. Gels were electroblotted onto polyvinylidene difluoride membrane filters (Millipore, Bedford, MA). Filters were blocked in TBST [10 mmol/liter Tris-HCl (pH 8.0), 150 mmol/liter NaCl, and 0.5% Tween 20] containing 3% nonfat milk and 1% BSA (Sigma Chemical Co.) for 1 h at room temperature and then incubated overnight at 4°C in TBST-1% nonfat milk, containing 1 μg/ml anti-r37LRP antibody or 1 μg/ml nonimmune rabbit immunoglobulins, as negative control. The reaction was revealed by incubating filters with horseradish peroxidase-conjugated goat antirabbit antibodies (Bio-Rad, Richmond, CA) diluted 1:2000 in TBST-1% nonfat milk and developed by enhanced chemiluminescence (Amersham), according to the manufacturer's specifications. Densitometer analysis of films was performed using a scanning densitometer (GS 300; Hoefer Scientific Instruments, San Francisco, CA).

Adhesion Assay. Ninety-six well plates (Costar, Cambridge, MA) were coated with 10 $\mu g/cm^2$ laminin-1/EHS (Collaborative Research., Bedford, MA) dissolved in PBS at 4°C overnight in the dark. After removal of excess coating buffer, one wash with PBS, one blocking step with PBS/1% heat-denaturated BSA (Sigma Chemical Co.) for 1 h at room temperature, and a second wash with PBS, AML cells suspended in Iscove's Modified Dulbecco's Medium (Life Technologies, Inc.) with 0.9 mM $CaCl_2$ and 0.5 mM $MgCl_2$ were seeded at 5×10^4 cells/well onto prewarmed 96-well plates (29). In the adhesion inhibition assay, as affinity purified anti-r37LRP polyclonal antibodies poorly recognized native 37LRP and 67LR and were not able to block 67LR-mediated cell binding to laminin, r37LRP was used. Therefore, AML cells were first incubated with 10 μg of r37LRP and then were seeded at 5×10^4 cells/well onto 96-well plates. Plates were incubated for 1 h at 37°C, 5% CO_2. The supernatant was removed, wells were gently washed three times with PBS, and attached cells were fixed with 3% paraformaldehyde for 10 min, followed by 2% methanol for 10 min, and finally stained with 0.5% crystal violet (Sigma Chemical Co.) in 20% methanol. After a 10-min washing with tap water and staining elution with 0.1 M sodium citrate (pH 4.2) in 50% ethanol, the amount of crystal violet staining viable cells that remained adherent to the wells was measured *in situ* at 540 nm absorbance using an automatic ELISA plate reader. Control AML cells seeded in BSA-coated wells were used as blank. All experiments were performed in triplicate. In control experiments, the His-tagged carbohydrate recognition domain of the subunit 1 of the rat hepatic asialoglycoprotein receptor, rCRD-RHL1 (30), was used as control recombinant protein. rCRD-RHL1 was purified by nickel affinity cromatography, dialyzed in 50 mM Tris (pH 7.5), 0.1% Triton X-100, adjusted to a concentration of 1 mg/ml and used at a concentration of 100 μg/ml, as r37LRP, in laminin adhesion inhibition assays. In some AMLs, cell counting of laminin-adherent cells was also performed. Briefly, nonadherent cells, harvested by pipetting, and adherent cells, removed with 0.05% trypsin, were counted in a hemocytometer. The number of adherent cells was expressed as a percentage of the total cell number and represented an average from two different experiments performed in triplicate.

Statistical Analysis. Relationships of 67LR expression to marker reactivity were estimated by the Student's *t* test. For comparison of clinical characteristics between two groups, the χ^2 test was applied; a P of < 0.05 was considered significant. The Kaplan-Meier product-limit was used for estimation of OS and DFS. OS was calculated from the date of diagnosis to the date of death or last follow-up. DFS was measured from achievement of CR until relapse or last follow-up. For comparison of survival and remission duration of two groups, the log-rank test was applied.

RESULTS

Expression of 67LR in HL-60 Cell Line Cultured with Differentiating Agents. We analyzed the maturation profile of HL-60 cells by examining 300 cells on MGG-stained smears after 5 days of culture with and without the differentiating agents ATRA or TPA, scoring the proportion of immature blast cells, cells at intermediate granulocyte or monocyte stage of differentiation, and mature granulocytes or macrophages. In control cultures, about 99% of cells were myeloblasts and promyelocytes, whereas in the presence of ATRA or TPA, there was an increase of granulocytes or monocytes to 49 ± 8 and 72 ± 5, respectively. HL-60 cells untreated or treated with ATRA and TPA, were also assayed for the expression of monocytic differentiation markers (NSE, CD11b, and HLA-DR). In control HL-60 cells we did not detect NSE-positive cells, whereas TPA treatment resulted in the induction of 56 ± 5 NSE-positive cells. ATRA did not induce HL-60 cells to acquire NSE activity. After incubation of HL-60 cells with TPA, a significantly higher proportion of cells expressing the monocyte antigens CD11b and HLA-DR was observed: 64% and 89%, respectively. In contrast, HL-60 cells remained unchanged in their monocytic profile in medium alone or after treatment with ATRA. A Western blot performed on cell extracts derived from the same cells, using identical protein amounts for each sample, demonstrated that HL-60 cells cultured with medium alone expressed levels of 67LR protein 8-fold lower than those expressed by the monocytic line THP-1 (Fig. 1*A*). The 67LR protein levels were almost undetectable in ATRA-treated HL-60 cells, whereas a clear induction of 67LR was detected after treatment with TPA. Densitometry scanning indicated that the protein levels of 67LR in TPA-treated HL-60 cells almost paralleled those observed in THP-1 cell line, whereas 37LRP expression was not affected by any treatment. A Western blot

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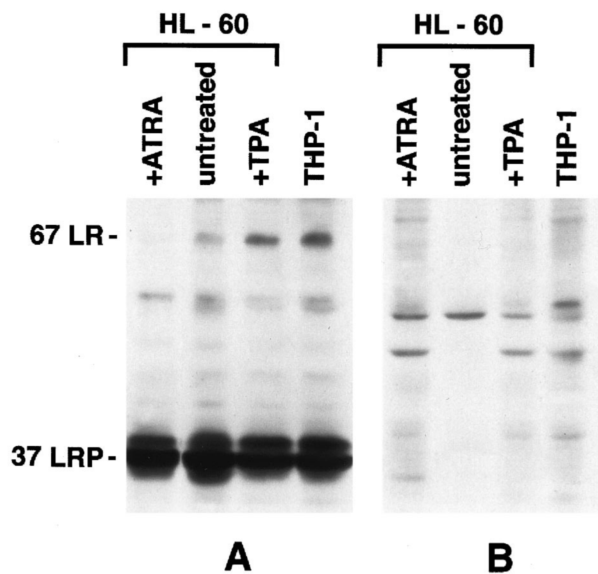

Fig. 1 Expression of 67LR as assessed by Western blotting in cell lines. Western blot with anti-r37LRP antibody (*A*) and nonimmune rabbit immunoglobulins (*B*). THP-1 cells were used as positive control. HL-60 cells were cultured for 4 days in the absence (*untreated*) or in the presence of 1 μM ATRA (+*ATRA*) or 20 nM TPA (+*TPA*). 67LR was preferentially expressed along the monocytic differentiation pathway of HL-60 cells.

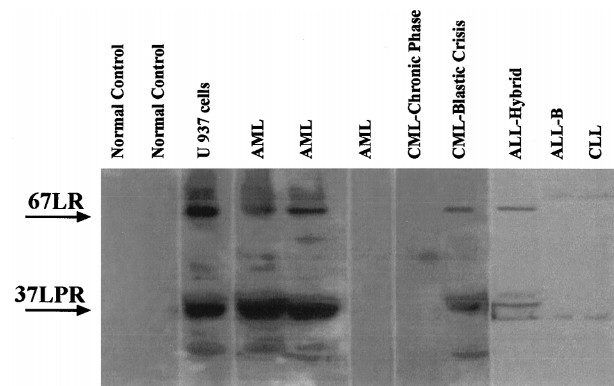

Fig. 2 Expression of 67LR in fresh BM cells in representative cases of normal donors or patients with leukemia of myeloid or lymphoid origin, as assessed by Western blotting. The U937 cell line was used as a positive control. ALL-B, B-lineage ALL. 67LR was differentially expressed in myeloid blast cells and was consistently associated with 37LRP expression.

Table 1 67LR[a] expression on BM cells of leukemic patients

	n	67LR+	67LR−
AML, total	57	24	33
M 0	2	2	0
M 1	15	7	8
M 2	15	1[b]	14
M 3	4	0	4
M 4	14	7	7
M 5	7	7[c]	0
ALL, B-lineage	4	0	4
ALL-hybrid	1	1	0
CLL	5	0	5
CML, cp	7	0	7
CML, myeloid bc	1	1	0

[a] 67LR detected by affinity purified anti-r37LRP antibody in a Western blot analysis.
[b] Statistical analysis (*t* test), $P = 0.01$.
[c] Statistical analysis (*t* test), $P = 0.02$.

performed on the same cell extracts with nonimmune rabbit immunoglobulins did not show reactivity with 37LRP or 67LR (Fig. 1*B*).

Expression of 67LR in *de Novo* AML and Correlation with the FAB Subtype. Expression of 67LR in normal and leukemic BM cells was analyzed by Western blotting, which was standardized using human HL-60 promyelocytic, THP-1 myelomonocytic, and U937 histiocytic cell lines expressing different amounts of 67LR (Figs. 1 and 2). As shown in Table 1 and Fig. 2, we did not detect 67LR in normal BM hematopoietic cells, in precursor-B ALL, in CLL, or in CML in chronic phase. By contrast, a variably enhanced expression of 67LR was found in AML: 24 (42%) patients were 67LR+ and 33 (58%) patients were 67LR−. Expression of 67LR was heterogeneously distributed among the various FAB subtypes (Table 1 and Fig. 3). A high frequency of 67LR expression (66%) was detected in monocytic-oriented leukemias (FAB M4, n = 7 of 14, 50%; FAB M5, $n = 7$ of 7, 100%; $P = 0.001$). Two cases of indifferentiated AML (FAB M0) showed strong expression of 67LR, and this marker was detected in 46% ($n = 7/15$) of AML without maturation (FAB M1). By contrast, only 7% ($n = 1$ of 15) of AML with maturation (FAB M2) showed 67LR reactivity, and leukemic samples from four cases of acute promyelocytic leukemia (FAB M3) were all negative.

Adhesion to Laminin of AML Cells Expressing 67LR. Because a significant number of AML cases showed enhanced expression of 67LR (67LR+AML), we investigated whether 67LR expression on fresh AML cells could mediate adhesion to immobilized laminin. Laminin binding was assessed by staining attached AML cells with crystal violet dye and measuring dye concentration at absorbance 540 nm. Absorbance values for AML cells adhering to BSA-coated wells in the absence of laminin averaged 0.008 ± 0.01 (SD). In the laminin-coated wells, absorbance values averaged 0.11 ± 0.03 and 0.05 ± 0.02 in 67LR+ AML (seven cases) and in 67LR– AML (four cases), respectively ($P = 0.017$; Fig. 4). Laminin-mediated adhesion in 67LR– AML was not statistically different from that of normal controls (absorbance, 0.03 ± 0.04; $P = 0.6$). AML cell laminin-binding was specifically mediated by 67LR– laminin interaction because *in vitro* treatment of AML cells with r37LRP, a cytoplasmic precursor containing both laminin-binding domains of mature 67LR, significantly reversed binding of 67LR+ AML cells (absorbance, 0.11 ± 0.03 *versus* 0.03 ± 0.03, $P = 0.002$ in 67LR+ AML; absorbance, 0.05 ± 0.02 *versus* 0.02 ± 0.02 in 67LR− AML, $P = 0.1$). In control experiments, laminin adhesion of AML cells was not affected by a control recombinant His-tagged protein, the rCRD-RHL1, used at the same concentration of r37LRP (data not shown). The percentage of laminin-adherent cells, was also performed by cell counting in

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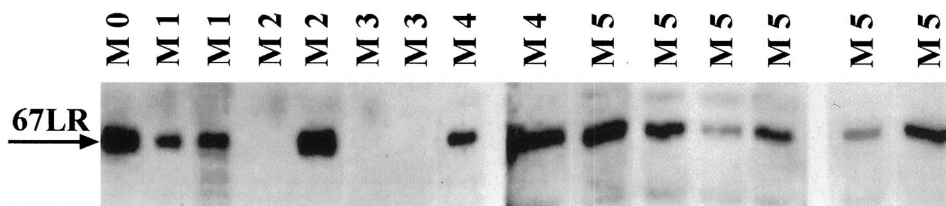

Fig. 3 Western blot analysis of 15 FAB-classified cases of AML. 67LR expression was more often observed in monocytic/monoblastic-oriented leukemias.

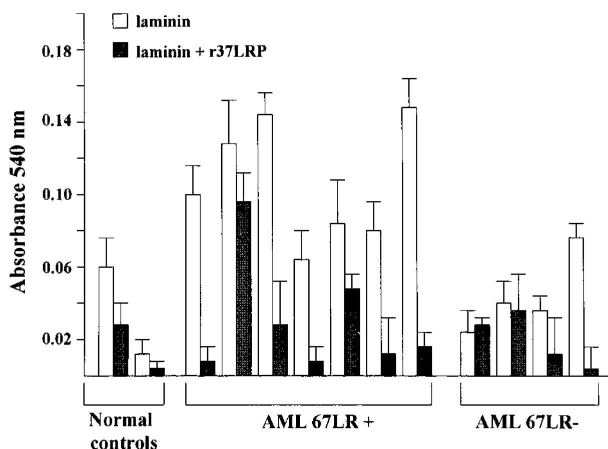

Fig. 4 Influence of 67LR expression on cell adhesion in 11 AML patients (67LR+, 7 patients; 67LR−, 4 patients) and 2 normal controls, as assessed by the crystal violet adhesion assay. AML cells were seeded on laminin-coated (10 μg/cm^2) surfaces at a density of 5×10^4 cells/well for 30 min at 37°C in the absence (□) or in the presence (■) of r37LRP, a cytoplasmic precursor-polypeptide containing both laminin-binding domains of cell surface 67LR. AML cells seeded in BSA-coated wells were used as a blank. Adherent stained cells were measured *in situ* and expressed as 540 nm absorbance. Histograms represent the means of triplicate wells ± SD. Student's *t* test was used to calculate *P*s. Reduction in adhesion to laminin induced by r37LRP was significant ($P < 0.05$). This experiment suggests that 67LR expression mediates specific adhesion of AML cells to laminin.

two 67LR+ AML and two 67LR– AML. The adherence of 67LR+AML cells to laminin-coated wells was 25.5% of plated cells (median, 12739 ± 420 cells/well), whereas the adherence of 67LR– AML cells was 4.2% of plated cells (median, 2099 ± 442 cells/well). When coincubation with r37LRP was performed, laminin-adhesion of 67LR+ AML cells was reduced to 10.4% of total cells plated (median, 5219 ± 530 cells/well), corresponding to a competition of 60% of total laminin binding. In 67LR– AML cells, coincubation with r37LRP reduced laminin adhesion to 3.4% of plated cells (median, 1699 ± 272 cells/well), corresponding to a competition of total laminin binding of 20%.

Correlations of 67LR Expression with Immunological Markers of AML. We considered AML leukemic populations as CD14+ and CD11a+ when staining exceeded that of controls by 20% (25). A correlation of borderline statistical significance emerged between 67LR+ and CD14+ AML ($P = 0.04$), as well as with CD11a+ AML ($P = 0.03$; Table 2). A high expression of HLA-DR+ on AML blasts (>80%) was significantly associated with the expression of 67LR ($P < 0.01$; Table 2). In no instance B-cell malignancies (ALL or CLL) expressed 67LR. 67LR was expressed in a single case of biphenotypic ALL (CD34+, CD13+, CD19+, CD24+) and in a case of myeloid blastic crisis of CML (Fig. 2). The expression of 67LR in AML was not correlated with differentiation antigens (CD13, CD7, CD33, and CD34), nor with integrin molecules (CD11b, CD11c, CD49d, and CD49e), or with CD45 phosphatase isoforms (CD45RA and CD45 R0; Table 2).

Relationship of 67LR Expression with Disease Characteristics at Diagnosis and with Outcome. The clinical features of patients with AML were analyzed in relation to 67LR expression. No statistically significant differences were found between 67LR expression and any hematological characteristics of the disease at diagnosis, including age, sex, hemoglobin, platelets, WBC, percentage of marrow and circulating blasts, and serum LDH. Finally, 67LR expression showed no prognostic significance for CR rate (85% *versus* 66%, $P = 0.24$), DFS ($P = 0.47$), and OS ($P = 0.92$; Fig. 5, *A* and *B*).

DISCUSSION

Differential expression of adhesion molecules on early hemopoietic stem cells and committed progenitors along the various lineages and stages of differentiation plays an important role in the regulation of normal hematopoiesis (1). Qualitative or quantitative changes in the adhesive properties of hematopoietic cells having undergone leukemic transformation may confer a proliferative advantage (2) and may affect retainment or release of blast cells from the BM microenvironment, thus, facilitating local proliferation or migration across the vessel wall into the circulation and subsequent development of extramedullary disease (31–32). A frequent feature of a number of cancer cells is the acquisition of aberrant membrane expression of 67LR, which seems to be associated with the ability of cells to proliferate and metastasize (7, 8). To date, few studies have dealt with the distribution and the biological significance of 67LR in human leukemias.

Using anti-67LR antibodies in Western blotting analysis, we found that untreated HL-60 cells and fresh BM promyelocytic blasts virtually lack 67LR expression; however, in HLA-60 cells, TPA-induced monocytic differentiation, but not ATRA-induced granulocytic differentiation, is associated with up-regulation of 67LR. These findings suggest a preferential expression of this receptor along the monocytic differentiation program of myeloid cells. Indeed, it has been reported that myeloid differentiation as seen in AML-M3 fresh blast cells is associated with low expression of other adhesion receptors such as CD11a, CD11b, CD11c, CD15, and CD54 (33). Moreover, it has been found that ATRA may exert a differential regulation of the adhesion molecule system in AML cells: it enhances *in vitro*

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Table 2 Correlation between 67LR expression and other differentiation antigens in AML[a] cells

	Mean percentage of stained blast cells ± SD			Cases considered positive for the marker[b]/ cases examined		
	67LR+	67LR−	P[c]	67LR+	67LR−	P[d]
DR	86.9 ± 16	63.2 ± 31	**0.002**	22/22	28/31	**0.03**
CD7	25.5 ± 30	20.5 ± 24	0.5	7/19	9/31	0.56
CD11a	89.1 ± 14	68.9 ± 32.5	**0.025**	16/16	20/23	0.13
CD11b	30.9 ± 31	22.7 ± 16	0.25	8/18	16/27	0.20
CD11c	52.4 ± 34	41.7 ± 28	0.33	11/15	13/18	0.94
CD13	40.1 ± 28	52.9 ± 30	0.15	17/21	25/33	0.63
CD14	15.1 ± 20	10.1 ± 13	0.29	7/22	3/31	**0.04**
CD33	77.0 ± 26	65.9 ± 28	0.15	19/21	31/31	0.87
CD34	30.3 ± 33	30.4 ± 30	0.9	8/21	16/31	0.45
CD45RO	30.2 ± 27	28.9 ± 26	0.80	10/17	9/29	0.25
CD45RA	73.5 ± 36	71.9 ± 28	0.87	14/17	21/26	0.48
CD49d	92.2 ± 9	88.3 ± 15	0.40	16/16	16/19	1.00
CD49e	81.3 ± 20	87.1 ± 87	0.13	14/15	17/17	0.27

[a] AML cases were defined 67LR+ if this protein was detected by Western blotting.
[b] More than 20% stained cells.
[c] Student's *t* test; significant data is in bold.
[d] χ^2 test; significant data is in bold.

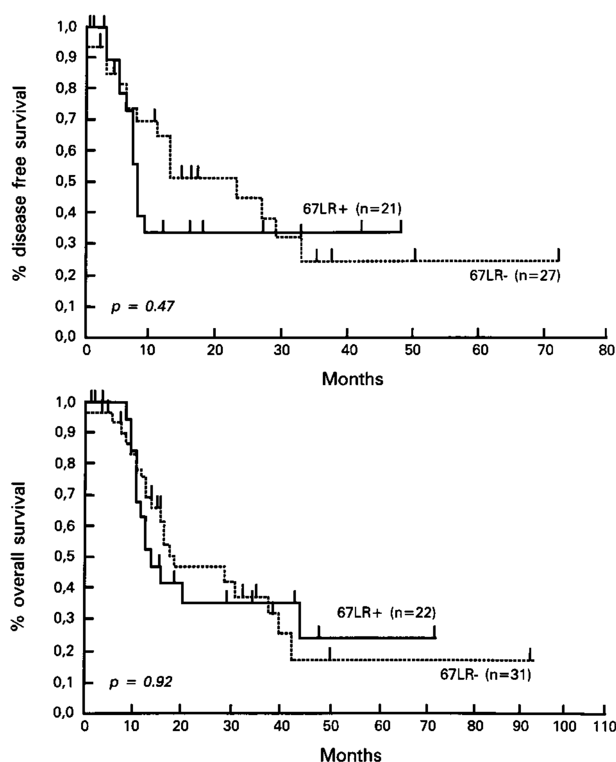

Fig. 5 DFS (*A*) and OS (*B*) in AML patients stratified for 67LR expression. The difference was not statistically significant in our small series (log-rank test).

expression of CD11b, CD11c, CD15 and CD54 in AML M3 and, to a lesser extent, in other AML subtypes, whereas it causes a down-regulation of CD49e and CD11a expression in AML M3 as well as in other AML subtypes (33). By contrast, TPA-induced monocytic differentiation of HL-60 cells results in an increase of CD11a expression and acquisition of CD11a-mediated homotypic adherence (34). In agreement with these results, by Western blotting analysis we found that fresh cells from AML patients display frequently enhanced expression of 67LR, the highest frequency being detected in monocytic-oriented AML (FAB M4 and M5). *Vice versa*, AML M2 was rarely associated with the expression of 67LR ($P < 0.05$). Interestingly, in a patient with AML M0 whose cells were 67LR+ at diagnosis, leukemic cells at relapse had typical M4 features. Cells from 67LR+ AML patients often showed strong positivity for the monocytic-associated antigens CD14 and CD11a. Changes in adhesion receptor expression have long been suspected to play a role in regulating leukemic cell spreading (2). In particular, β-1 and β-2 integrin cell-surface receptors and their respective ligands have been reported to mediate the egress of leukemic cells from marrow and to drive certain leukemic phenotypes to selectively infiltrate extramedullary sites such as skin and the central nervous system (31, 32). CD14 and CD11a antigens are involved in normal leukocyte migration, and they are frequently expressed in extramedullary infiltration of AML (34–36). A correlation between 67LR expression and monocytoid differentiation of leukemic cells further suggests a critical role for this molecule in the dissemination of the disease. However, although the expression of integrins is frequently higher in AML with monocytic differentiation (35), there was no correlation of 67LR expression with other integrin molecules, including CD11b, CD11c, CD49d, CD49e CD45RA, and CD45 R0. Although strong expression of 67LR was observed in the only two cases of AML M0 studied and was associated with a marked expression of HLA-DR and CD11a, it did not identify a stem cell-like phenotype, as indicated by the absence of correlation with early antigens such as CD7, CD34, CD33 (Table 2), CD10, and CD19 (data not shown), further suggesting a lineage-committed progenitor cell origin for AML-expressing 67LR (36–38).

67LR expressed by AML cells was functionally active; indeed, we found that a strong expression of 67LR was associated with increased adhesiveness to laminin. However, since *in*

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vitro addition of recombinant 37LRP, which is the cytoplasmic precursor of 67LR containing both laminin-binding domains of the cell surface receptor, only partially reversed laminin adhesion, it seems that adhesion of AML cells to laminin does not depend exclusively on 67LR expression. It has recently been shown that adhesion to laminin of very early hematopoietic progenitors also relies on the integrin laminin receptor CD49f (39).

AMLs expressing CD49d and CD49e were found to identify leukemia subsets with peculiar biological and clinical features, such as higher values of marrow and circulating blasts, as well as total WBC and enhanced proliferative growth *in vitro* (2). In our series of AMLs, no stastistically significant correlation was found between 67LR expression and any clinical feature of AML at presentation. Whereas in solid tumors mounting evidence indicates that increased expression of 67LR is an unfavorable prognostic factor (15, 16), our data demonstrate that 67LR expression in leukemic cells is not of predictive value in relation to disease outcome.

In conclusion, our study shows that the expression of 67LR, as detected by anti-r37LRP antibody on immunoblots of leukemic cell lines and fresh acute or chronic leukemic cells, is enhanced in some subsets of AML cells, allowing them to adhere to laminin and may be a useful additional lineage-associated antigen to identify monocytic oriented AML. Additional studies will clarify whether the expression of 67LR requires the association with other integrin or nonintegrin LRs to identify clinical risk groups in AML, as documented in nonhematopoietic tumors (40).

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