Constitutive integrin activation on tumor cells contributes to progression of leptomeningeal metastases[1]

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Leptomeningeal metastases are a serious neurological complication in cancer patients and associated with a dismal prognosis. Tumor cells that enter the subarachnoid space adhere to the leptomeninges and form tumor deposits. It is largely unknown which adhesion molecules mediate tumor cell adhesion to leptomeninges. We studied the role of integrin expression and activation in the progression of leptomeningeal metastases. For this study, we used a mouse acute lymphocytic leukemic cell line that was grown in suspension (L1210-S cell line) to develop an adherent L1210 cell line (L1210-A) by selectively culturing the few adherent cells in the cell culture. β_1, β_2, and β_3 integrins were in a constitutively high active state on L1210-A cells and in a low, but inducible, active state on L1210-S cells. Expression levels of these integrins were comparable in the two cell lines. Static adhesion levels of L1210-A cells on a leptomeningeal cell layer were significantly higher than those of L1210-S cells. All mice that were injected intrathecally with L1210-A cells died rapidly of leptomeningeal leukemia. In contrast, 45% long-term survival was seen after intrathecal injection of mice with L1210-S cells. Our data indicate that constitutive integrin activation on leukemic cells promotes progression of leptomeningeal leukemia by increased tumor cell adhesion to the leptomeninges. We argue that an aberrantly regulated inside-out signaling pathway underlies constitutive integrin activation on the adherent leukemic cell population. *Neuro-Oncology 8, 127–136, 2006 (Posted to Neuro-Oncology [serial online], Doc. 05-079, March 13, 2006. URL www.dukeupress.edu/neuro-oncology; DOI: 10.1215/15228517-2005-013)*

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[4]Abbreviations used are as follows: BSA, bovine serum albumin; CSF, cerebrospinal fluid; DMEM, Dulbecco's modified Eagle's medium; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis tetraacetic acid; FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule-1; IgG, immunoglobulin G; LM, leptomeningeal metastases; MoAb, monoclonal antibody; PBS, phosphate-buffered saline; PMA, phorbol 12-myristate 13-acetate; PSG, penicillin–streptomycin–L-glutamine; VCAM-1, vascular cell adhesion molecule 1.

Cancer cells can invade the subarachnoid space filled with cerebrospinal fluid (CSF)[4] and seed to the leptomeninges, a complication known as leptomeningeal metastases (LM). Any cancer can metastasize to the leptomeninges, but the most common cancer cell types are leukemia, lymphoma, breast cancer, small cell lung cancer, and melanoma (Bleyer and Byrne, 1988; DeAngelis, 1998). Nowadays, LM occurs in up to 8% of patients with solid tumors, and its incidence is increasing: 5% to 15% of patients with acute lymphocytic leukemia and 5% of patients with non-Hodgkin's lymphoma develop LM (Bleyer and Byrne, 1988; Colocci et al., 2004). The overall prognosis for patients with LM derived from solid tumors is poor. Untreated patients die within six to eight weeks of their diagnosis (Olson et al., 1974; Rosen et al., 1982), whereas aggressive chemotherapy and radiotherapy increase median survival to four to six months (Grant et al., 1994). The median duration of meningeal remission in patients with leptomeningeal leukemia or lymphoma is approximately two years, and long-term remission can be achieved in 35% of the patients (Balis et al., 1985; Frick et al., 1984). Pathophysiologically, tumor cells can gain access to the subarachnoid space in several ways: hematogenous dissemination, direct extension from bony or parenchymal metastases, perineural migration, or iatrogenic seeding of the meninges during surgical extirpation of brain metastases (Kokkoris, 1983; van der Ree et al., 1999). Once malignant cells enter the subarachnoid space, the CSF flow deposits them to distant sites within the neuraxis. The most common sites of tumor deposition are the basal cisterns and the cauda equina, possibly because gravity and sluggish CSF flow promote adhesion of tumor cells to these sites. Both autopsy of LM patients and animal models of LM show that tumor cells adhere to the leptomeninges and form tumor cell layers or nodules (Reijneveld et al., 1999). However, the role of tumor cell adhesion to the leptomeninges in LM and its mediating adhesion molecules are largely unknown.

Integrins have been identified as principal mediators of tumor cell intravasation, arrest in the blood vessel, extravasation, and infiltration in the target tissue (Ruoslahti, 1999). Integrins comprise a family of at least 24 transmembrane adhesion receptors composed of noncovalently linked α and β subunits, which interact with cellular adhesion molecules (e.g., intercellular adhesion molecule-1 [ICAM-1] and vascular cell adhesion molecule-1 [VCAM-1]) or extracellular matrix proteins like collagen, fibronectin, and vitronectin (Hynes, 1992). Integrins are known to exist in distinct activation states, being regulated by *inside-out* signaling pathways: Extracellular stimuli (e.g., chemokines) induce intracellular signal transduction pathways that subsequently activate integrins (Hynes, 1992; Schwartz et al., 1995). An increase in activation state is determined by two processes: a change in conformation of the integrin (affinity) and/or clustering of integrins on the cell membrane (avidity). Both integrin expression and activation on tumor cells have been linked to tumor progression (Chan et al., 1991; Felding-Habermann et al., 2001; Gosslar et al., 1996). In LM, in vitro studies pointed out a role for integrins in tumor cell adhesion to the leptomeninges. Giese et al. (1998) showed that static adhesion of glioma cells to human arachnoidea could be blocked by antibodies against α_2, α_3, and β_1 integrin subunits. We demonstrated that the interaction of $\alpha_4\beta_1$ integrin on tumor cells and VCAM-1 on leptomeningeal cells mediates initial melanoma cell tethering to the leptomeninges under flow conditions (Brandsma et al., 2002).

To study the role of integrin expression and activation on tumor cells in LM in vivo, we used a mouse acute lymphocytic leukemic suspension cell line (L1210) and generated a derivative, adherent leukemic cell line. Using this model, we show that constitutive integrin activation on leukemic cells contributes to increased in vitro leukemic cell adhesion to the leptomeninges and rapid progression of leptomeningeal leukemia in vivo. Our findings point to an abberantly regulated integrin inside-out signaling pathway in tumor cells as a mechanism of LM progression.

Materials and Methods

Antibodies and Peptides

Purified rat monoclonal IgGs against mouse L-selectin (CD62L, clone MEL-14), mouse VCAM-1 (CD106, clone 429 [MVCAM.A]), mouse integrin β_1 chain (CD29, clone 9EG7; fluorescence-activated cell sorting analysis), mouse integrin β_1 chain (CD29, clone Ha2/5; static adhesion assays), mouse integrin β_2 chain (CD18, clone GAME-46), mouse CD44 (clone KM114), and mouse integrin α_v chain (CD51, clone RMV7) were all purchased from Pharmingen (San Diego, Calif.). Purified rat monoclonal IgG against mouse α_{IIb} chain (CD41, clone MWReg30) was obtained from Santa Cruz Biotechnology (Santa Cruz, Calif.). Purified hamster monoclonal IgGs against mouse ICAM-1 (CD54, clone 3E2) and mouse integrin β_3 chain (CD61, clone 2C9.G2) and purified fluorescein isothiocyanate (FITC)–conjugated mouse anti-rat IgG_{2a} (clone G28-5) and anti-hamster and anti-rat $IgG_{1/2b}$ (clone G70-204 and G94-56) were also obtained from Pharmingen. R-phycoerythrin-conjugated goat F(ab')2 antihamster IgG (H + L) mouse/rat adsorbed second-step reagent was obtained from Southern Biotechnology Associates, Inc. (Birmingham, Ala.). Antibody concentrations were used as recommended by the manufacturer. Vitronectin was purified according to the method described by Yatohgo et al. (1988). Fibronectin was obtained from Harbor Bio-Products (Norwood, Mass.). Collagen type I was obtained from Sigma (St. Louis, Mo.). Recombinant mouse ICAM-1 Fc chimera was purchased from R&D Systems (Minneapolis, Minn.).

Reagents

Tissue culture supplies (culture media, antibiotics, and trypsin) were obtained from Gibco Biocult (Grand Island, N.Y.). Ethylenediaminetetraacetic acid (EDTA) was purchased from Riedel de Haen (Seelze, Germany).

Ethylene glycol-bis tetraacetic acid (EGTA) was and phorbol 12-myristate 13-acetate (PMA) were obtained from Sigma. Magnesium (II) chloride and manganese (II) chloride hexahydrate were obtained from Merck Biosciences (Bad Soden, Germany).

Mouse L1210 Leukemia Cells

The L1210 mouse lymphocytic leukemia cell line was obtained from the Netherlands Cancer Institute (Amsterdam). As the majority of cells are grown in suspension, this leukemic cell line is called L1210-S (suspension) cell line. We developed an adherent leukemic cell line—named the L1210-A (adherent) cell line (see Results)—by selectively culturing the few adherent cells from the L1210-S line. Both the L1210-S and the L1210-A cell lines were cultured in noncoated flasks in RPMI, supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin–L-glutamine (PSG), and 60 μM β-mercaptoethanol. Cells were incubated in 5% CO_2–95% air at 37°C. L1210-A cells were treated with 10 mM EDTA (pH = 7.5) for 5 min, centrifuged at 1500 rpm for 5 min, and resuspended in the culture medium for cell passaging. The L1210-S cell line was maintained as a suspension culture.

Mouse Leptomeningeal Cells

Primary cultures of mouse leptomeningeal cells were obtained as described previously (Brandsma et al., 2002). Briefly, leptomeninges were dissected from the cortical surface of two-day-old neonatal DBA/2 cortex and treated with 0.25% trypsin for 30 min at 37°C. After trypsin was neutralized, cells were centrifuged (1250 rpm, 5 min, room temperature), resuspended, and plated on poly-L-lysine-coated plates. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing sodium pyruvate and nonessential amino acids (Gibco), supplemented with 10% fetal bovine serum, 1% PSG, and 0.1% amphotericin. Cells were incubated in 5% CO_2–95% air at 37°C and passaged two or three times before use.

Cerebrospinal Fluid

We obtained fresh CSF samples from a single patient with a normal pressure hydrocephalus who had CSF drained via an external lumbar drain (Department of Neurosurgery, University Medical Center Utrecht). Cell count, protein, and glucose levels were within normal limits in these CSF samples.

Immunofluorescence

Immunofluorescence flow cytometry was used to measure expression levels of surface adhesion molecules. L1210 cells were treated with 10 mM EDTA (pH 7.5; 5 min), centrifuged, and washed twice in phosphate-buffered saline (PBS) at 4°C. Cells were resuspended in PBS/1% bovine serum albumin (BSA) (4°C) and distributed in a concentration of 1–2 × 10^5 cells/sample in a 96-well plate. They were centrifuged (1250 rpm, 3 min, 4°C) and incubated in 35 μl of appropriately diluted antibody in PBS-1% BSA (60 min, 4°C). Subsequently, cells were washed three times in PBS-1% BSA and incubated for another 30 min in 35 μl of the appropriately diluted, FITC-labeled, second-step antibody (4°C). After washing twice with PBS-1% BSA, stained cells were analyzed on a FACScalibur flow cytometer (Becton Dickinson, San Jose, Calif.). The mean fluorescence intensity was measured for each sample. Samples that were first incubated with isotype control antibodies and subsequently with FITC-labeled antibodies served as negative controls.

Static Adhesion Assays

For in vitro adhesion assays on matrix proteins, mouse ICAM-1, or leptomeningeal cells, L1210-A and L1210-S cells were washed with PBS, treated with 10 mM EDTA (pH 7.5, 5 min), and washed with PBS again. Cells were resuspended in DMEM without phenol red and sodium pyruvate (Gibco Biocult) and labeled fluorescently by incubation with 5 μM calcein (Molecular Probes, Leiden, The Netherlands) for 15 min at 37°C. Cells were centrifuged (1250 rpm, 5 min, room temperature) after labeling, washed two times with PBS, and resuspended in DMEM without phenol red and sodium pyruvate. Adhesion assays were performed in triplicate by administering 5 × 10^5 L1210 cells (>95% viability) per well in a 96-well plate coated with matrix proteins or recombinant mouse ICAM-1. Coating with matrix proteins (vitronectin, 10 μg/ml; collagen, 5 μg/ml) or recombinant mouse ICAM-1 (5 μg/ml) was performed overnight at 4°C. Subsequently, wells were incubated with 2.5% BSA-PBS for 1 h at room temperature. Noncoated wells that were incubated with 2.5% BSA-PBS served as controls. Static adhesion assays were performed for 30 min at 37°C, whereafter the fluorescence per well was measured with a Cytofluor II fluorometer (PerSeptive Biosystems, Framingham, Mass.). Wells were washed three times with washing buffer (20 mM HEPES, 140 mM NaCl, 2 mg/ml glucose, 1 mM EGTA, and 1 mM Mg^{2+}, pH 7.4), and the fluorescence per well was measured again. The ratio of the latter fluorescent signal and the initial fluorescent signal was calculated, representing the percentage of adhered cells per well. The effect of PMA stimulation, integrin-blocking monoclonal antibodies (MoAbs), or dRGD-w peptide on leukemic cell adhesion was determined by preincubation of leukemic cells with PMA (100 ng/ml), MoAbs (10 μg/ml), or dRGD-w peptide (100 μM) for 30 min at 37°C, before static adhesion assays were performed. The divalent cations Mg^{2+} (5 mM) or Mn^{2+} (0.5 mM) were added to the leukemic cell suspension, just prior to performing the adhesion assay.

For adhesion assays of leukemic cells on a leptomeningeal cell layer, adhered leukemic cells were fixed with 2% paraformaldehyde. Five FITC images (1.3 mm^2/image) of the central area of the leptomeningeal cell layer were obtained by using a fluorescence microscope (Leica DM IRHC; Leica Microsystems, Rijswijk, The Nether-

lands). The confluence of the leptomeningeal cell layer was confirmed by light microscopy. The number of adhered L1210 cells per FITC image was determined by quantitative analysis using Leica WIN software (Leica Microsystems), and high-magnification light microscopic pictures were made.

Proliferation Assays

For proliferation assays, L1210-S and L1210-A cells were seeded at a density of 2×10^4 cells per noncoated well in a 48-well plate. Cells were cultured in either normal culture medium (RPMI, 10% fetal calf serum, PSG) or fresh CSF supplemented with 60 μM β-mercaptoethanol for 72 h. At 24, 48, and 72 h, the number of leukemic cells was counted by using a cell counter (Coulter particle counter, Becton Dickinson). For counting, cells were incubated with 10 mM EDTA (5 min, 37°C) and resuspended in 10 ml Isoton (Baker-Mallincrodt, Deventer, The Netherlands). The absence of residual adherent cells on the culture plates was confirmed by light microscopy. The mean number of cells of six wells (for culture medium) or three wells (for CSF) was calculated for each culture condition. Cell viability was determined by trypan blue dye exclusion in a separate well.

Induction of Leptomeningeal Metastases

Eight-week-old male DBA/2 mice were purchased from the Central Laboratory Animal Institute (Utrecht, The Netherlands). Leptomeningeal leukemia was induced as described previously for melanoma LM (Reijneveld et al., 1999). Briefly, L1210 leukemia cells were washed twice with PBS and suspended in Hanks Balanced Salt Solution (Gibco). Cell viability was determined by trypan blue exclusion (>95% for all experiments). For survival studies of L1210-A and L1210-S leptomeningeal leukemia, 2×10^5 leukemic cells were injected in a volume of 10 μl into the cisterna magna. Neurological symptoms and survival were recorded every 24 h. Mice were defined as symptomatic when they showed more than 10% weight loss in combination with either (1) lethargy, (2) an arched back with a stretched neck, or (3) rotatory movements when lifted by the tail. For histologic studies, mice injected with L1210-A or L1210-S cells were sacrificed three or eight days after tumor inoculation. The brains, livers, spleens, and femurs were excised and directly fixed in 4% paraformaldehyde for at least 24 h. Formalin-fixed and paraffin-embedded 5-μm brain and femur sections were stained with hematoxylin-eosin for morphological studies.

Statistical Analysis

Statistical analysis of data obtained by immunofluorescence flow cytometry and static adhesion assays was performed using the Student's *t*-test for independent samples. For comparison of survival data, a log-rank analysis of Kaplan-Meier curves was performed. *P* values of <0.05 were considered significant.

Results

Selection of an Adherent Leukemic Cell Population from the L1210 Acute Lymphocytic Leukemic Suspension Cell Line

When culturing the L1210 acute lymphocytic leukemic suspension cell line (L1210-S cell line), a few cells (<1%) were noted to adhere strongly and spread on the bottom of noncoated culture flasks (Fig. 1A). These adherent cells were selectively cultured by aspirating the suspended L1210 cells every day for two weeks. Eventually, this resulted in the adherent L1210 cell line (L1210-A cell line), which formed extensive filopodia and lamellipodia on the bottom of the noncoated culture flask (Fig. 1B). When L1210-A cells were grown to confluence, suspended L1210-A cells appeared that adhered and spread again after they were placed in a new noncoated culture flask. In the L1210-S cell line, which was maintained as a suspension cell line, a few adherent cells (<1%) remained after each passage. Proliferation rates of the L1210-A and L1210-S cell lines in culture medium on noncoated wells were similar (Fig. 1C). Neither cell line proliferated in CSF on noncoated wells (Fig. 1D). Viability of the two leukemic cell lines was more than 95% during cell proliferation in both culture medium and CSF.

L1210 Cell Adhesion and Spreading on a Leptomeningeal Cell Layer

To investigate whether the two leukemic cell lines differed in capacity to bind to the leptomeninges, we performed static adhesion assays of L1210-S and L1210-A cells on leptomeningeal cell layers. A significantly higher number of L1210-A cells (327 ± 38 cells) adhered to a confluent leptomeningeal cell layer after 30 min of static adhesion as compared to the L1210-S cells (110 ± 6 cells) ($P < 0.01$, Fig. 2A). Similar results were found after 60 min of static adhesion: 357 ± 39 adhered L1210-A cells versus 155 ± 22 adhered L1210-S cells ($P < 0.01$ [Fig. 2A]). High magnification studies of the adhered leukemic cells after static adhesion during 60 min showed that all L1210-S cells were rounded up (Fig. 2B), whereas most of the adhered L1210-A cells were spread out, forming filopodia and lamellipodia on the leptomeningeal cell layer (Fig. 2C).

Significant Survival Difference Between L1210-A and L1210-S Leptomeningeal Leukemia

To determine whether a difference in the capacity of the two leukemic cell lines to adhere to the leptomeninges in vitro translated into a more aggressive behavior in vivo, we performed survival studies of mice with leptomeningeal leukemia. All mice that were injected intrathecally with L1210-A cells (2×10^5 cells) died of LM within 12 days (median survival time, 10 days; n = 22, obtained in four experiments with at least five mice per group). In contrast, intracisternal L1210-S cell injection resulted in

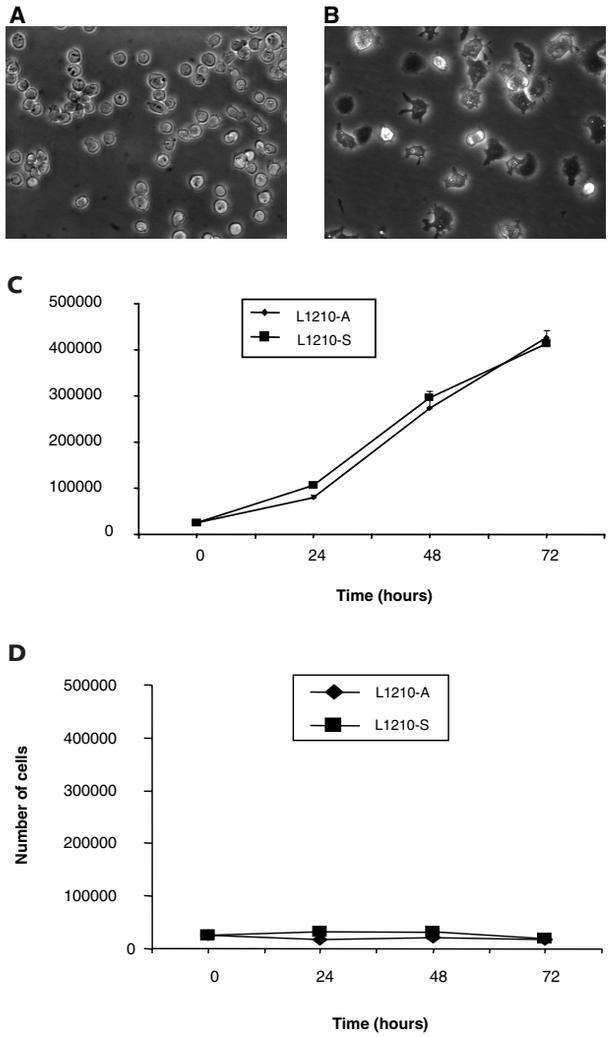

Fig. 1. Morphological and proliferation characteristics of L1210-S and L1210-A cells. A and B. Light-microscopic pictures of L1210-S and L1210-A cells. Panel A shows the L1210 acute lymphocytic leukemic suspension cell line (L1210-S cell line). A few cells (<1%) strongly adhere and spread on the bottom of noncoated culture flasks. The adherent leukemic cells were selectively cultured by aspirating the suspended L1210 cells every day for two weeks, resulting in the adherent L1210 cell line (L1210-A), shown in panel B. These leukemic cells form extensive filopodia and lamellipodia on the bottom of the noncoated culture flask. C. Proliferation of L1210-S and L1210-A cells in culture medium. Leukemic cells were seeded at a density of 2×10^4 cells on noncoated wells of a 48-well plate in culture medium (RPMI, 10% fetal calf serum, 60 μM β-mercaptoethanol). At 24, 48, and 72 h, the number of leukemic cells was counted with a cell counter. The mean number of leukemic cells of six wells (± SEM) was plotted. One representative experiment out of six is shown. D. Proliferation of L1210-S and L1210-A cells in CSF. L1210-A and L1210-S cells were seeded at a density of 2×10^4 cells on noncoated wells of a 48-well plate in CSF supplemented with 60 μM β-mercaptoethanol. At 24, 48, and 72 h, the number of leukemic cells in three wells was counted with a cell counter. The mean number of leukemic cells (± SEM) is plotted. One representative experiment out of three is shown.

prolonged survival, with 45% of the mice as long-term survivors (median survival time, 16 days; n = 22; hazard ratio of L1210-A vs. L1210-S, 16.5; 95% confidence interval, 4.7–58; $P < 0.001$; Fig. 3). No leukemic infiltration of bone marrow was seen in any of the mice and spleen, and the weight of liver and spleen of mice with leptomeningeal leukemia was not increased as compared to that of normal mice (data not shown).

Adhesion Molecule Expression on L1210-A and L1210-S cells

To find the cellular mechanism or mechanisms that underlie the increased adhesive capacity of L1210-A cells as compared to that of L1210-S cells, we studied the expression levels of a number of adhesion molecules on the two leukemic cell lines by using immunofluorescence flow cytometry. Both cell types showed similar, low expression levels of β_2 integrin subunits (CD18) and ICAM-1 (CD54). Similar high expression levels of CD44 (hyaluronate receptor, phagocyte glycoprotein, or Pgp-1) were found on both leukemic cell lines. L-selectin (CD62L) was not expressed on either cell line. The β_1 integrin subunit (CD29) and β_3 integrin subunit (CD61) expression levels were low in both cell lines, but slightly

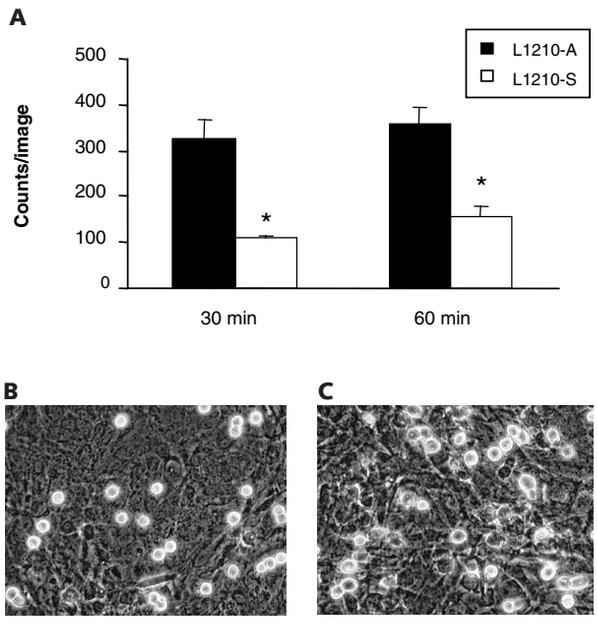

Fig. 2. L1210-A cells adhere and spread more efficiently to a leptomeningeal cell layer than do L1210-S cells. Adhesion assays of fluorescently labeled leukemic cells to determine adhesion to a leptomeningeal cell layer were performed at 37°C for 30 or 60 min, whereafter nonadherent cells were washed away and adhered cells were fixed with 2% paraformaldehyde. A. The mean number of leukemic cells (± SEM) that adhered to the leptomeningeal cell layer after 30 and 60 min of static adhesion is plotted. Two independent experiments were performed in quadruplicate. B and C. Light-microscopic pictures of L1210-S cells (B) and L1210-A cells (C) that adhered to a leptomeningeal cell layer after 60 min of static adhesion. 40×.

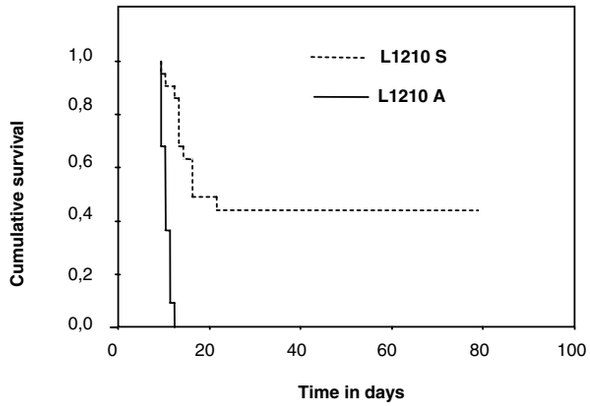

Fig. 3. Significant difference in survival of mice with L1210-A and mice with L1210-S leptomeningeal leukemia. Kaplan-Meier survival curves of mice intrathecally injected with L1210-A cells (solid line; n = 22) or L1210-S cells (dashed line; n = 22). Leukemic cells (2×10^5) were injected into the cisterna magna of the mice, and survival was recorded.

higher in L1210-A cells than in L1210-S cells (Table 1). Furthermore, low expression levels of the α_v integrin subunits were found in the L1210-A cells (mean fluorescence intensity = 7.8 ± 0.4) as compared to the L1210-S cells (mean fluorescence intensity = 4.9 ± 1.2; n = 2), whereas no expression of the α_{iib} integrin subunit was found on either leukemic cell type (data not shown).

Constitutively Active β_1, β_2, and β_3 Integrins on L1210-A Cells

Not the integrin expression level but in particular the integrin activation state determines cell adhesion (Diamond and Springer, 1993; Lum et al., 2002). Therefore, we studied the activation state of β_1, β_2, and β_3 integrins in the two leukemic cell lines. We performed static adhesion assays using wells coated with ligands for β_1 integrin (collagen), β_2 integrin (mouse ICAM-1), and β_3 integrin (vitronectin) in the presence of extracellular factors that activate integrins (Mg^{2+}, Mn^{2+}, or PMA) or agents blocking integrin-ligand interactions (integrin-blocking MoAbs, dRGD-w peptide, or EDTA). Levels of L1210-A cell binding to collagen (Fig. 4A), mouse ICAM-1 (Fig. 4B), and vitronectin (Fig. 4C) were significantly higher than L1210-S cell binding levels; 47% ± 2% of the L1210-A cells versus 18% ± 4% of the L1210-S cells adhered to collagen, 38% ± 1% of the L1210-A cells versus 23% ± 4% of the L1210-S cells adhered to mouse ICAM-1, and 47% ± 2% of the L1210-A cells versus 12% ± 1% of the L1210-S cells adhered to vitronectin after 30 min of static adhesion. Leukemic cell binding on all matrix proteins was blocked completely by 10 mM EDTA, which prevents integrin-ligand interaction by capturing divalent cations. β_1 integrin-blocking MoAb completely blocked leukemic cell binding to collagen. Leukemic cell binding to mouse ICAM-1 was fully prevented by β_2 integrin-blocking MoAb. Finally, both dRGD-w peptide and β_3 integrin-blocking MoAb completely prevented leukemic cell binding to vitronectin. No effect on leukemic cell binding to collagen, mouse ICAM-1, or vitronectin was seen for the isotype controls of the integrin-blocking MoAbs. Levels of L1210-S cell binding to collagen, mouse ICAM-1, and vitronectin were significantly increased by PMA and Mn^{2+}. Moreover, L1210-S cell binding to collagen was also significantly increased by Mg^{2+}. Both Mg^{2+} and Mn^{2+} are known to force the integrin in a high-affinity state (Hogg and Leitinger, 2001; Mould et al., 1995), whereas PMA can induce integrin clustering on the cell membrane (avidity change) in a protein kinase C–dependent way (Peter and O'Toole, 1995; van Kooyk and Figdor, 2000). However, the extracellular factors Mg^{2+}, Mn^{2+}, or PMA did not further enhance L1210-A cell binding to collagen, mouse ICAM-1, or vitronectin.

Table 1. Expression levels of adhesion molecules on L1210-A and L1210-S cells*

Adhesion Molecule	L1210-A cells	L1210-S cells
Rat IgG_{2a} /rat FITC	3.2 ± 0.3	4.1 ± 0.7
Rat $IgG_{1/2b}$/rat FITC	4.7 ± 0.6	6.1 ± 1.7
Hamster IgG_1/hamster FITC	7.3 ± 1.0	9.5 ± 1.1
β_1 integrin/rat FITC (rat IgG_{2a})	26.8 ± 1.6	20.3 ± 1.3
β_2 integrin/rat FITC (rat $IgG_{1/2b}$)	11.5 ± 1.1	13.6 ± 1.6
β_3 integrin/hamster FITC	17.0 ± 0.9	8.3 ± 0.4
ICAM-1/hamster FITC	26.0 ± 1.7	23.9 ± 3.6
L-selectin/rat FITC (rat IgG_{2a})	3.6 ± 0.2	4.6 ± 0.8
CD44/rat FITC (rat $IgG_{1/2b}$)	66.1 ± 10.8	42.5 ± 6.6

Abbreviations: FITC, fluorescein isothiocyanate; ICAM-1, intercellular adhesion molecule 1; IgG, immunoglobulin G.

*The expression levels of β_1, β_2, and β_3 integrin subunits, ICAM-1, L-selectin, and CD44 were determined by immunofluorescence flow cytometry. The mean of the mean fluorescence intensity ± SEM is indicated as measured in three or more experiments.

Effect of Mn^{2+} on L1210-S Cell Adhesion and β_3 Integrin on L1210-A Cell Adhesion

To determine whether changes in integrin activation state influence the capacity of leukemic cells to adhere to the leptomeninges, we performed in vitro static adhesion assays of L1210-S and L1210-A cells by using confluent primary mouse leptomeningeal cell layers in the presence of Mg^{2+}, Mn^{2+}, or PMA. L1210-S cell binding to mouse leptomeningeal cell layers was significantly increased by Mn^{2+}, but not by Mg^{2+} or PMA. However, Mg^{2+}, Mn^{2+}, or PMA did not further enhance L1210-A cell binding to mouse leptomeningeal cells. L1210-A cell binding to leptomeningeal cells was strongly inhibited by blocking β_1, β_2, and β_3 integrins with MoAbs and to a slightly lesser extent by dRGD-w peptide as compared to the isotype control MoAbs. No further significant decrease in L1210-S cell adhesion to the leptomeningeal cells was seen in the presence of integrin-blocking MoAbs or dRGD-w peptide (Fig. 5A). To further dissect the individual roles of the β_1, β_2, and β_3 integrins, adhesion assays on primary leptomeningeal cells were performed in the presence of MoAbs against the single β integrin subunits. Figure 5B shows that the adhesion

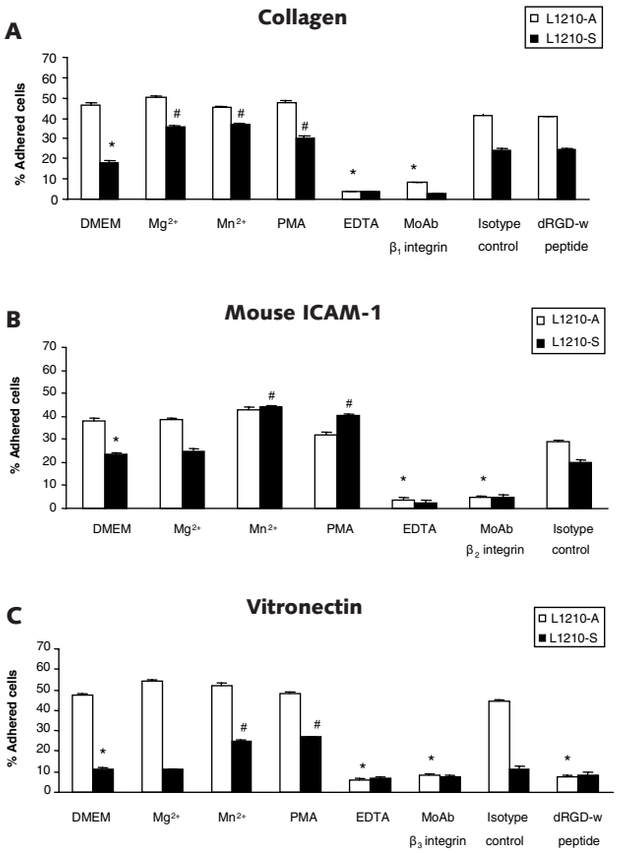

Fig. 4. β_1, β_2, and β_3 integrins are constitutively active on L1210-A cells. Static adhesion assays of leukemic cells were performed on collagen (A), mouse ICAM-1 (B), and vitronectin (C). Assays were done without extracellular stimulation (DMEM); in the presence of Mg^{2+} (5 mM), Mn^{2+} (0.5 mM), or EDTA (10 mM); or after pretreatment of leukemic cells with PMA (100 ng/ml), integrin-blocking or isotype control MoAbs (10 μg/ml), or dRGD-w peptide (100 μM) for 30 min at 37°C. The percentage of adhered cells after 30 min of static adhesion and three washing steps is plotted on the *y*-axis. White and black bars represent the mean percentage of adhered L1210-A and L1210-S cells (± SEM), respectively. Data were obtained from more than three independent experiments performed in triplicate. *Significantly different compared to the mean percentage adhered L1210-A cells in DMEM. #Significantly different compared to the mean percentage adhered L1210-S cells in DMEM.

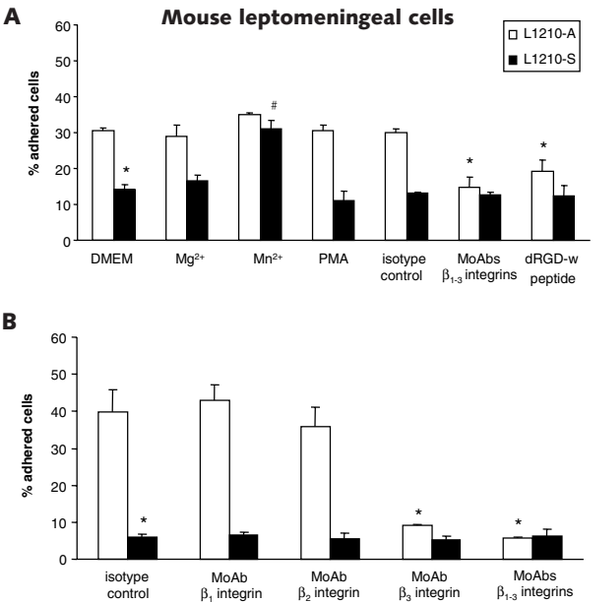

Fig. 5. Mn^{2+} induces L1210-S cell adhesion to a leptomeningeal cell layer and L1210-A cell adhesion is β_3 integrin dependent. A. Static adhesion assays of leukemic cells were performed on confluent mouse leptomeningeal cell layers without extracellular stimulation (DMEM); in the presence of Mg^{2+} (5 mM) or Mn^{2+} (0.5 mM); or after pretreatment of leukemic cells with PMA (100 ng/ml), β_1, β_2, β_3 integrin-blocking or isotype control MoAbs (10 μg/ml), or dRGD-w peptide (100 μM) for 30 min at 37°C. B. Static adhesion assays of leukemic cells on confluent mouse leptomeningeal cell layers were performed after pretreatment of leukemic cells with MoAbs against the single β_1, β_2, or β_3 integrin subunits (10 μg/ml) or all three β integrin chains, and results were compared to results for pretreatment with the isotype control MoAbs (10 μg/ml) for 30 min at 37°C. The percentage of adhered cells after 30 min of static adhesion and three washing steps is plotted on the *y*-axis. White and black bars represent the mean percentage of adhered L1210-A and L1210-S cells (± SEM), respectively. Data were obtained from at least three independent experiments performed in triplicate. *Significantly different compared to the mean percentage adhered L1210-A cells in DMEM or after pretreatment with isotype control MoAbs. #Significantly different compared to the mean percentage adhered L1210-S cells in DMEM.

of the L1210-A cells to the leptomeningeal cell layer is almost completely β_3 integrin dependent ($P < 0.03$ compared to isotype control antibodies).

Discussion

We show that constitutive integrin activation on leukemic cells contributes to leptomeningeal leukemia. For this, we used adherent and suspension forms of a leukemic cell line, which had similar proliferation rates in culture medium but differed in adhesion and spreading capacity on a leptomeningeal cell layer in vitro. We found that the adherent leukemic cell population, but not the suspension leukemic cell population, led to rapid death in a leptomeningeal leukemia mouse model. We showed that β_1, β_2, and β_3 integrins are in a constitutively high activation state on the L1210-A cells and in a low, but inducible activation state on L1210-S cells. The suspension cell line was converted to the adherent phenotype by activating the integrins with divalent cations (matrix proteins and mouse leptomeningeal cells) or PMA (matrix proteins). Our data point to an abberantly regulated inside-out signaling pathway of integrins in tumor cells as a novel mechanism of LM progression. Integrin activation on hematopoetic cells is a tightly regulated process under physiological circumstances (Cal-

vete, 1994; Ley, 2002). Circulating leukocytes maintain their integrins in a low-activity state, which can rapidly be changed into an intermediate or a highly active state by chemokine-triggered inside-out intracellular signaling pathways (Hynes, 1992; Shimizu et al., 1999). Under pathological conditions, such as an infection, chemokines are present on the endothelial cells and activate integrins on leukocytes, which subsequently leads to leukocyte adhesion to the endothelium and transmigration into the tissue. It has been shown that only a small percentage of activated integrins is needed to reach maximum levels of leukocyte adhesion, which renders the expression level of integrins as less important than their activation state (Diamond and Springer, 1993; Lum et al., 2002). Therefore, the small differences in β_1 and β_3 integrin expression levels of the two leukemic cell lines may contribute to the survival difference, but the difference in activation state of the β_1, β_2, and β_3 integrins is considered to be more important. The β_1 integrins potentially mediating leukemic cell adhesion to collagen are the $\alpha_1\beta_1$ and $\alpha_2\beta_1$ integrins, since these integrins are known to be expressed on leukocytes and interact with collagen in a non-RGD-dependent way, as was found for the L1210-A cells (Ben Horin and Bank, 2004; Gendron et al., 2003). $\alpha_L\beta_2$ integrins on the leukemic cells most likely recognize ICAM-1, because these integrins are expressed on lymphocytes, whereas $\alpha_M\beta_2$ integrins are present only on leukocytes of the myeloid lineage (Li, 1999; van Kooyk and Figdor, 2000). Several integrins ($\alpha_v\beta_5$, $\alpha_v\beta_3$, $\alpha_v\beta_1$, $\alpha_8\beta_1$, and $\alpha_{iib}\beta_3$) potentially interact with vitronectin (Hynes, 2002). We consider it most likely that for the L1210 cells $\alpha_v\beta_3$ integrins mediate the adhesion to vitronectin, as leukemic cell adhesion was largely β_3 integrin dependent, and low expression levels of the α_v integrin subunit but not α_{iib} integrin subunit were found on the L1210-A cells.

Several studies suggest that the inside-out signaling of integrins in tumor cells can be dysregulated, which can lead both to adhesion defects due to integrin inactivity and to increased adhesion caused by constitutive integrin activation. Geijtenbeek et al. (1999) demonstrated $\alpha_L\beta_2$ integrin and $\alpha_4\beta_1$ integrin–mediated adhesion defects in leukemic cells isolated from bone marrow of patients with B-lineage acute lymphoblastic leukemia. Trusolino et al. (1998) found that $\alpha_v\beta_3$ integrins on thyroid carcinoma were highly active and enriched at focal contacts, mediating tight adhesion, whereas these integrins were in a latent state on normal thyroid cells, which could not form cytoskeletal connections and promote cell adhesion. An autocrine loop of the hepatocyte growth factor/scatter factor and a constitutively tyrosine phosphorylated receptor were thought to be responsible for the high $\alpha_v\beta_3$ integrin–activated state in the thyroid carcinoma cells. Felding-Habermann et al. (2001) showed that constitutively activated $\alpha_v\beta_3$ integrins, but not the nonactivated form, promoted distant metastases of mammary carcinoma. This finding was attributed to $\alpha_v\beta_3$ integrin–mediated interaction of tumor cells with platelets, which supports tumor cell arrest to the blood vessel wall.

Here we show that constitutive integrin activation on leukemic cells contributes to leptomeningeal leukemia. We attribute this finding to an increased integrin-mediated leukemic cell adhesion to the leptomeninges, which was mostly β_3 integrin dependent as determined in in vitro assays to determine the adhesion of leukemic cells to a primary leptomeningeal cell layer. Three hypotheses were formulated to explain integrin-mediated LM progression: (I) direct integrin-ligand interactions between adhered cells and leptomeningeal cells/matrix proteins lead to survival or proliferation signaling, (II) adhered cells proliferate faster than cells in suspension, because the leptomeningeal vasculature provides nutrients, growth factors, and oxygen to the adhered cells more efficiently, and (III) proliferating, adhered leukemic cells can form tumor masses that induce angiogenesis. Our finding that leukemic cells do not proliferate in the CSF underscores the relevance of tumor cell adhesion to the leptomeninges in LM progression. No data were found to support the first hypothesis, because binding of leukemic cells to either collagen or vitronectin could not induce leukemic cell proliferation in CSF (data not shown). The second and third hypotheses are therefore more likely to explain integrin-mediated LM progression.

Integrin activation is a combination of integrin affinity and avidity changes. The constitutively activated state of β_1, β_2, and β_3 integrins on L1210-A cells is likely to be caused by an increase in both integrin affinity and integrin avidity, since Mn^{2+} (affinity change) as well as PMA (avidity change) significantly increased adhesion of L1210-S cells to collagen, mouse ICAM-1, and vitronectin. Only L1210-S cell binding to collagen was induced by Mg^{2+}, known to be less potent in changing the affinity state of integrins than is Mn^{2+}. Surprisingly, L1210-S cell binding to mouse leptomeningeal cells was induced only by Mn^{2+} and not by PMA, which suggests that integrin affinity is more important than integrin avidity for tumor cell adhesion to the leptomeninges.

It is tempting to speculate about the intracellular factor(s) being dysregulated in tumor cells with constitutively active integrins. R-ras, a member of the Ras family of small GTP-binding proteins, and its downstream effector, Raf-1, are interesting proteins in this respect, because they are involved in both integrin activation and oncogenesis (Hughes et al., 1997; Sethi et al., 1999). The Ras-related GTPase protein, Rap-1, a protein that has been shown to be a key regulator of integrin activation in leukocytes, may be another interesting candidate (Katagiri et al., 2000; Reedquist et al., 2000; Shimonaka et al., 2003). Future research will focus on unraveling the intracellular inside-out signaling defects that lead to constitutively activated integrins on tumor cells. Ultimately, this research should lead to the development of agents that efficiently block tumor cell adhesion in order to prevent LM progression.

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