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Chapter 3

Targeted disruption of the β1 integrin gene in a lymphoma cell line greatly reduces metastatic capacity

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Targeted Disruption of the β1 Integrin Gene in a Lymphoma Cell Line Greatly Reduces Metastatic Capacity

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ABSTRACT

Integrins have been implicated in tumor metastasis. To investigate this, we generated β1 integrin-negative double knockout (DKO) mutants of the highly metastatic ESB murine T-lymphoma cell line. The in vivo growth capacity of the mutants, which had lost αβ1 and αβ1 expression, was not altered, but their metastatic capacity was greatly reduced. Tail vein injection of 10^6 ESB and single-knockout cells led to death of all animals within 9–11 days. In contrast, only one-half of the animals injected with 10^6 DKO cells died, but much later, after 20–60 days. The other one-half remained disease-free for up to 100 days. Whereas ESB and single-knockout cells disseminated predominantly to liver and spleen, metastasis of DKO cells to these organs was rare, even after this prolonged period. Instead, skeletal muscles were invaded extensively. Metastatic capacity was largely restored in a DKO clone, which had been transfected with β1 cDNA and expressed β1 at similar levels as ESB cells. We conclude that β1 integrins are essential for efficient liver and spleen colonization by the ESB lymphoma.

INTRODUCTION

Integrins are transmembrane proteins composed of noncovalently associated α and β subunits (1). To date, 16 α and 8 β chains have been identified that can form at least 22 heterodimers. Integrins mediate adhesion of cells, predominantly to extracellular matrix proteins, but also to cell surface proteins on other cells. The ability of integrins to bind ligand is not constitutive but highly regulated, particularly in blood cells, by so-called inside-out signaling (2). In recent years, integrins have been shown to transmit signals to the interior of the cell, which can lead to cytoskeletal reorganization and activation of mitogen-activated protein kinase pathways (3, 4).

Metastasis is a complex process, with multiple steps, starting with the dislodgement of cells from the primary tumor and ending with the extravasation and growth at the secondary site (5). Acquisition of a more malignant phenotype by tumor cells is often associated with altered expression of adhesion molecules. Integrins, in particular, have been suggested to play a major role in the invasion of cells from the primary tumor into the surrounding tissue as well as the invasion of blood-borne tumor cells into tissues (6). To demonstrate this involvement, α subunit cDNAs have been transfected into tumor cell lines in which heterodimers were formed with endogenous β subunits. For instance, expression of αβ1 by a rhabdomyosarcoma cell line resulted in increased metastasis, both from a s.c. tumor and by i.v. injected cells (7). In contrast, expression of αβ1 by melanoma cells resulted in reduced metastasis from a s.c. tumor but had no effect on metastasis formation after i.v. injection (8), suggesting that αβ1 inhibits the dislodgement of cells from the primary tumor. Although these results are compelling, the introduced integrin is not necessarily relevant for the tumor type investigated, because it is apparently not normally expressed. A more direct approach was to block metastasis by anti-integrin antibodies or RGD peptides in vivo (9–12), which reduced metastasis formation. However, both antibodies and peptides can have multiple effects, e.g., on the immune system and on platelet aggregation, and it is not certain that invasion was blocked. Previously, we have generated mutants of a T-cell hybridoma that were deficient in expression of the integrin αβ2 or LFA-1; 2 These mutants had virtually lost metastatic capacity (13). However, because the mutants were generated by chemical mutagenesis, it could not be excluded that the mutations had affected other relevant proteins as well.

In this study, we used a more direct approach: the disruption of both alleles of the β1 integrin gene by homologous recombination in the highly metastatic near-diploid murine T-cell lymphoma ESB, the LFA-1 levels of which are low. In contrast to the T-cell hybridomas, ESB cells express αβ1. The disruption of the β1 alleles led to a large reduction in metastatic capacity and an apparent change in the tissue distribution of the residual metastases. Metastatic capacity could be largely restored by re-expression of β1 integrins in the DKO cells. We conclude that the β1 integrins of this cell line, quite probably mainly αβ1, are indispensable for efficient colonization of certain tissues, in particular the spleen and the liver.

MATERIALS AND METHODS

Generation of Targeting Constructs. Standard techniques for DNA manipulations were used (14). A DBA/2-derived genomic library (Clontech, Inc., Palo Alto, CA) was screened with a β1 cDNA probe representing exons 2–4 (15). A 6.5-kb XhoI-Sall fragment containing exons 2 and 3, was subcloned in pGEM-7Zf(+) (Promega Corp., Madison, WI) and used to generate the targeting constructs. Exon 2, which starts with the ATG start codon, is located 3′ of a unique BstEII site and 5′ of a unique NheI site. These sites were used to exchange exon 2 for the promotorless β-galactosidase-neomycin fusion DNA (geo) cassette fused to exon 2, present in pKOgeo21 (16). Thus, the nonisogenic β/ gene DNA in this construct was replaced by isogenic DBA/2 DNA. To generate a promoterless hygromycin cassette, a 0.6-kb HindIII-SacI fragment containing exon 2 was subcloned in pBluescript KSII(−) (Stratagene, La Jolla, CA). The fragment contains the unique BstEII site 5′ and a unique XbaI site 93 bp 3′ of exon 2. The plasmid was cut with BstEII and XbaI, and the part consisting of the vector and two small β/gene fragments was isolated (fragment 1). Into this fragment, the hygromycin cassette (made from fragments 2–6, described below) was cloned between the BstEII and XbaI sites. After having sequenced part of the intron located 5′ of exon 2, two primers were designed (AATAGGT- GAAAAGTAAACATC and GACAGACCTGCCGTAGTTCCAGG- TTTTCATCTATAACGAAATGFTATTG) to amplify the 3′ part of the intron upstream of exon 2. The first primer contains the BstEII site (underlined), and the reverse primer matches the intron-exon boundary plus the ATG start codon (underlined) and also contains the first nine codons of the hygromycin B resistance gene, including the AarII site (underlined). After PCR with Pfu DNA polymerase (Stratagene), the 180-bp product was cut with BstEII and AarII (fragment 2). The plasmid pPGKhyg (17) was digested with AarII and Tru9I (the latter enzyme cuts after the stop codon and before the polyadenylation signal) (fragment 3). The plasmid pPGK-neoNTRkpa (18) was cut with Psp1406I and NsiI to obtain the fragment containing the IRES of the encephalomyocarditis virus (19), the thymidine

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2 The abbreviations used are: LFA-1, leukocyte function associated antigen-1; DKO, double knockout; DKO-β1, DKO cells re-expressing β1 integrins; SKO, single knockout; IRES, internal ribosome entry site; mAb, monoclonal antibody; VCAm-1, vascular cell adhesion molecule 1; ICAM-1, intercellular adhesion molecule-1.
kinase gene of the herpes simplex virus, and two polyadenylation signals (fragment 4). To connect the hygromycin gene and the IRES sequence, an adapter was used (TAACCCCTCTCTCCCCCCTCCCTCTAATA and CTGTAGGGGGGGGGGGAGAGAGGGG) that has a 5' TrpG and a 3' PspI1406I overhang and regenerates the most 5' sequence of the IRES (fragment 5). Two oligonucleotides containing the loxp sequence were annealed (GTATCCAGGAGTCAGGTGCTAATAGTGTGCTGCATACGAGTTCTCTAGAGCTCA: GTATCGGATCCCTCTAGGAA-TAACCTGGTATAAGCTATTATACTATATGTACATCGTG), resulting in an adapter with a BesEII overhang and containing a Nil site 5' and a XbaI and BamHI site 3' of the loxp sequence (restriction sites underlined). The adapter was cut with NsiI and XbaI (fragment 6). The fragments 1–6 were ligated in a hexamolecular reaction. After confirming the correct assembly of the construct by sequencing, the cassette was cut out of the plasmid with BesEII and XbaI and ligated between the BesEII and Xhel site of the 6.5-kb Xhel-Sall fragment of the Bl integrin gene. Finally, the 5' loxp sequence was introduced into the BesEII site.

Cell Lines and Culture Conditions. ESb (20) and mutant cells were cultured in enriched RPMI 1640 (Hybridoma medium; Ref. 13). RAC-11P cells were cultured in DMEM supplemented with 10% FCS and TK1 cells in RPMI 1640 with 10% FCS and 0.1 mM 2-mercaptopethanol. Media and FCS were from Life Technologies, Inc. (Paisley, United Kingdom).

Generation of Knockout Cells and Southern Analysis. Two 10^7 ESb or similar knockout cells in 800 μl RPMI 1640 were electroporated using Bio-Rad Gene Pulser (360V, 960 μF) in the presence of 100 μg of targeting construct (released from vector sequences). Cells were seeded in 48-well plates (2 × 10^5 cells/well). Selection was started after 24 h (1 mg/ml G418, Life Technologies, Inc., Paisley, United Kingdom; 1.2 mg/ml hygromycin B, Calbiochem-Novabiochem Corp., La Jolla, CA). For Southern blot analysis, DNA was isolated from the clones (21), digested with KpnI or BamHI, and 10 μg were loaded on a 0.7% agarose gel. After electrophoresis, DNA was transferred to a Nytran 13 N membrane (Schleicher & Schuell, Inc., Dassel, Germany) and hybridized with a 150- bp HindIII-PvuII CDNA probe of exon 4 (Fig. 1A).

Antibodies and FACS Analysis. The hybridoma producing rat anti-mouse αL mAb MA17/4 was obtained from the American Type Culture Collection (Rockville, MD), and the rat anti-mouse β2 M18/2 hybridoma was from Dr. T. A. Springer (Harvard Medical School, Boston, MA). The hamster anti-mouse β3 mAb 2C9-G2, the hamster anti-rat β1 mAb Ha25, and the rat anti-mouse α4 mAb 9C10 were purchased from Pharmingen (San Diego, CA). Rat anti-human α6 mAb GoH3 (22) was kindly provided by Dr. A. Sonnenberg, and the rat anti-mouse α5 mAb Sc6 (23) was provided by Dr. C. Fidgior. The rat anti-mouse CD44 mAb KM201 (24) was a gift of Dr. P. Kincade, and the polyclonal rabbit antisera D4 (25) was kindly provided by Marcantonio and Hyne (25). The rat anti-mouse CD44 mAb KM201 (24) was a gift of Dr. P. Kincade, and the polyclonal rabbit antisera D4 (25) was kindly provided by Marcantonio and Hyne (25).

Cell lines were surface-labeled with 125I (Amersham International, UK) by the lactoperoxidase method, washed, and solubilized in lysis buffer containing 1% (v/v) NP40, 25 mM Tris-HCl pH 7.5, 2 mM CaCl_2, 100 mM NaCl, 50 μM aprotinin, 0.5 mM pefabloc SC, and 10 μg/ml leupeptin at 1 h at 4°C. Lysates were clarified at 14,000 rpm and precleared overnight at 4°C by incubation with Protein A-Sepharose CL-4B (Pharmacia LP, Uppsala, Sweden). Precleared cell lysates were added to Protein A-Sepharose beads previously incubated with rabbit-anti-rat/75 IgG and the precipitating antibodies. After incubation at 4°C for 2 h, the beads carrying the immune complexes were washed in three times in lysis buffer and two times in PBS, incubated in SDS sample buffer at 100°C for 5 min, and proteins were analyzed by SDS-PAGE on a 5% polyacrylamide gel under nonreducing conditions.

In Vitro Adhesion Assays. Microtiter plates were coated overnight at 4°C with 100 μl of rat fibronectin (40 μg/ml, diluted in H2O), 100 μl murine laminin (20 μg/ml, diluted in PBS), 100 μl recombinant soluble murine ICAM-1, purified from the supernatant of transfected cells, kindly provided by Dr. P. Takel (28) (2 μg/ml, diluted in PBS), or 100 μl VCAM-1-immuno-globulin fusion protein containing the first two immunoglobulin domains of VCAM-1 (kindly provided by Drs. P. Newman and M. Humphries) (10 μg/ml, diluted in PBS). Unbound sites were subsequently blocked with Tris buffer (20 mM Tris, 150 mM NaCl, 6 mM KCl, 6 mM p-glucose, pH 7.2) containing 0.5% ovalbumin for 2 h at room temperature. ESb and variant cells were labeled with 100 μM 325Cr (Amersham) per 10^6 cells in 250 μl of culture medium for 45 min. Each well contained 5 × 10^5 cells in a final volume of 100 μl of Tris buffer supplemented with either 1 mM Ca^2+ and 1 mM Mg^2+ or 2 mM Mn^2+. After incubation for 30 min in 5% CO2 at 37°C, nonadherent cells were removed by washing three times with 100 μl Tris buffer supplemented with 1 mM Ca^2+ and 1 mM Mg^2+ and inverting the plate. The cells were lysed in 100 μl of 1 N NaOH, and the percentage of bound cells was determined by counting the radioactivity. All measurements were done in triplicate. Percentages were corrected for spontaneous release of 32Cr, and background binding of cells to wells coated with only 0.5% ovalbumin.

In Vivo Growth, Experimental Metastasis, and Histology. To compare the capacity of the parental and mutant cell lines to grow in vivo, 7-week-old syngeneic DBA/2 mice were injected i.p. with 10^6 or 10^6 cells in 200 μl of PBS with 1 mM Mg^2+ and 1 mM Ca^2+. After 16 or 9 days, respectively, ascites fluid was collected, and the number of cells were counted. For s.c. tumor growth, 10^6 cells were injected in the flank, and animals were monitored for...
tumor growth every week. To assess the metastatic capacity, $10^8$ cells in 100 μl PBS with 1% CMC and 1% Muc were injected into a lateral tail vein. Animals were autopsied when moribund or after a fixed time period, and their livers were weighed. Metastasis formation was determined both macroscopically and microscopically. Tissues were fixed in ethanolic-acetic acid-formal saline fixative and embedded in paraffin; 5-μm sections were mounted onto slides and stained with H&E according to standard procedures.

**Transfection of DKO Cells with β1 and ESb Cells with LacZ cDNA.** To transfet β1 cDNA into DKO cells, $10^7$ DKO 18/22 cells were electroporated (as described above) with 100 μg of XhoI-linearized pBSJ1 (15). This construct contains a purumycin resistance gene and the β1 cDNA with the endogenous polyadenylation signal, both under the control of a pgk-1 promoter. Cells were seeded in 24-well plates (5 x $10^5$ cells/well), and after 48 h, 1.2 μg/ml purumycin (Clontech Inc., Palo Alto, Ca) were added. Resistant clones were analyzed for β1 expression by FACScan analysis using the mAb MB1.2 (29). To produce ESb-geo cells stably expressing the β-galactosidase-neomycin (geo) fusion protein, we generated a pMFG-LacZ-neo vector (30). The plasmid was used to produce geo-transducing retroviruses in BOSC23 packaging cells as described by Peer et al. (31). ESb-geo cells were obtained after cocultivation of 2 x $10^5$ ESb cells and 5 x $10^5$ BOSC23 cells. Positive clones were selected by LacZ staining according to standard techniques.

**RESULTS**

**Targeted Disruption of the β1 Integrin Gene in ESb Cells.** In this study, we have analyzed the role of β1 integrins in metastasis formation by a T-cell lymphoma. We sequentially disrupted the two β1 alleles in the highly metastatic murine T-cell lymphoma ESb, which expresses αβ1 (LVA-4) and αεβ1 (LVA-6). Gene targeting in embryonic stem (ES) cells has become a routine procedure in many laboratories, but in more differentiated cells, it is much less efficient. However, we and others have shown that this approach is feasible (32-36). To increase the ratio of homologous recombination to random integration, we used isogenic DNA (37) and promoterless selectable marker genes (38, 39). To disrupt the two alleles of the β1 gene in ESb cells, which originate from a DBA/2 mouse, a 6.5-kb fragment of genomic DBA/2 DNA was used to generate the targeting constructs. The fragment contains exon 2, which has a size of 67 bp, begins with the ATG start codon, and contains the leader sequence (Fig. 1A). To disrupt the first allele, the hygromycin B resistance gene (hyg), fused to the IRES of the encephalomyocarditis virus and the thymidine kinase gene (tk) of the herpes simplex virus, was inserted in frame at the ATG start codon. Two loxP sequences were introduced that flank the cassette, the 5' loxP site −100 bp 5' from exon 2 to prevent interference with proper splicing of exon 2 (Fig. 1A). Although not used in this study, expression of the Cre recombinase in these cells will lead to deletion of the cassette containing the hyg and tk genes, and cells in which this has occurred can be selected using ganciclovir.

After transfection of the construct by electroporation, 74 clones were obtained, in two of which a β1 integrin allele had been disrupted (SKO 18 and 43; Fig. 1B). To target the second allele, we used essentially the same construct as Fässler et al. (16), with the β-galactosidase-neomycin fusion gene inserted in frame at the ATG start codon in the β1 gene fragment, with the difference that we had replaced the β1 gene fragments with the corresponding fragments from DBA/2 DNA. Electroporation of SKO 18 cells with the geo construct resulted in 24 G418-resistant clones, of which three had two disrupted alleles (Fig. 1B). Southern blot analysis with probes representing the selectable marker genes revealed that only one copy of each of the constructs was integrated in the genome of the DKO cells (data not shown).

To demonstrate that the disruption of the β1 integrin alleles had led to the loss of β1 integrin expression, we immunoprecipitated lysates from $^{125}$I surface-labeled ESb, SKO, and DKO cells with antibodies against the β1, α4, and α6 subunits and antibodies against CD44 as a control. Disruption of one allele did not result in reduced surface expression of αβ1 and αεβ1, but on the surface of the DKO cells no heterodimers could be detected (Fig. 2A). A longer exposure did reveal low surface levels of α4 and α6 subunits. However, no protein was precipitated by a polyclonal antibody directed against the β1 subunit, which did precipitate integrins in ESb and SKO cells, and no β subunits were visible in the precipitates obtained with the α4 and α6 mAbs. Conceivably, the presence of the α subunit at the surface may have been due to up-regulation of β4 and β7 subunits and formation of α6β4 and α4β7 heterodimers. However, immune precipitations and FACScan analysis with the β7 mAb M293 and M298 (40), using TK1 cells (41) as a positive control, and the β4 mAb 346-11A (42), with RAC-11P cells (43) as positive control, showed that this was not the case (data not shown). These results suggest that in the absence of appropriate β subunit partners, some α4 and α6 subunits can reach the
cell surface, apparently not associated with other surface proteins, as has in fact been described before for the α4 subunit (44). These results were confirmed by flow cytometry. Surface levels of β1 integrins on ESb and SKO cells did not differ, but on DKO cells, B1 could not be detected. In contrast, low levels of α6 and moderate levels of α4 were detected (Fig. 2B). Flow cytometry analysis further revealed that the parental cell line expresses, besides α4β1 and α6β1, the β2 integrins αLβ2 (LFA-1) and αMβ2 (Mac-1), αβ3, and CD44. Surface levels of the β3 and β2 subunits were low and moderate, respectively, and similar on all cell lines (Fig. 2B). Surface levels of αβ3 and αLβ2 (LFA-1) were low, and the level of αMβ2 (Mac-1) was moderate but did not differ between the cell lines (Fig. 2B and data not shown).

In Vitro Adhesion of ESb and Mutant Cell Lines. The β1 integrins α6β1 and α4β1 bind to laminin and to both fibronectin and VCAM-1, respectively. The two β2 integrins bind to ICAMs and αMβ2 also to several other proteins including fibrinogen. The αβ3 integrin has multiple ligands including fibrologin, collagen, and vitronectin (1). We assessed the ability of ESb and mutant cell lines to adhere to immobilized laminin, fibrologin, and recombinant VCAM-1. Integrins on ESb cells are not active, and the cells hardly bind spontaneously. We induced adhesion by the addition of Mn2+•, which activates integrins, probably by inducing a conformational change in the ligand-binding domain of the integrin (45). ESb and SKO cells did not differ in adhesion to these ligands. In contrast, none of the three DKO cells bound to laminin or VCAM-1 (Fig. 3). However, the DKO cells did bind to fibronectin. Adhesion of ESb and SKO cells to fibronectin was inhibited to a similar extent by the mAb 9C10 against the α4 subunit and the mAb 2C9.G2 against the β3 subunit, indicating that both α4β1 and αβ3 contribute to this adhesion (data not shown). The residual binding of DKO cells to fibronectin was completely blocked by mAb 2C9.G2 and is, therefore, mediated only by αβ3 (Fig. 3). All cells adhered very poorly to ICAM-1 (data not shown).

In Vivo Growth and Experimental Metastasis. To investigate the ability of cells to grow in vivo, 10⁴ or 10⁶ ESb, SKO, and DKO cells were injected i.p. into DBA/2 mice. After 9 or 16 days, the ascites fluid was collected, and the cells were counted. The growth rates, with an average doubling time of ~24 h, were not different. All animals showed local tumor growth in the peritoneum at the site of injection, including the mice injected with DKO cells. s.c. injection of 10³ cells led to tumor formation, and again no differences were observed between ESb, SKO, and DKO cells (data not shown).

We performed two metastasis assays. In the first, 10⁶ ESb, SKO 18, or DKO 18/22 cells were injected into a tail vein of 7-week-old syngeneic DBA/2 mice. For the parental line, this dose leads to metastasis formation in 100% of the mice. After 9 days, most of the animals injected with the parental and SKO cells were dead, and those remaining were moribund. All animals had developed extensive liver and spleen metastasis, as determined macroscopically. Histological analysis confirmed that the tumor cells formed multiple foci in the liver and also spread diffusely between the hepatocytes. In the spleen, focus formation was confined to the red pulp. In contrast, in mice injected with the DKO 18/22 cells and sacrificed at day 9, no liver or spleen metastasis was observed. Liver metastasis formation by the ESb and SKO cells had led to a 2-fold increase in liver weight as compared with the animals injected with the DKO cells (Fig. 4).

In the second assay, we measured the survival time of the mice injected with, in this case, all three DKO cell lines. Animals were killed when moribund, and autopsies were performed. Approximately one-half of the 50 mice injected with the three DKO cell lines, taken together, survived for 100 days without metastasis formation. The other half became ill between days 20 and 60 (Fig. 5). Whereas ESb and SKO cells formed multiple foci and spread diffusely in liver and spleen (Fig. 6, A and B), DKO cells hardly metastasized to these organs. Of the animals that became moribund, 30% had developed liver metastasis, but this was restricted to a few foci or only one very large nodule, and only 10% had foci in the spleen. In contrast, skeletal muscles in these mice were massively invaded by tumor cells between the muscle fibers. Many mice showed paralysis of the hind legs, due to tumor invasion into muscles along the vertebral column and muscles of the pelvis region. Also intercostal muscles were sometimes heavily infiltrated (Fig. 6C). In addition, the meninges (brain membranes; Fig. 6D) and the periosteu (the membrane around bones) contained large numbers of infiltrated tumor cells. In ESb-injected
mice, some invasion of the periostea and the meninges was also observed but much less than in DKO-injected mice, obviously because of the much shorter survival time. No metastasis was observed in lymph nodes, bone marrow, lungs, or kidneys, although many tumor cells were often seen in the vessels. The differences in mortality between the mice injected with the three DKO cell lines were not significant (log-rank $\chi^2$ test; $P = 0.054$), and we observed no differences in the pattern of dissemination between the three DKO cells.

**Rescue of the Phenotype by Re-expression of $\beta_1$ Integrins on DKO Cells.** To obtain definite proof that the reduced metastatic capacity of the DKO cells was due to the absence of $\beta_1$ integrins, we transfected $\beta_1$ cDNA into DKO 18/22 cells (15). In one of the clones, the surface levels of $\beta_1$ (Fig. 7A), $\alpha_4$, and $\alpha_6$ (data not shown) were comparable to ESb and SKO cells. The adhesion of this DKO-$\beta_1$ transfected to laminin, fibronectin, and VCAM-1 after stimulation with Mn$^{2+}$ was restored to wild-type levels (data not shown), demonstrating that the transfected $\beta_1$ was functional.

Next, we investigated the metastatic capacity of the transfected by injecting $10^4$ DKO-$\beta_1$ cells into a tail vein of DBA/2 mice, as well as SKO 18 and DKO 18/22 cells for comparison. As observed before, animals injected with SKO cells became ill at day 9 or 10. Animals injected with the DKO-$\beta_1$ cells had many foci in the liver and the spleen at day 9, whereas animals injected with DKO cells had no foci in the liver and no or only a few foci in the spleen on day 9 (data not shown). Surprisingly, however, mice injected with DKO-$\beta_1$ cells that were not sacrificed on day 9 survived. Further analysis showed that foci were no longer present in these mice, after 11–13 days. This suggested that the DKO-$\beta_1$ cells were rejected by the immune system. The first possibility that we envisaged was immunogenicity caused by the LacZ-neo (geo) fusion protein, which is only present in the DKO and DKO-$\beta_1$ cells. To study this, we generated ESb-geo cells that expressed high levels of the geo protein. The metastatic capacity of these ESb-geo cells did not differ from parental cells, ruling out this possibility. It is, therefore, most likely that rejection was due to a CTL response against ESb cells, as described by Schirmacher et al. (46). If the metastatic capacity of the DKO-$\beta_1$ cells would be slightly reduced compared to ESb and SKO cells, this rejection might be sufficient to eradicate the reduced number of metastatic cells. If so, this problem should be overcome by using a higher dose of cells. Therefore, we injected $5 \times 10^4$ cells instead of $10^4$ cells. Indeed, most of the animals (85%) injected with DKO-$\beta_1$ cells did become moribund but 2–4 days later than animals injected with SKO cells (Fig. 7B). All SKO and DKO-$\beta_1$-injected animals had foci in the liver and spleen and in addition skeletal muscle was invaded in all DKO-AL animals. In contrast, only 15% of the animals injected with the corresponding DKO clone became moribund due to muscle invasion, and only after a prolonged period of 30–55 days. Thus, the DKO-$\beta_1$ cells exhibited a greatly increased metastatic capacity, compared with the DKO clone from which they were derived, demonstrating that the presence of $\beta_1$ integrins greatly increased the efficiency of metastasis formation.

**DISCUSSION**

Integrins play a pivotal role in the migration of leukocytes into inflamed tissues. These leukocyte integrins need to be activated by chemokines and other factors at the inflamed site (47, 48). Leukocyte trafficking through noninflamed tissues is less well defined, but evidence is accumulating that the regulation is similar. For instance, migration of B cells into spleen follicles and into Peyer's patches in the intestine is greatly reduced in mice lacking the putative chemokine receptor BRL1 (49). Furthermore, the chemokine SDF-1, which is expressed in many noninflamed tissues, is a potent chemokine receptor for T cells and may regulate the migration of T cells through those tissues (50).

We have proposed that neoplastic leukocytes use comparable mechanisms to invade noninflamed tissues where metastases are formed. This notion was supported by our finding that hybridomas generated from activated T cells and nonmetastatic T-lymphoma cells, which retained the invasive capacity of the T cells, were highly metastatic (51). These cells require the $\beta_2$ integrin LFA-1 to metastasize (13), and LFA-1 activity during in vitro invasion is regulated by pertussis toxin-sensitive $\gamma_2$-proteins (52). We showed recently that expression of the catalytic subunit of pertussis toxin, which had no effect on proliferation, blocked metastasis of these T-cell hybridomas, particularly to the liver (53). This strongly supports the notion that lymphoma metastasis depends on local integrin-activating factors that signal through $\gamma_2$ proteins.

We show here that ESb lymphoma cells, which express only low
levels of LFA-1, require β1 integrins for metastasis, in particular to liver and spleen. In this context, it is of interest that β1 integrins are absolutely required for influx of hematopoietic progenitor cells into the fetal liver and are, therefore, essential for hematopoiesis (54). The dominant role of β1 integrins in this organ, and also in the spleen, is further illustrated by the fact that in β1-null chimeric mice, the liver and spleen are the only two organs in which no β1-null cells were found (55).

The ESb cells contain two β1 integrins, the fibronectin and VCAM-1 receptor α4β1, and the laminin receptor α6β1. It is most likely that α4β1 is the crucial molecule. The ESb cells metastasize most rapidly and extensively to the liver and the red pulp of the spleen. In the liver, no basement membrane is present under the endothelium of microvessels from which lymphoma cells invade this organ (56). In the spleen, arterial capillaries terminate open-ended in the reticular meshwork of the red pulp (57). This meshwork, as well as the space of Disse between liver microvascular endothelium and hepatocytes, contains abundant fibronectin with the alternatively spliced CS1 segment (58, 59) to which α4β1 binds (60). Furthermore, the other α4β1 ligand, VCAM-1, is present in the red pulp of the spleen and on part of the endothelial cells in the liver (61, 62). We propose, therefore, that ESb cells bind to VCAM-1 and fibronectin to arrest, invade and be retained in the two organs. In addition, or alternatively, interaction with these ligands may be required for sustained proliferation and cell survival. Given the absence of laminin at these sites, a role for α6β1 is less likely. This is further supported by the lack of extravasation in the lungs, in which metastasis formation of other tumor cell types depends on the interaction of α6β1 with the laminin-containing basement membranes (10, 63).

Despite the presence of α4β1, ESb cells do not metastasize to the bone marrow, whereas transfection of α4 cDNA, and consequent expression of α4β1, was sufficient for Chinese hamster ovary cells to generate bone marrow metastasis (64). The reason for this difference is not clear, but a likely explanation is that integrins are constitutively
active on Chinese hamster ovary cells but require activation in ESB cells. ESB cells may not have the receptors for, or be responsive to, integrin-activating factors in the bone marrow. Our results seem to contradict the observation by Gossler et al. (65) that transfaction of α4 cDNA into the LB T-cell lymphoma blocks metastasis. However, the cell type is quite distinct: LB cells disseminate mainly to secondary lymphoid organs and express the αEβ7 integrin, which is a marker for a minor lymphocyte subset and is involved in homing to intestinal tract epithelium (66). How α4 interferes with metastasis has not been explained. The difference with our cells further illustrates that the role of particular integrins is determined by the cellular context.

In the presence of the integrin activator Mn²⁺, the DKO cells no longer bound to VCAM-1 but still adhered to fibronectin, the other α4β1 ligand. This adhesion was mediated by the integrin αVβ3. Based on blocking antibodies, this integrin was reported to be involved in liver metastasis by a large cell lymphoma (67), and the residual metastasis of DKO cells to the liver may, therefore, be due to αVβ3. If so, this is clearly much less efficient, possibly because of differences in activation. The αVβ3 integrin may also be responsible for invasion of meninges, peristeme, and muscles. Another candidate is the hyaluronic acid receptor CD44, which is expressed at high levels in ESB cells. However, we have shown previously that MDAY-D2 lymphosarcoma cells, in which the CD44 gene was mutated by targeted disruption, invaded muscle in a similar fashion (36), and therefore, a role for CD44 seems less likely.

In T-cell hybridomas, invasion of liver, spleen, and many other organs depends on LFA-1 (αLβ2; Ref. 13). ESB cells express LFA-1 at low levels, and Mn²⁺-induced adhesion to the LFA-1 ligand ICAM-1 was limited and variable. Analysis of revertants of LFA-1-deficient T-cell hybridoma mutants with different LFA-1 levels showed that relatively high levels are required for metastasis (68). It is, therefore, unlikely that LFA-1 is important for metastasis formation of ESB cells. In a recent paper by Rocha et al. (69), metastasis of ESB cells was not impaired by an anti-α4 blocking mAb, in apparent contradiction to our results. However, the ESB subline used had much higher surface levels of LFA-1 than our cells, suggesting that in those cells the role of LFA-1 was more important.

The greatly enhanced metastatic capacity of the β1 transfectant of one of the DKO clones, with restored α4β1 and α6β1 surface levels, provided the ultimate proof for the role of β1 integrins. However, metastatic capacity was not fully restored, as demonstrated by the rejection of metastases that developed from 10⁴ injected cells and by a 2–4-day delay in death from 5 × 10⁴ cells. This difference is probably due to clonal variation in one of the many other properties required for metastasis formation.

In ESB and SKO-injected mice, muscle invasion was very limited. This may have been due to the early death of the animals, so that there was not sufficient time for extensive proliferation in the muscles. Indeed, at the higher dose, and after the 2–4-day delay, we observed hind leg paralysis caused by muscle invasion in all animals injected with the DKO-β1 cells. In DKO-injected mice, this occurred much later and only in 15% of the animals. This shows that β1 integrins contribute to invasion of skeletal muscle as well.

Targeted disruption of genes in whole organisms is a powerful method to unravel the functions of individual proteins in complicated in vivo phenomena. The study of the behavior of tumor cells in vivo, and of metastasis in particular, has relied heavily on correlations, and direct experimental evidence is scarce. Disruption of genes in tumor cell lines would seem to be an obvious option but has rarely been used. However, we and others have shown that this approach is feasible (35, 36). Previously, we have thus shown that CD44 is not required for metastasis of the MDAY-D2 lymphosarcoma (36). In the present study, the frequency of homologous recombination was relatively high. It is not clear as yet whether this was due to the use of promotorless constructs or to particular properties of the ESB cell line. ESB and other lymphoid cell lines have the additional advantage that, in contrast to other tumor cell types, the cells are often near-diploid (70), so that disruption of two alleles is usually sufficient. We intend to elucidate other aspects of metastasis mechanisms by this approach.

In summary, we have shown that β1 integrins, quite probably α4β1, are required for efficient metastasis formation by ESB lymphoma cells, in particular to spleen and liver. This demonstrates the importance of integrins in metastasis formation. It is clear, however, based on our previous results and those of others, that the particular integrin involved differs between lymphoma subtypes. Furthermore,
expression of an integrin is not sufficient, because it requires activation by tissue factors, to which the tumor cells must be responsive.

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REFERENCES


Chapter 3


