Targeted Disruption of CD44 in MDAY-D2 Lymphosarcoma Cells Has No Effect on Subcutaneous Growth or Metastatic Capacity

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Abstract. CD44 splice variants have been shown to be involved in metastasis of carcinomas. In addition, the standard form of CD44 has been implicated in metastasis, particularly of melanomas and lymphomas. To investigate this, we have generated a CD44-negative mutant of the highly metastatic murine MDAY-D2 lymphosarcoma. The two CD44 alleles of this diploid cell line were sequentially disrupted by homologous recombination, using isogenic CD44 genomic constructs interrupted by a neomycin or hygromycin resistance-conferring gene. The resulting double knockout (DKO) cells had completely lost the capacity to bind to immobilized hyaluronic acid, but did not differ from MDAY-D2 cells in integrin expression or in vitro growth. Subcutaneous (s.c.) growth potential and metastatic capacity of MDAY-D2 and DKO cells were assessed by s.c. and i.v. injection of the lowest cell dose (10^3 or 10^4, respectively) that gave rise to tumor formation by MDAY-D2 cells in ~100% of the mice. Quite unexpectedly, we observed no difference at all in either s.c. growth rate or local invasion into surrounding tissues between MDAY-D2 cells and the CD44-negative DKO cells. Also hematogenous metastasis formation upon i.v. injection was similar: both parental and DKO cells metastasized extensively to the spleen, liver, and bone marrow. We conclude that, at least for these MDAY-D2 lymphosarcoma cells, the standard form of CD44 is dispensable for tumor growth and metastasis. Our results show that targeted disruption of genes in tumor cells is a feasible approach to study their role in tumorigenesis and metastasis.

CD44 is a cell surface protein expressed by many different cell types (for reviews see references 21 and 42) that can act as a receptor for the extracellular matrix component hyaluronic acid (HA) (2) and the proteoglycan serglycin (41). CD44 is required for in vitro lymphopoiesis, particularly of B cells (24), that use CD44 to bind to the abundant HA. In addition, CD44 can act as co-stimulator in lymphocyte activation (32) and the induction of lymphocyte effector functions (5, 11, 28). In addition to the standard 85–95-kD form, various larger CD44 variants can be generated by alternative splicing of at least 10 exons (40).

CD44 has been proposed to play a major role in metastasis of different types of tumor cells (for review see reference 31). The most compelling evidence concerns a splice variant of CD44 containing exon v6 that upon transfection into a rat pancreatic carcinoma cell line conferred metastatic potential (12). In these cells, the standard form of CD44 is apparently not involved. In contrast, there is extensive evidence for a role of standard CD44 in metastasis of melanomas and lymphomas. CD44 was shown to be involved in the invasiveness of melanoma cells in vitro (8). Human melanoma cells expressing standard CD44 and sorted for high expression showed enhanced lung colonization (4), and an anti-CD44 antibody inhibited melanoma metastasis (13). Furthermore, CD44-HA interaction stimulated subcutaneous (s.c.) growth of a melanoma cell line (3).

In non-Hodgkin lymphomas, high CD44 levels correlate with tumor aggressiveness and extensive tumor spread (17, 25). More direct evidence for CD44 involvement was provided by Sy et al. (34), who transfected the standard form of CD44 into Namalwa lymphoma cells, which grew very poorly when injected subcutaneously. The transfectants did grow well and metastasized. This enhanced tumorigenicity was suppressed by a CD44–immunoglobulin fusion protein (35).

A role for CD44 in invasion of tissues would be consistent with two observations on normal lymphocytes. First, CD44 on lymphocytes is required for optimal contact allergic responses, as shown by surface modulation of CD44 before adoptive transfer (5). This suggested that CD44 is essential for optimal migration of lymphocytes into the skin, independent of the other adhesion molecules expressed,
possibly because CD44 can mediate motility on hyaluronic acid (39), a major component of the dermis (19). Second, among cytotoxic T cell clones directed against the malaria parasite *Plasmodium yoelii*, which were all highly active in vitro, only cells expressing high CD44 levels were effective in vivo. Moreover, CD44-negative cells sorted from a protective clone lost in vivo activity, and this was independent of the expression of other relevant adhesion molecules, in particular leukocyte function-associated antigen 1 (LFA-1) and α2β1 (28). Since this in vivo activity involves lysis of infected hepatocytes and thus requires migration into the liver parenchyma, this strongly suggested that CD44 is required for migration. This requirement could also explain the correlation between CD44 and spread of hematopoietic tumors, in particular to the liver.

To further investigate the mechanisms of CD44 function in s.c. tumor growth and metastasis, we have generated a CD44-negative mutant of the highly metastatic murine MDAY-D2 lymphosarcoma cell line. MDAY-D2 was originally described as a sarcoma (18), but the cells express the integrin α2β1 (LFA-1) and are therefore of hematopoietic origin. MDAY-D2 cells express high levels of CD44 and bind to immobilized HA in vitro. To generate the mutant, we inactivated both alleles of the CD44 gene by homologous recombination. We show here that CD44-negative MDAY-D2 double knockout (DKO) cells readily form s.c. tumors and, upon i.v. injection, metastasize extensively to the liver, spleen, and bone marrow, comparable to the parental MDAY-D2 cells. Hence, for this cell line, CD44 is dispensable for metastasis and s.c. growth.

**Materials and Methods**

**Cell Lines and Culture Conditions**

Parental MDAY-D2 (18) and variant cells were cultured in enriched RPMI 1640 medium (Hybridoma medium [29]).

**Antibodies**

Antibodies were obtained from the following sources: anti-CD44 mAb KM201 from Dr. P. Kincade; α2 integrin (CD49d) mAb GoH3 from Dr. A. Sonnenberg; hybridomas producing α2-integrin (CD49d) mAb FS2 and α1-integrin (CD11a) mAb M174 from the American Type Culture Collection, Rockville, MD; the α2-integrin (CD49b) mAb MF53 from Pharmingen, San Diego, CA; and mouse anti-rat IgG mAb conjugated to FITC from Zymed Laboratories, South San Francisco, CA.

**Generation of Targeting Constructs**

A genomic library of the murine DBA/2-derived ESB cell line was constructed from 18-23-kb fragments obtained by fractionation of partial Sau3A-digested genomic DNA on 10–40% sucrose gradients. This DNA was ligated to BamHI-digested EMBL-3 bacteriophage half-site arms (Promega Biotec, Madison, WI), and after packaging, 106 independent plaques were plated on Escherichia coli strain KW251 (Promega Biotec). CD44 genomic clones were identified using a murine CD44 cDNA probe (kindly provided by Dr. I. Hart, ICRE, London, UK) and were mapped with partial CD44 cDNA probes to determine the location of exon 1.

To generate the targeting construct, we used a 7-kb Sall-SacI fragment, cloned into pBluescript KS− (Stratagene, La Jolla, CA). Into the unique Ncol site, at the transcription start site in exon 1, we introduced a pgk-1 neo cassette that had been digested with EcoRI and Sall and to which a Ncol-EcoRI adaptor (upper strand: 5'CATGGGCAACGAAGGTG3' and lower strand: 5'AAATCCACCTTCGTGCC3') and an XhoI-Ncol adaptor (upper strand: 5'TCGAGCGGTACCGGATCC3', lower strand: 5'AATTCACCTTCGTGCC3') had been ligated. The 3' XhoI-Ncol adaptor contains a BamHI site (see Fig. 1 A). The hygromycin B resistance gene-containing vector was constructed by digestion of the pBluescript/CD44.neo targeting vector with Sphi (site in the pgk-1 promoter) and BglII (site downstream of the polyA signal), removal of the pgk-1 neo insert and ligation of an Sphi-BglII-digested pgk-1 hygromycin fragment (38). Targeting constructs, digested with Sall and SaeI, were separated from vector sequences by gel electrophoresis and purified by electrolution.

**Immunofluorescence Analysis**

Antibody incubations and washing steps were performed at 4°C in PBS containing 0.5% BSA, 0.02% Na-azide, 1 mM Mg2+, and 1 mM Ca2+.

**Generation of Knockout Cells**

2 × 106 MDAY-D2 cells in 800 μl RPMI medium were electroporated in the presence of 100 μg of targeting construct, using a gene pulser (Bio-Rad Laboratories, Richmond, CA) (0.42 kV, 960 μF). Cells were seeded in 48-well tissue culture dishes at 5 × 104, 1 × 104, and 2 × 103 cells/well in medium with 20% FCS. Drug selection (1 mg/ml G418; Gibco, Paisley, UK) or 1.2 mg/ml hygromycin B (Calbiochem-Novabiochem, Corp., La Jolla, CA) was started after 1 d. CD44-negative cells were sorted using a FACScan®.

**DNA and RNA Analysis**

Genomic DNA (15 μg) was digested with BamHI and loaded on a 0.7% agarose gel for Southern analysis. RNA was isolated with the Ultraspec kit according to the manufacturer’s protocol (BIOTEX, Houston, TX). 20 μg was loaded on a 1.0% agarose formaldehyde gel. After electrophoresis, material was transferred to Nytran 13N membrane (Schleicher and Schuell, Inc., Dassel, Germany) and hybridized using standard techniques.

**Adhesion to Hyaluronic Acid**

MDAY-D2 cells were seeded in 96-well U-bottom plates (model 650101; Greiner, Frickenhausen, Germany) with 100 μl HA (1 mg/ml Sigma Chemical Co., St. Louis, MO) in PBS overnight at 4°C. Plates were washed three times before the addition of cells. Cells were labeled with 100 μCi 51Cr (American International, Little Chalfont, UK) per 10 × 104 cells in 250 μl culture medium for 45 min. To test inhibition of adhesion by the KM201 mAb, cells were preincubated with 10 μg/ml mAb for 15 min at room temperature. Each well contained 5 × 104 cells in a final volume of 100 μl RPMI. After incubation for 30 min at 5% CO2 and 37°C, nonadherent cells were removed by washing three times with 100 μl PBS containing 1 mM Ca2+ and 1 mM Mg2+ and inverting the plate. The percentage of bound cells was determined by lysing the cells with 1 N NaOH and counting the radioactivity. Percentages were corrected for spontaneous release of 51Cr and background binding to wells. Results are the mean of triplicate wells.

**Subcutaneous Growth and Experimental Metastasis**

DBA/2 mice were injected subcutaneously in the flank with 105 cells in 200 μl PBS with 1 mM Mg2+ and 1 mM Ca2+. s.c. tumor growth was monitored once a week. Animals were killed when the tumor was larger than 2 cm3 or when ulceration of the tumor occurred. Metastatic capacity was assessed by injecting 104 cells in 200 μl PBS with 1 mM Mg2+ and 1 mM Ca2+ into the tail vein of syngeneic DBA/2 mice.

**Histology**

Tissues were fixed in ethanol-acetic acid-formol-saline fixative, embedded in paraffin, and 5-μm sections were mounted onto slides and stained with hematoxylin and eosin according to standard procedures.

**Results**

Our aim was to study the role of the CD44 protein in me-
tastasis of the MDAY-D2 lymphosarcoma cell line. Targeted disruption of genes in embryonal stem cells for the generation of knockout mice has rapidly become a routine procedure. For other cell types, this is more difficult. However, since others have shown that this is feasible (6, 9, 15), we attempted to use this approach to generate well-defined CD44-negative mutants of the MDAY-D2 cells. To increase the frequency of homologous recombination, we used isogenic DNA (37).

The targeting constructs were generated from a 7-kb SalI-SacI fragment of the CD44 gene, isolated from a DBA/2-derived genomic library. Either the neomycin-resistance or the hygromycin-resistance (38) gene, under control of the pgk-1 promoter (1), was inserted into the translation initiation site in exon 1 (Fig. 1A). First, MDAY-D2 cells were transfected with the construct containing the neomycin cassette. Disruption of one CD44 allele was expected to result in a reduced CD44 surface level. Therefore, the 186 G418-resistant clones obtained were analyzed for CD44 expression by FACS® analysis; 24 clones with 20–45% decreased CD44 surface levels were examined by Southern analysis. Because of the introduced BamHI site in the construct (Fig. 1A), correct targeting should result in hybridization with a 4.0-kb BamHI restriction fragment, in addition to the 12.5-kb fragment of the wild-type allele. One of the clones showed this hallmark (Fig. 1B) and was termed single knockout (SKO). The CD44 surface level of SKO cells was 62% of that of parental MDAY-D2 cells (see Table I).

To disrupt the second CD44 allele, the SKO cells were transfected with the hygromycin-containing fragment, and CD44-negative cells were sorted from a bulk culture of transfected cells. Approximately 0.08% of the population was sorted and plated as single cells; 50% of these clones were CD44 negative, and Southern analysis revealed that 29 of the 30 CD44-negative clones were genuine DKO (Fig. 1B). In one of the CD44-negative clones, the second allele was not disrupted. Apparently, this was a spontaneous mutant (SM). Because the DKO cells were sorted from the bulk population, they cannot be considered independent clones. Therefore, the SM clone was used as a second independent CD44-negative cell line in some experiments.

Northern analysis (Fig. 1C) of MDAY-D2 cells revealed the 1.6-, 3.3-, and 4.0-kb mRNA bands of the standard CD44 transcript (21). RNA levels were reduced in the SKO cells, and in the DKO cells no CD44 mRNA was detected. Hybridization of this blot with a probe specific for variant exons 4–10 of CD44 (40) did not reveal any bands, even after prolonged exposure (not shown). Thus, MDAY-D2 cells express only the standard CD44 isoform.

**Proliferation and Integrin Expression**

Proliferation rates of the cell lines in vitro were found to be identical. Also, the expression levels of integrins that are potentially relevant for metastasis were determined. Fibronectin receptors have been implicated in metastasis of cells to the liver (33), the α6β1 integrin may be involved in melanoma metastasis to the lungs (30), and we have found that for T cell hybrids, the α1β2 integrin (LFA-1) is required for metastasis (29). MDAY-D2 cells express the fibronectin receptors α5β1 and αvβ3, and in addition the integrins α6β1 and α4β1 (LFA-1) (Table I). MDAY-D2, SKO, DKO, and DKO cells were analyzed for CD44 expression by FACS® analysis; 24 clones with 20–45% decreased CD44 surface levels were examined by Southern analysis. Because of the introduced BamHI site in the construct (Fig. 1A), correct targeting should result in hybridization with a 4.0-kb BamHI restriction fragment, in addition to the 12.5-kb fragment of the wild-type allele. One of the clones showed this hallmark (Fig. 1B) and was termed single knockout (SKO). The CD44 surface level of SKO cells was 62% of that of parental MDAY-D2 cells (see Table I).

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**Figure 1.** Constructs used for CD44 targeting, and results of Southern and Northern analysis of MDAY-D2 parental, SKO, and DKO cells. (A) CD44 targeting constructs. CD44 locus around exon 1. The neo and hygromycin cassettes were inserted into the NcoI site, located at the translation initiation start site in exon 1. The probe used to detect homologous recombination at this locus is the indicated 0.8-kb NcoI fragment. (B) Southern analysis of genomic DNA of MDAY-D2, SKO, and DKO cells, digested with BamHI and hybridized with the 0.8-kb NeoI probe. In the parental cells, a 12.5-kb BamHI fragment is detected. In the correctly targeted gene, an additional BamHI site is present in the 3′ adaptor, used to ligate the pgk-1 neo cassette into the NcoI site so that a 4-kb fragment is detected. (C) Northern analysis of genomic DNA of MDAY-D2, SKO, and DKO cells, digested with BamHI and hybridized with the 0.8-kb NeoI probe. In the parental cells, a 12.5-kb BamHI fragment is detected. In the correctly targeted gene, an additional BamHI site is present in the 3′ adaptor, used to ligate the pgk-1 neo cassette into the NcoI site so that a 4-kb fragment is detected. (C) Northern analysis of MDAY-D2, SKO, and DKO cell lines, using the 1.4-kb standard CD44 cDNA probe. CD44 mRNAs of 4.0, 3.3, and 1.6 kb were observed in MDAY-D2 and SKO cells, but not in the DKO cells. GAPDH hybridization is shown as a measure for the amount of RNA in the samples.
Adhesion to Immobilized Hyaluronic Acid

Lesley et al. (20) have shown that CD44 can exist in three functional states with respect to HA binding: nonactivatable, activatable (by, e.g., the CD44 mAb IRAWB [20]), and constitutively active. As shown in Fig. 2, 38% of MDAY-D2 cells bound to immobilized HA without activation. Therefore, CD44 is in the active conformation. Binding of MDAY-D2 cells to HA was blocked completely by the KM201 antibody against CD44 (24), and thus CD44 is the only HA receptor present on MDAY-D2 cells. Indeed, the DKO cells did not bind HA at all (Fig. 2). The SKO cells bound almost as well as parental MDAY-D2 cells (34%), despite the lower CD44 surface level (Fig. 2).

Subcutaneous Tumor Take and Growth

Sy et al. (34, 35) and Bartolazzi et al. (3) showed that transfection of CD44 into certain lymphomas and melanomas greatly increased s.c. tumor take and growth. To test the role of CD44 for MDAY-D2 cells, we injected the parental and mutant cells s.c. into syngeneic mice at the lowest dose of MDAY-D2 cells that reproducibly gave rise to metastasis in almost 100% of the mice. The results of the first experiment are summarized in Table II. Extensive metastasis to liver and spleen was seen in almost all mice, including those injected with the DKO cells: seven out of seven for MDAY-D2 cells, six out of seven for SKO, seven out of nine for DKO, seven out of nine for SM, and six out of nine for DKO cells. We conclude that s.c. growth of the CD44 negative DKO and SM cells did not differ from that of the SKO or MDAY-D2 cells.

Experimental Metastasis

Metastatic potential was assessed by injecting 10⁶ cells into the tail vein of syngeneic mice. This was the lowest dose of MDAY-D2 cells that reproducibly gave rise to metastasis in almost 100% of the mice. The results of the first experiment are summarized in Table II. Extensive metastasis to liver and spleen was seen in almost all mice, including those injected with the DKO cells: seven out of seven for MDAY-D2 cells, six out of seven for SKO, and seven out of eight for both SKO and DKO cells. The animals injected with MDAY-D2 cells showed symptoms of illness between days 13 and 17 after injection, with SKO or DKO cells after 15–22 d, so no difference was seen in survival between the CD44-positive SKO and CD44-negative DKO cells.

The distribution of metastases was assessed by histology in a second experiment in animals killed after a fixed period of 15–16 d. Lungs, thymus, lymph nodes, liver, spleen, bone marrow, kidneys, intestines, brain, and blood were examined macroscopically and by histology. Many MDAY-D2 cells were found circulating in the blood and therefore in blood vessels of lungs, meninges, and kidneys, but not in extravascular locations. However, in the spleen, tumor cells were located extravascularly in the red pulpa areas, and only when the metastases grew bigger, the cells invaded the white pulpa area (not shown). In the liver, diffuse and nodular infiltrates were observed, as shown in Fig. 3. In the bone marrow, tumor cells sometimes totally replaced the normal cell population (Fig. 3). The tissue distribution and invasion patterns of the DKO cells did not differ at all from SKO and MDAY-D2 cells. Therefore, we conclude that CD44 is dispensable for both metastasis formation and s.c. growth of MDAY-D2 lymphosarcoma cells.

Discussion

The most direct approach to elucidate the role of a specific protein in a complicated in vivo phenomenon is the study of mutants that no longer express this protein. Previously, we have thus provided evidence for a decisive role of LFA-1 in metastasis of a T cell hybridoma, by showing...
that LFA-1–deficient mutants hardly metastasized (29). However, these mutants were generated by chemical mutagenesis, and therefore it cannot be excluded that alterations in expression of other genes have contributed to the phenotype. A better approach is the targeted disruption of the genes of interest. However, this was generally considered unfeasible for mammalian cells other than embryonal stem cells because of the low frequency of homologous recombination. Recently, however, the generation of targeted mutants of different cell types has been reported (6, 9, 15). Our present results confirm this and show that targeted disruption of genes is a viable and potentially very powerful approach to elucidate mechanisms of metastasis, at least of diploid tumor cell lines.

We anticipated a strongly reduced s.c. growth potential and metastatic capacity of the CD44-negative MDAY-D2-DKO cells. This was based on the documented role of CD44 in in vitro invasion (8, 23), the correlation between CD44 levels and tumor spread (4, 17, 25), as well as on direct evidence from in vivo studies with anti-CD44 mAb and CD44-transfectants (3, 13, 34, 35). These results all pertained to the standard form of CD44 in lymphomas and

Figure 3. Invasive capacity of CD44-negative MDAY-D2 DKO cells. (A) Invasion of cells from the subcutaneously implanted tumor into muscular (M) and fat (F) tissue. Bar, 40 μm. (B) Higher magnification; bar, 20 μm; isolated tumor cells have invaded between the muscle fibers (M). (C) i.v. injected tumor cells (T) have invaded the liver parenchyma (P). Tumor cells are also present in the larger vessel. Bar, 40 μm. (D) Tumor nodule in sternal bone marrow. B, bone. Bar, 40 μm.
MDAY-D2-DKO mutant cells grew readily in a s.c. environment and not slower than the parental cells. Particularly striking was the extensive local invasion of the DKO cells from the tumor site, through the surrounding tissues, and into the thorax or abdomen, similar to parental cells. This involved, e.g., extensive migration between HA-rich muscle tissue (2,19), as shown in Fig. 3. The distribution of metastases after i.v. injection, and the extent and pattern of invasion into the affected tissues (i.e., liver, spleen, and bone marrow) were also not different. The observation on spleen metastasis is consistent with a recent report by Zahalka et al. (43) that CD44 mAbs did not affect spleen metastasis of a lymphoma, whereas they did affect metastasis to lymph nodes, which are not affected by our MDAY-D2 cells.

The results cannot be explained by redundancy, at least with respect to HA binding. Motility of several cell types on HA has in fact been ascribed to a different HA receptor: RHAMM (14), which has also been found on blood cells (27). However, adhesion of MDAY-D2 cells to HA was completely blocked by anti-CD44 mAb, and indeed with respect to HA binding. Motility of several cell types involved, e.g., extensive migration between HA-rich muscle tissue, into the thorax or abdomen, similar to parental cells. This is not essential for invasion of lymphocytes into the skin, but rather for the local proliferation and activation of these cells.

Table II. Metastatic Potential of MDAY-D2, SKO, and DKO Cells

<table>
<thead>
<tr>
<th>Group</th>
<th>MDAY-D2 (n)</th>
<th>SKO (n)</th>
<th>DKO (n)</th>
</tr>
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<tbody>
<tr>
<td>Liver</td>
<td>7/7</td>
<td>7/8</td>
<td>7/8</td>
</tr>
<tr>
<td>Spleen</td>
<td>7/7</td>
<td>7/8</td>
<td>7/8</td>
</tr>
<tr>
<td>Ill on day</td>
<td>13-17</td>
<td>15-22</td>
<td>16-22</td>
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5-md-old BDA/2 mice were injected i.v. with 10^8 cells, and autopsy was performed when animals became ill after the indicated time period. n, number of animals with metastases in the liver and the spleen detected upon gross examination.

pressed by MDAY-D2 cells (not shown) and was recently described to act as HA receptor in the liver (22).

A possible explanation for the discrepancy between our and previous results may be based on the function of CD44 as a transmitter of extracellular signals rather than on its presumed role in invasion. Anti-CD44 mAbs can act as costimulators for T cell activation (7,16,26,32) and can induce natural killer cell (36) activity. This probably mimics signals triggered by the CD44 ligand HA, since both anti-CD44 mAbs and HA itself can induce cytotoxic T lymphocyte activity through CD44 (10,11). It is therefore conceivable that, e.g., in contact allergic responses (5), CD44 is not essential for invasion of lymphocytes into the skin, but rather for the local proliferation and activation of these cells.

If this hypothesis is correct, the CD44 dependence of growth of certain tumors may also be explained, if these tumors still require extracellular signals for proliferation. Thus, e.g., the Namalwa human lymphoma cell line may require signals induced by CD44-HA interaction in the HA-rich s.c. environment for rapid growth (34,35). The highly malignant MDAY-D2 cells may be growth autonomous in this respect and not require such signals. Also, the established correlation between CD44 expression and spread of lymphomas may be explained if some lymphomas require CD44-transmitted signals for proliferation at sites other than lymphoid tissues. Thus, our results suggest that not all highly malignant lymphomas require CD44 to grow and spread.

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