Absence of the CD44 Gene Prevents Sarcoma Metastasis

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ABSTRACT

To test the role of the CD44 gene in tumorigenesis, mice with the min mutation of the APC gene or with the tm1 mutation of the p53 gene were crossed with CD44 knockout mice. The absence of CD44 gene products did not affect tumor incidence or survival; however, mice with disruption of the CD44 gene showed virtually aborted metastasis formation of osteosarcomas. This is in agreement with the role attributed to CD44 variants in the spread of cancer. Therefore, CD44 gene products are not essential for tumor incidence and growth but are important in regulating metastasis formation.

INTRODUCTION

The transmembrane glycoprotein CD44 is expressed on lymphocytes and macrophages. It serves as a homing receptor that mediates binding to high endothelial venules and has also been implicated in lymphoid development. Lymphocyte activation results in the expression of multiple, alternatively spliced products of the CD44 gene, which are generated by the insertion of ≤10 variant exons into the extracellular domain. In pathophysiology, aberrant expression of certain CD44 splice variants has been connected causally to the spread of diverse malignant cells (1, 2) and may distinguish metastasizing from nonmetastasizing tumors. This function is mediated by the cytokine ligand osteopontin (3). Additional roles in carcinogenesis have been attributed to various forms of CD44. Expression of this receptor on tumor cells may support tumor growth (4, 5), possibly after adhesion to hyaluronate, and signal transduction through CD44 can induce oncogenes, such as ras (6). In contrast, the standard form that lacks variant exons may exert suppression of tumor growth and dissemination (7). The contributions of these diverse CD44 functions to carcinogenesis are not fully elucidated.

Despite ample experimental evidence for a role of some forms of CD44 in malignancy, clinical studies relating expression of CD44 or its variants to prognosis in diverse cancers have remained controversial (8). Nonconclusive results in patients may have been obtained because of insufficient sensitivity or specificity of the analyses. Thus, the measurements of mRNA for specific CD44 variants expressed by tumors do not detect posttranslational modifications that may affect function (9). Furthermore, the CD44 receptor expressed on tumors represents one component of a functional pair. Specific ligands may bind to selective splice variants, so that the availability of these ligands contributes to determining the phenotype. Similarly, many conventional rodent models of malignancies are compromised because they rely on the injection of preformed tumor cells, often in nonphysiological locations, rather than on the generation of endogenous tumors. We set out to analyze the role of CD44 in a genetically defined and homogeneous system, which most closely resembles the pathophysiology of human cancers. Mouse models, where the role of individual genes is tested by breeding the relevant gene-targeted mice with mice that have high susceptibility to tumors because of mutations in tumor suppressor genes, have provided substantial insights. We used two endogenous tumor models using mice with point mutations in tumor suppressor genes with or without targeted deletion of the CD44 gene: Mice with the APC+/−/min genotype display a predisposition to multiple intestinal neoplasia. The murine min mutation is a nonsense mutation, which is analogous to mutations found in human autosomal dominantly inherited familial adenomatous polyposis, as well as in sporadic colorectal cancers (10). APC+/−/min mice develop multiple benign intestinal tumors, whose growth reflects early steps of transformation. Mutations of the p53 gene contribute to the pathogenesis of a large percentage of human cancers. Similarly, mice with one mutant allele of the p53 gene are susceptible to a larger spectrum of tumors, predominantly sarcomas and lymphomas. These mice allow the investigation of malignant dissemination.

MATERIALS AND METHODS

Mice. Mice with point mutations in tumor suppressor genes, APC+/−/min bred on C57Bl/6 background or trp53+/−/min on C57Bl/6 background, were obtained from The Jackson Laboratory. Either APC+/−/min mice or trp53+/−/min mice were mated with CD44+/− mice that had been back-crossed from 129 to C57BL/6 for four generations (10). The genotypes were assessed using PCR from genomic DNA (10–12), and CD44 expression was confirmed by flow cytometry from blood samples using the pan-CD44 antibody IM7 (PharMingen). Siblings were housed in groups of one to four per cage at the Redstone Animal Facility (DFCI) in alternate 12-h light and dark cycles. A diet of pelleted chow (Agway, Prolab 3000) and bottled water was administered ad libitum, and room temperature was kept at 25°C. The colony was tested frequently for endoparasitic and ectoparasitic infections, as well as for bacterial and viral infections by the Charles River Labs (Wilmington, MA). No infection was detected during the course of this study. Permission to exceed a tumor diameter of 2 cm was granted by the institutional animal care and use committee, and the mice were seen frequently by a veterinarian.

Inheritance. Mice with disrupted CD44 genes were mated with heterozygotes for point mutations of the relevant tumor suppressor gene, yielding mice that were hemizygous for CD44 and either wild type or heterozygous for the tumor suppressor gene. Those two genotypes were interbred, which is expected to result in equal 12.5% representation of the genotypes of interest according to Mendelian inheritance (the remaining 2 × 25% are CD44+/−). The litters from this second generation mating were screened. In the p53-related part of the study, 292 mice were analyzed, of which 16 were trp53+/−/CD44+/−, 36 mice were trp53+/−/CD44−/−, 24 mice had the genotype trp53+/−/CD44+/−, and 26 mice had the genotype trp53−/−/CD44−/−. In the APC-related part of the study, 217 mice were screened with the distribution of APC+/−/CD44+/− 21 mice, APC+/−/CD44−/− 20 mice, APC−/−/CD44+/− 10 mice, and APC−/−/CD44−/− 15 mice.

Because of the incomplete back-crossing from 129 to C57Bl/6, it is formally possible that a polymorphic modifier, linked to the CD44 locus, segregates with it and supersedes the influence of CD44 on tumor development. The tumor susceptibility locus Scc1 might be a candidate (13). This is unlikely, because quantitative trait loci, including Scc-1, depend strongly on interlocus interactions.
interactions for influencing tumorigenesis (14). Cosegregation of one modifier could not affect the phenotype. It is generally improbable that genomic heterogeneity would account for the observations described here, because 97% of the genome are derived from the C57Bl/6 strain.

**Necropsy.** The animals were checked at least every 12 h, and total necropsy was performed on mice found moribund. Organs were fixed in formalin, and H&E slides were prepared for histological analysis. Histological evaluation was performed by an investigator blinded to the CD44 status of the samples.

Osteosarcoma metastases were observed in livers, lungs, and occasionally spleens. Enumeration of osteosarcoma metastases was performed on step sections from livers and lungs. For this purpose, every 10th microtome cut corresponding to a step thickness of 60 μm was analyzed.

**Immunohistochemistry.** CD44 expression on osteosarcomas was assessed by immunohistochemistry with antibody KM114 after antigen retrieval with citrate. Staining was done with 3,3′-diaminobenzidine. Staining of all tumor samples with secondary antibody served as negative control, and a normal mouse spleen was used as a positive control (data not shown).

**Enumeration of Intestinal Polyps.** Entire intestines from stomach to rectum were extracted, washed in PBS, and fixed in 10% buffered formalin, and the number of polyps was counted under a dissection microscope. As controls, intestines from 3 APC−/−CD44+/− mice at the ages of 420–442 days and 3 APC+/−CD44−/− mice at the ages of 433–442 days were examined for spontaneous polyps.

**Statistical Evaluation.** The data sets were analyzed for statistically significant differences at 95% confidence by t test (after confirmation of normal distribution and equal variance) and by Wilcoxon Mann-Whitney test (after testing for equal distribution). The prerequisites for applicability of either test were not fulfilled for the metastasis data. They were analyzed for equal variance by the Cochran test.

**RESULTS**

**Absence of CD44 Prevents Tumor Metastasis.** Because aberrant expression of CD44 splice variants may confer a malignant phenotype to tumor cells, we asked whether the targeted deletion of the CD44 gene was sufficient to suppress the dissemination of solid tumors. Osteosarcomas developed mostly on the lower back. One trp53+/tm1CD44−/− mouse had an osteosarcoma of the skull. Metastases were detected in the lungs and livers from trp53+/tm1 mice with osteosarcoma. Step sections from livers and lungs identified 28 metastases in 6 CD44+/− mice and 1 metastasis in 4 CD44−/− mice (Fig. 1). One CD44+/− mouse also displayed a macroscopically visible metastasis in the spleen. All 6 CD44+/− mice had multiple osteosarcoma metastases, whereas in 4 CD44−/− mice, only one individual lung metastasis was detected. Consistently, CD44 expression was prominent in the osteosarcomas of CD44+/− mice (Fig. 2).

**Absence of CD44 Does Not Alter the Phenotype of Benign Tumors.** The intestinal polyps caused by mutation in the APC gene grow noninvasively but express various splice variants of CD44. This occurs at the earliest stages of transformation diagnosed as aberrant crypt foci with dysplasia (15). Histology was performed on the largest intestinal polyps from each APC+/−/min mouse to assess malignancy. Consistent with previous reports, these tumors are noninvasive as judged by intact basement membranes in all cases (Fig. 3). No metastases were observed in other organs (three histological sections per organ). These results were not affected by the presence or absence of CD44. Histological findings included ectopic hepatic hematopoiesis and bone marrow...
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Fig. 2. Expression of CD44. A and B, immunohistochemistry for CD44 expression in osteosarcomas from a trp53^{+/H11001} CD44^{+/+} (A) and a trp53^{+/H11001} CD44^{--/} mouse (B). In C and D, typing of the mice was performed by flow cytometry with FITC-anti-CD44 on spleen cells and lymph node cells (data not shown) and by PCR on genomic DNA with published primers (11); C, CD44^{+/+}; D, CD44^{--/}.

CD44 Does Not Affect Tumor Incidence. We tested whether deletion of the CD44 gene alters tumor incidence as judged by the number of intestinal polyps in mice with one mutated APC allele. All APC^{+/min} mice succumbed to intestinal polyposis. At the time of death, APC^{+/min} mice had developed around a mean of 66 polyps in CD44^{+/+} background and 58 polyps in CD44^{--/} background (Fig. 4), suggesting that the tumor development in this model does not depend on the presence of CD44 gene products.

Mice with the trp53^{+/H11001} genotype developed, predominantly, sarcomas and lymphomas. The mesenchymal tumors were diagnosed as fibrosarcomas, osteosarcomas, hemangiosarcomas, and histiocytic sarcomas. Their incidence, associated life span, and tumor weight on death were not affected by the presence or absence of the CD44 gene (Table 1). Like the osteosarcomas, the fibrosarcomas were located mostly on the lower back.

siderosis in several mice, which are likely attributable to blood loss through the intestinal polyps.

Fig. 3. Intestinal polyps caused by the min mutation of the APC gene are not invasive. Histology of representative intestinal polyps from APC^{+/min} mice. The largest polyp from each intestine was sectioned in the middle and stained with H&E for histological assessment of signs for malignancy. Regardless of the presence of the CD44 gene, the basement membrane remains intact (arrows). A, CD44^{+/+}; B, CD44^{--/}.

Fig. 4. Tumor incidence. The numbers of intestinal polyps were counted in APC^{+/min} mice at the end of their life span (average 209 days for 10 APC^{+/min}CD44^{+/+} mice and 236 days for 15 APC^{+/min}CD44^{--/} mice). The incidence of spontaneous polyps was assessed at an average of 433 days for 3 APC^{+/+}CD44^{+/+} mice and 437 days for 3 APC^{+/+}CD44^{--/} mice. Symbols, individual data points; mean values are presented as horizontal lines.
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Table 1 Characterization of solid tumors in trp53+/−tm1 mice. Incidence, associated life span, and tumor weight at the time of death are specified for each histologic type of sarcoma for CD44+/+ and CD44−/− genetic background. Life span and tumor weight are indicated as mean ± standard error.

<table>
<thead>
<tr>
<th>Tumor</th>
<th>Incidence</th>
<th>Life span</th>
<th>Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Osteosarcoma</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>trp53+/−tm1CD44+/+</td>
<td>6 (25%)</td>
<td>532 ± 30 days</td>
<td>7.7 ± 2.4 grams</td>
</tr>
<tr>
<td>trp53+/−tm1CD44−/−</td>
<td>4 (15%)</td>
<td>467 ± 56 days</td>
<td>6.1 ± 1.7 grams</td>
</tr>
<tr>
<td>Fibrosarcoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trp53+/−tm1CD44+/+</td>
<td>7 (29%)</td>
<td>410 ± 23 days</td>
<td>21.9 ± 5.0 grams</td>
</tr>
<tr>
<td>trp53+/−tm1CD44−/−</td>
<td>7 (27%)</td>
<td>403 ± 30 days</td>
<td>12.3 ± 2.9 grams</td>
</tr>
<tr>
<td>Hemangiosarcoma</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>trp53+/−tm1CD44+/+</td>
<td>3 (12%)</td>
<td>304 ± 51 days</td>
<td>10.7 ± 8.1 grams</td>
</tr>
<tr>
<td>trp53+/−tm1CD44−/−</td>
<td>0 (0%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Histiocytic sarcoma</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>trp53+/−tm1CD44+/+</td>
<td>1 (3%)</td>
<td>595 days</td>
<td>2.9 grams</td>
</tr>
<tr>
<td>trp53+/−tm1CD44−/−</td>
<td>2 (8%)</td>
<td>589/590 days</td>
<td></td>
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<tr>
<td>trp53+/−tm1CD44+/+</td>
<td>1 (4%)</td>
<td>420 days</td>
<td></td>
</tr>
<tr>
<td>trp53+/−tm1CD44−/−</td>
<td>1 (6%)</td>
<td>600 days</td>
<td></td>
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</table>

*S f = female; m = male.

Sporadic carcinomas also occurred in trp53+/−tm1 mice independently of their CD44 status with one case of squamous cell carcinoma in a trp53+/−tm1CD44+/+ mouse and one incident of lung carcinoma among the trp53+/−tm1CD44−/− mice (Fig. 5).

There were four cases (17%) of lymphoma, typical of those observed in p53−/−tm1 mice (12, 16), in trp53+/−tm1CD44+/+ mice with an associated mean life span of 445 days. In comparison, there were six cases (23%) of lymphomas, resembling anaplastic large cell lymphoma (17), in trp53+/−tm1CD44−/− mice with an associated life span of 503 days (data not shown). The morphology of the lymphoid malignancies in CD44−/− mice appeared unusual but requires further characterization.

Five mice with one mutant p53 allele had multiple tumors. In trp53+/−tm1CD44+/+ mice, one osteosarcoma occurred together with a histiocytic sarcoma. Frequently, lymphomas were diagnosed in conjunction with solid tumors. One trp53+/−tm1CD44+/+ mouse had lymphoma and osteosarcoma. In trp53+/−tm1CD44−/− mice, lymphoma was seen in conjunction with osteosarcoma, fibrosarcoma, and histiocytic sarcoma in one case each.

**CD44 Does Not Affect Survival.** The mice with one mutant allele of the APC gene developed symptoms of ruffled fur, bloated abdomen, and black stools followed by lethargy and succumbed around a mean of 209 days (range 133–350 days) of age, for CD44+/+ background, or 236 days (range 96–326 days) of age, for CD44−/− background (Fig. 6A). At the end of their life span, 2 of the CD44−/− mice also suffered from rectal prolapse. None of the APC+/+ mice died during the 420-day period of observation, regardless of their CD44 status.

Mice with one mutant p53 allele developed various tumors with a predominance of sarcomas and lymphomas and had a 50% survival of ~470 days of age regardless of their CD44 genotype (Fig. 6B). The period of observation was limited to 600 days, at which point 1 of 24 trp53+/−tm1CD44+/+ mice (4%) was still alive and 6 of 26 trp53+/−tm1CD44−/− mice (23%) survived. In the control groups, 14 of 16 trp53+/−tm1CD44+/+ mice (87%) and 30 of 36 trp53+/−tm1CD44−/− mice (83%) were alive. Conversely, 1 trp53+/−tm1CD44+/+ mouse (4%), 3 trp53+/−tm1CD44−/− mice (11%), 1 trp53+/−tm1CD44+/+ mouse (6%), and 5 trp53+/−tm1CD44−/− mice (14%) died without detectable signs of malignancies. Although five of them were diagnosed with histological signs of inflammation (abscess, periarteritis nodosa, glomerulonephritis, dermatitis, and pneumonia), the contribution of these conditions to the death of the mice is unknown. The higher incidence of deaths unrelated to neoplasms (8 CD44−/− mice of 50 when disregarding the trp53 status, compared with 2 CD44+/+ mice of 40) implies that the lack of the CD44 gene may increase the susceptibility to other pathogenic influences.

**DISCUSSION**

Diverse roles in cancer have been ascribed to various CD44 gene products, but their contributions to endogenous tumors have not been studied. Here, we have tested the consequences of targeted deletion of the CD44 gene in the development of endogenous tumors caused by mutations in two distinct tumor suppressor genes, which are also mutated frequently in human cancers. We show that the absence of the
CD44 gene products virtually abrogate osteosarcoma metastasis. In contrast, we did not find evidence for a role of CD44 in tumor growth or survival.

In various cancers, expression of CD44 splice variants is necessary and sufficient to cause metastasis formation (1, 2). In the present study, the role of CD44 in malignancy of nonhematopoietic origin was limited to inducing dissemination (Fig. 1) and corroborating the role of CD44 as a metastasis gene in solid tumors. The expression of CD44 is sufficient to confer metastatic properties to primary osteosarcoma cells, but the expression of multiple CD44 variants on intestinal adenomatous polyps (15) is not associated with invasive growth. This may be accounted for by the dominance of metastasis suppressor gene products over gene products that induce dissemination (18). Intestinal cells frequently express the adhesion molecule DCC, which may prevent CD44-mediated invasion. Alternatively, metastasis formation by the intestinal polyps may be suppressed by elevated β-catenin, secondary to loss of APC protein function. This decreases enterocyte crypt villus migration (19) and may prevent invasive behavior. Osteosarcoma cells, in contrast, do not display any prominent expression of metastasis suppressor genes so that the expression of CD44 variants is sufficient to cause a malignant phenotype.

Various genetic influences can affect tumor multiplicity in APC+/min mice. They include genes for cell cycle control, DNA repair, and metalloproteinases. The genetic modifier Mom1 encodes a secretory phospholipase, PLA2g2a, expressed throughout the intestinal tract. The active allele of PLA2g2a leads to a reduction in the growth rate and multiplicity of intestinal adenomas (20). APC+/min mice homozygous for a null allele of p53 developed significantly more intestinal adenomas than those homozygous for the wild-type allele of p53. Similarly, the intact DNA mismatch repair gene Pms2 reduces the number of intestinal tumors as compared with mice with a targeted deletion of this gene (21). In contrast, deletion of the gene for the metalloproteinase Matrilysin leads to substantial reduction in intestinal tumors, despite a lack of destruction of the basement membrane by these polyps (22). The intestinal polyps caused by the APC gene mutation express various splice variants of CD44 at the earliest stages of transformation, diagnosed as aberrant crypt foci with dysplasia (15); however, the contributions by CD44 gene products to the pathogenesis of the intestinal polyps were unknown. In this study, the numbers of polyps and associated life spans were not influenced by the absence of CD44 gene products. The size of the individual polyps did not appear to be compromised.

The expression of CD44 on tumors has been described to not only affect metastatic spread but also tumor growth (4, 5) and induction of oncogenes, such as ras (10). This opened the possibility that deletion of the CD44 gene might influence disease progression. In APC+/min mice, the incidence of polyps and associated life spans were, however, not altered. Similarly, incidence, survival, and tumor weight of sarcomas in trp53+/min mice were not influenced by the absence of CD44, arguing against a prominent role for CD44 in early transformation or tumor growth. In contrast, the dissemination of osteosarcomas was virtually abrogated by the absence of CD44 gene products (29 microscopically and macroscopically identified metastases in 6 CD44+/− mice, compared with 1 metastasis identified in 4 CD44+/− mice). We have found previously metastasis gene products to constitute a unique group of cancer-related biomolecules, which is distinct from growth controlling oncogene or tumor suppressor gene products. They are dysregulated in cancer at the levels of gene expression or mRNA splicing (18). The present results confirm the role of CD44 as a metastasis gene and refine our insights into the contributions of CD44 to cancer.

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REFERENCES


hematopoietic progenitor distribution, granuloma formation, and tumorigenicity.


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