CD44-Deficient Mice Develop Normally with Changes in Subpopulations and Recirculation of Lymphocyte Subsets

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CD44-Deficient Mice Develop Normally with Changes in Subpopulations and Recirculation of Lymphocyte Subsets

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Cell adhesion molecules are considered to be pivotal elements required for proper embryo development. The transmembrane glycoprotein CD44, which is expressed in numerous splice variants on the surface of many different cell types and tissues, has been suggested to be involved in several physiological processes such as cell-cell interactions, signal transduction, and lymphocyte homing and trafficking during embryogenesis and in the adult organism. Some splice variants are thought to play an important role in tumor progression. To investigate the physiological roles of CD44 in vivo, we abolished expression of all isoforms of CD44 in mice by targeted insertion of a lacZ/neo cassette into the reading frame of the leader peptide. CD44-deficient mice are viable without obvious developmental defects and show no overt abnormalities as adults. However, CD44-deficient lymphocytes exhibit impaired entry into the adult thymus, although lymphocyte development is apparently unaltered. Our data indicate that all splice variants of CD44 are dispensable for embryonic development and implicate a critical function for CD44 in lymphocyte recirculation. The Journal of Immunology, 1999, 163: 4917–4923.

Several physiological and pathological processes, such as cell migration, development of the immune system, embryogenesis, and tumor development depend on cell-cell and cell-matrix interactions. These interactions are mediated via cell surface receptors such as integrins, selectins, cadherins, members of the Ig superfamily, and the CD44 splice variants (1–3).

The CD44 family of transmembrane glycoproteins is encoded by a single gene consisting of 21 exons, of which 12 can be differentially spliced (4–6). At least 17 different CD44 proteins generated through this great variability in splicing can be identified, including the predominant isoform, sCD44, which is a product of standard exons 1–5, 16–18, and 20 (7, 8).

The region encoded by the variant exons is very hydrophilic and is associated with several potential protein modification sites. Through these sites, CD44 has been proposed to bind multiple ligands including collagens, fibronectin (9), heparin-binding growth factors (10), osteopontin (11), and chondroitin sulfate (12). The major physiological interaction partner of CD44, hyaluronic acid (HA)3 (13, 14), has been described to bind to regions within standard exons s2 and s5, which are suggested to form a common domain stabilized through disulfide bridges (15, 16). Hyaluronate is a major component of the extracellular matrix surrounding proliferating and migrating cells in embryonic tissues. In the earliest embryonic developmental stages, cell proliferation and hyaluronate synthesis have been shown to be simultaneous events (17).

CD44 as a mediator of HA uptake and degradation has been indicated by several lines of evidence (18). CD44 has been shown to be functionally significant in hematopoiesis, lymphocyte homing, and formation of tumor metastasis (19–23). However, its expression is strongly increased during differentiation of T cells to memory cells (24). In addition, CD44 expression is enhanced during proliferation and differentiation of B cells (25). Mice depleted of thymic cells by irradiation display CD44 expression on the bone marrow-derived cells repopulating the thymus, an effect that can be inhibited by anti-CD44 Abs, suggesting CD44 as an essential player in this repopulation process (26, 27).

Tumor cell and leukocyte migration are thought to utilize similar mechanisms in the detachment of the cells from their primary locus; their travel through the lymph or blood stream; and their attachment to, invasion into, and settlement at secondary organs (28). The CD44 rat isoform p-Meta1, containing v4–v7, is not detected in normal tissues but is involved in the metastatic spread of tumors (29). The isoform p-Meta1 is closely related to a CD44 variant containing v6, which is required for the lymphatic spread of tumor cells and is believed to be involved in the process of lymphocyte activation (30). This observation supports the paradigm that metastasizing tumor cells mimic lymphocyte behavior.

In addition to its various functions within the immune system, a role for CD44 has been implicated in development of the mouse (31), as well as in a wide range of adult tissues (32). A detailed study on CD44 expression during wild-type mouse embryogenesis has been performed (33, 34), demonstrating that during embryogenesis, CD44 is strongly expressed in the forming heart, somites, and condensing limb bud mesenchyme during morphogenesis. This suggests an essential role of CD44 in cellular interactions during early development and pattern formation, an observation that has been confirmed independently (35).

To study the biological role of CD44 in vivo, we generated mice lacking all isoforms of CD44 by an in-frame insertion of the lacZ/neo (β-galactosidase and neomycin resistance genes) reporter cassette into the CD44 leader peptide, thus abolishing CD44 expression. We analyzed the role of CD44 in development, differentiation, and pattern formation of specific cell types and tissues during embryogenesis, especially where, based on previous results,
lack of CD44 was likely to lead to irregularities. CD44 homozygous mutant mice are viable and fertile and do not exhibit any overt phenotype. Although the development of the lymphoid system is not affected in the absence of CD44, mutant lymphoid cells exhibit an impaired homing to the thymus and lymph nodes in the adult animals.

Materials and Methods

Generation of CD44 mutant mice

The targeting construct was generated as follows. A phage clone was isolated from a mouse genomic library (strain 129/Sv) containing 5′ sequences of the CD44 gene. A fragment including sequences of about 2 kb 5′ of the first standard exon to 5′, or the first intron was subcloned into the EcoRV and XbaI sites of pBlueScript II KS(+)(Stratagene, La Jolla, CA). The thymidine kinase gene of pMC1/TK (36) was inserted into the XbaI Site of the construct. A linker was introduced into the NcoI site connected with the CD44 start codon of exon s1, and another linker was introduced into the XbaI/SacI sites of pGNA (37); both linkers contained an SgrI restriction site. Using SgrI restriction, the lacZneo cassette of pGNA was inserted in frame directly 3′ of the ATG start codon of exon s1. After linearization with NotI, the targeting construct was electroporated into 1.2 × 10⁶ R1 embryonic stem cells (38).

Electroporation of the vector, embryonic stem cell culture, G418 and gancyclovir selection, injection, and transfer of blastocysts were performed as described previously (39). Using a 5′ flanking probe, an endogenous fragment of 15 kb and a recombinant 5′-kb fragment were detected. Homozygous recombinant embryonic stem (ES) cells and germine transmission of the CD44 mutation were screened by Southern blot analysis of EcoRI-digested genomic DNA.

DNA and RNA analysis

Preparation of genomic DNA and Southern blot analysis to identify targeted clones and to genotype mice was performed as described previously (40). Embryos of El13.5 of gestation were homogenized and total RNA preparation and Northern blot analysis was performed as described (40).

Flow cytometry analysis

mAbs against the following structures were used (all from PharMingen, San Diego, CA, unless specified otherwise): CD3 (145-2C11-PE), CD4 (RM4-5-FITC; GK1.5; American Type Culture Collection (ATCC), Manassas, VA), CD8 (53-6.7-FITC-PE; 2.43; ATCC), CD25 (7D4-biotin), CD16/32 (F(ab′)2), CD44 (IM7-CyChrome; KM201; ATCC), CD45R/B220-PE, CD62 (MEL14-FITC), CD69-PE, CD112 (TM–B1-PE), DX5-FITC, TCR-αβ-biotin, TCR–γδ (GL3-PE), I-Ab.d-FITC, Goat-anti-rat-FITC (Vector Labs., Burlingame, CA) and streptavidin-PE or streptavidin-CyChrome were used as second-step reagents where applicable. Cell suspensions from the respective organs were prepared from mice 3 mo of age and stained using standard procedures; data were collected using CellQuest on a FACSort cytometer (Becton Dickinson, Mountain View, CA) and analyzed with WinMDI (J. Trotter, Scripps Institute, La Jolla, CA).

Homing assay

Single-cell suspensions were prepared from spleen and thymus of CD44-deficient and wild-type mice and stained with PKH26 (Sigma, St. Louis, MO) or FITC (Molecular Probes, Eugene, OR) as described previously (41). Stained cells were introduced into CD44 homozygous and heterozygous mutant and wild-type recipients via injection into the tail vein. Donor and recipient mice were 5 wk old and originated from inbreds between sibling parents. Mice were sacrificed 2 or 24 h later, and the spleen, thymus, and peripheral lymph nodes removed and analyzed by flow cytometry.

Results

Targeted inactivation of CD44 gene locus

To inactivate the CD44 gene through homologous recombination, a targeting vector was constructed in which the leader peptide of CD44 was disrupted by an in-frame fusion of the lacZneo cassette directed behind the ATG start codon, resulting in total loss of CD44 transcription and function. Transcription and translation of the targeted allele subsequently lead to the synthesis of the lacZ protein under control of the 5′ regulatory elements of the CD44 locus in all cells and tissues normally expressing one or several of the CD44 isoforms. The targeting construct (Fig. 1A) was introduced into R1 embryonic stem cells (38) of passage 14 by electroporation. Colonies resistant to G418 and gancyclovir were screened for homologous recombination by Southern blot analysis using a 0.75-kb EcoRI/EcoRV probe (Fig. 1A) located 5′ of the targeting vector. In addition to the endogenous 15-kb EcoRI fragment, the 5′ probe detects a 5-kb fragment in targeted ES clones. Three different CD44 heterozygous mutant ES cell clones could be identified, resulting in a gene-targeting frequency by homologous recombination of 1 in 150.

All three clones were injected into blastocysts of C57BL/6 mice. Chimeras generated from two different cell clones (6a and 9-13) resulted in germline transmission of the mutant CD44 allele when crossed into a C57BL/6 background. Mice heterozygous for the CD44 mutation were intercrossed to generate CD44-deficient animals. CD44 homozgyous mutant mice showed no overt phenotype after over 2 yr of observation time, were healthy and fertile, and, consistent with a normal Mendelian segregation, were detected with a frequency of approximately 25% in the F₂ generation (Fig. 1B and Table I).

The absence of CD44 expression in homozgyous mutant mice was confirmed by Northern blot analysis using total RNA of E13.5 wild-type, heterozygous, and homozgyous mutant embryos (Fig. 1C). A cDNA probe consisting of CD44 exons s1–s5 detected a 4.0-kb transcript in wild-type mice corresponding to the sCD44 isoform, which makes up the major part of isoforms expressed in this stage of embryonic development. It was reduced in heterozygous mutant mice to about half of the wild-type level, and was undetectable in homozgyous mutant CD44 embryos, demonstrating that no RNA products are synthesized by inappropriate splicing events. Furthermore, using flow cytometry analysis, no CD44 expression was detected in several organs, e.g., thymus, spleen, and lymph nodes, of CD44 homozgyous mutant mice, whereas heterozygotes showed normal levels of CD44 surface expression, comparable with wild-type animals (Fig. 2, data for wild-type not shown).

No developmental defects in lymphoid organs of CD44-deficient mice but impaired trafficking abilities of CD44-deficient lymphocytes

To test for potential phenotypic and functional deviations of the immune system such as lymphocyte homing and T cell activation of CD44 homozgyous mutant mice, the following issues were investigated: 1) the cellular composition of various lymphoid organs (thymus, peripheral lymph nodes, and spleen) in adult mice to detect potential irregularities in the subset distribution; 2) the phenotype of cells in fetal thymus at E16.5–17.5, when CD44 expression was reported to be highest in this organ; and 3) trafficking of isolated and labeled lymphoid cells in wild-type and CD44 heterozygous and homozgyous mutant mice.

No subset deviation in adult mice in the absence of CD44 protein

Thymus, spleen, and peripheral lymph nodes were removed from adult CD44 wild-type, homozgyous, and heterozygous mutant animals, and single-cell suspensions were stained for CD44 expression and analyzed using flow cytometry. T cell subsets appeared to be normal both in numbers and in the CD4/CD8 expression patterns in various lymphoid organs of CD44 homozgyous mutant mice and comparable with those of CD44 heterozygous mutant mice (Fig. 3), indicating that T cells of CD44-deficient mice develop normally. Abs against other markers, which are generally used to further characterize these subsets, such as TCRαβ and CD25, also did not reveal any deviations (data not shown).
No gross abnormalities in E16.5–17.5 fetal thymus

Fetal thymus was removed from E16.5–17.5 embryos originating from (CD44<sup>−/−</sup> × CD44<sup>+/−</sup>) matings. Single-cell suspensions were prepared and analyzed using a broad panel of mAbs. Staining with anti-CD44 Abs was performed to determine the genetic background of the samples. Again, no major difference was detected between homozygous and heterozygous mutant (and wild-type) mice either in cell yield or in cell subset distribution (data not shown). However, we repeatedly encountered an interesting phenomenon in CD44-deficient mice compared with wild-type mice: both CD4<sup>+</sup> and CD8<sup>+</sup> cells had lost a CD25<sup>low</sup> population (Fig. 4).

Impaired trafficking of CD44-deficient lymphocytes into the thymus

CD44 has been described to play a role in thymus colonization of T cells. It is expressed on bone marrow cells repopulating the thymus of irradiated mice and in long-term bone marrow reconstitution. Anti-CD44 Abs inhibit thymus reconstitution by bone marrow progenitors (26, 27). A similar effect would be expected for CD44-deficient lymphocytes. This expectation was indeed confirmed for the homing of CD44 homozygous mutant lymphocytes to the thymus and peripheral lymph nodes after tail vein injection of equal numbers of mixed fluorescent-labeled CD44-deficient and wild-type lymphocytes.

The ratio of CD44-deficient and wild-type fluorescent-stained donor cells from various recipients recovered 2 or 24 h after reintroduction via the tail vein should be ~1 in an ideal situation in

Table I. Offspring analysis from CD44 heterozygote intercrosses

<table>
<thead>
<tr>
<th>No. of Litters</th>
<th>No. of Offspring</th>
<th>Offspring Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>22</td>
<td>214</td>
<td>+/+ 58 (27.1%) +/− 102 (47.7%) −/− 54 (25.2%)</td>
</tr>
</tbody>
</table>

*Analysis of CD44 genotypes in offspring generated from heterozygote intercrosses demonstrates transmission of the CD44 alleles according to Mendelian rules.
which no homing difference exists. The ratio would be <1 in the case of a selective disadvantage of the homozygous mutant lymphocytes compared with wild-type cells. A ratio of 1 is reasonably approximated in spleens at all times in all recipients, irrespective of the recipients’ genetic background (Fig. 5). Peripheral lymph node ratios are generally lower at the early time point (2 h) but are then (over)compensated later on; this suggests a delayed entry of CD44 homozygous mutant cells into peripheral lymph nodes, supporting the proposed functional role of CD44 in lymph node homing.

Striking differences were found between CD44-deficient and wild-type cells in their ability to participate in thymic homing, irrespective of the recipients’ background: homozygous mutant cells entered about 10–20 times less efficiently into the thymus. This remained unchanged even when, in a few additional cases, the infused suspension contained twice as many homozygous mutant cells (data not shown). The ratio is somewhat higher at 24 h, also suggesting a delayed immigration at this time point. Taken together, these results support the notion that CD44 is important in lymphocyte trafficking, most notably to the thymus and peripheral lymph nodes.

Discussion

CD44 deficiency does not affect embryonic development and differentiation and does not lead to spontaneous tumor development

This work demonstrates that complete loss of CD44 from the very beginning has no overt effect on mouse development, viability, and differentiation of tissues and organs. These results are unexpected,
because it was suggested from previous studies (33, 32, 35, 42) that the loss of CD44 would interfere with normal embryonic and adult development. In addition, former reports display evidence for a role of CD44 during tumor development (43–46), whereas our data indicate that total loss of CD44 information in mice has no effect on tumorigenicity over an observation period of more than 2 yr. Mice were checked frequently by autopsy and by occasional histological examination of specific organs. Despite the lack of any overt phenotype, the CD44 homozygous mutant mice described in this report show various changes in the lymphoid system.

Lymphoid organs deficient in CD44 develop normally but lymphocyte trafficking is impaired

No deviations of T cell subsets both in number and phenotype were detected in any lymphoid organs of adult CD44-deficient mice. In addition, fetal CD44-deficient thymus displayed no gross abnormalities in cell yield or cell subset distribution. However, an interesting phenomenon was the loss of a small, transient, CD25low population in both CD41 and CD81 cells in the thymi of CD44 homozygous mutant embryos. Light scatter properties suggested that these CD4/8 CD25low cells, which are absent in deficient mice, may undergo apoptosis, a well-characterized, fundamental process in T cell homeostasis. The fact that this subset difference was specifically detected only by the anti-IL-2R (CD25) mAbs may also favor this possibility, because IL-2 and IL-2R are thought to be associated with the deletion of lymphoid cells (47). We thus speculate that CD44 influences the apoptotic deletion of immature T cells. Along this line, it is also quite intriguing that in IL-2R-deficient (CD25 homozygous mutant) mice a consistent up-regulation of CD44 has been documented, accompanied with enlargement of various lymphoid compartments (48).

Although CD44-deficient mice develop no deviations of T cell subsets in comparison with heterozygous or wild-type mice, lymphocyte trafficking of reintroduced CD44-deficient T cells into the thymus is impaired, irrespective of the recipients’ genetic background. In CD44 homozygous mutant animals, T cell precursors may have no problems in colonizing the thymus during embryonal development, but upon reinfusion into adult mice, CD44-/- cells encounter a selective disadvantage in comparison with wild-type cells. These results support the notion of CD44 as a receptor for lymphocyte homing, a process that is orchestrated by cooperation with several other molecules, for example L-selectin, ICAM-1, and LFA-1 (49, 50). In wild-type T cells, both CD44 and the integrin LFA-1 have been reported to function as adhesion molecules and costimulators of resting T cells. Because Abs against LFA-1 reduce the migration capability of lymphocytes to lymph nodes by 40–60%, and CD44-mediated lymphocyte adhesion has been shown to be triggered by interaction with LFA-1 (50, 51), we propose a mechanism in which CD44 and LFA-1 cooperate for efficient homing of lymphocytes, although each molecule alone is sufficient to conduct the process at a reduced level in the absence of the other. Accordingly, we attribute the reduction of homing capability of CD44-deficient T cells to the suspended interaction between CD44 and LFA-1.
Putative substitute molecules for CD44 are required to bind to HA and fibronectin

Because CD44 seems to act through different binding interactions, different mechanisms for CD44 substitution are conceivable. First, in either cell migration or pattern forming and organ differentiation processes in embryonic development, CD44 has been demonstrated to interact through hyaluronic binding. Second, lymphocytes have been described as using CD44 to bind to fibronectin but not to HA (52). Furthermore, in addition to CD44, αβ integrin-mediated adhesion of colony-forming cells to a heparin-binding domain of fibronectin can be blocked by anti-CD44 Abs, suggesting that αβ integrin and CD44 cooperate in establishing progenitor cell adhesion (53). Thus, compensation of the homing function of the CD44 molecule could be achieved in its absence by the described adhesion molecules. However, our lymphocyte homing experiments demonstrate that the proposed compensation does not occur to a full extent: in a competitive situation between homozygous mutant and wild-type CD44 cells, the wild-type cells possess a clear advantage in homing capability over the mutant cells, at least when the targets are the thymus or the peripheral lymph nodes.

Putative CD44 substitutes are not only presumed to interact with fibronectin, but also with HA, as, for example, lectin, aggrecan, selectin, and link protein do (54, 16), supposedly via their B(X7)β motif (16). This motif is predicted to be a minimal binding requirement for HA and occurs in all HA binding proteins described to date. Whether candidate substitutes for the CD44 function contain both kinds of binding specificities (the one to HA and the one to fibronectin) or whether different individual molecules would override the respective functions remains to be determined. Detection of the identity of the putative proteins substituting for CD44 function and comparison of their expression pattern and role in developmental and differentiation processes with that of CD44 would be interesting to provide important new insights into the action of CD44 and its participation in interaction networks.

Acknowledgments

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References


