DNA vaccination with CD44 variant isoform reduces mammary tumor local growth and lung metastasis

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Abstract

We have shown recently that cDNA vaccination, using a virtual lymph node, ameliorates experimental allergic encephalomyelitis. Successful cure from mammary tumor requires resolution of local tumor growth and metastases. We have examined whether targeting of CD44 cell surface adhesion molecule by cDNA vaccination plays a role in resolving mammary tumor development. We show here that CD44 cDNA vaccination decreases the tumor mass and metastatic potential in experimental mammary tumor of BALB/c mice. Vaccination of mice, inoculated with the mammary tumors, by cDNA of CD44 variant (CD44v) but not by cDNA of standard CD44, markedly reduced local tumor development and lung metastasis. Concomitantly, transfection of CD44 antisense into a highly metastatic mammary tumor cell line disrupted the CD44 expression of the cells and reduced their ability to establish local tumors as well as metastatic colonies in the lung. Moreover, when CD44v, but not standard CD44 sense cDNA, was transfected into the poorly metastatic cell line, tumor development was markedly enhanced. It is possible therefore that DNA vaccination with a specific CD44v construct could induce an immune resistance to mammary tumor progression. [Mol Cancer Ther 2008; 7(6):1615–23]

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Introduction

Essential elements of tumor spread, including the dissemination of malignant lymphomas, are related to their motile capacity in both the vasculature and the extracellular matrix as well as to their transvascular migration and ability to anchor in extravascular tissues. All these activities are highly dependent on multiple functions, including the activity of metalloproteases (1), receptor tyrosine kinases (2), and cytoskeleton components (3–5), and on cell surface adhesion molecules such as selectins, addressins, integrins, members of the immunoglobulin superfamily (6), and CD44 (7–11). CD44 is a single-chain glycoprotein that uses 10 constant exons and 10 alternatively spliced variant exons to give rise to many different isoforms. These include the standard CD44 (CD44s), containing the constant exons only, and CD44 variants (CD44v), generated by differential utilization of the variant exons. The multistructural nature of CD44 dictates its ability to interact with many ligands, hyaluronic acid being the principal one, and to be involved in many biological activities, such as cell migration and cell localization in target organs (9, 12). Indeed, we (12, 13) and others (14) have shown that targeting of CD44 by passive immunization with anti-CD44 monoclonal antibodies (mAb) markedly reduces the malignant activity. Vaccination in general and DNA vaccination in particular have some advantages over passive immunization. They provide prolonged antigen expression that continuously stimulates the immune system and offers a unique opportunity to manipulate the antigenicity of a protein at the cDNA level (15). This argument and our previous experience with CD44 cDNA vaccinatin, leading to the amelioration of experimental allergic encephalomyelitis (16) and type 1 diabetes (17), prompted us to examine whether the successful reduction in tumor activity by CD44 targeting with specific antibodies (9) could be reproduced by genetic vaccination against CD44. To design the appropriate CD44 cDNA vaccine, we first analyzed the DA3 mammary tumor CD44 structure (CD44s or CD44v)-function relationship. As the CD44v has been proven to be responsible for tumor progression, we tailored a DNA vaccine that would express this specific cDNA variant. Indeed, the DNA vaccine markedly reduced mammary tumor local growth and metastases.

Materials and Methods

Mice

Female BALB/c mice 8 to 10 weeks old were obtained from Harlan and maintained under specific pathogen-free conditions in the Animal Unit of The Hebrew University-Hadassah Medical School. The institution's Guidelines for the Care and Use of Laboratory Animals were adhered to.

mAbs and Other Reagents

Rat KM81 (IgG2b) anti-mouse pan CD44 mAb (constant region-specific) was generated from the American Type Culture Collection hybridomas (18). Rat 9A4 (IgG1) anti-mouse CD44v6 mAb (19) was provided by Dr. J. Moll (Karlsruhe Institute of Genetics). Rat anti-mouse cell surface immunoglobulin idiotype 4D2 (IgG2b; ref. 20) was provided by Dr. J. Haimovich (Tel Aviv University) and was used as an irrelevant isotype-matched control mAb. The hybridomas were injected into the peritoneal cavity of nude mice and the mAbs were purified from the ascitis fluid by protein S-Sepharose chromatography as described previously (21). FITC-conjugated $F(ab)_2$ goat anti-rat [goat IgG (H&L)] was obtained from Jackson Immunoresearch Laboratories. The following GAG were obtained from Sigma: hyaluronic acid (H5388) and chondroitin 4,6-sulfate (C4384). *Eco*RI (R6011) and *Bam*HI (R6021) restriction enzymes were purchased from Promega.

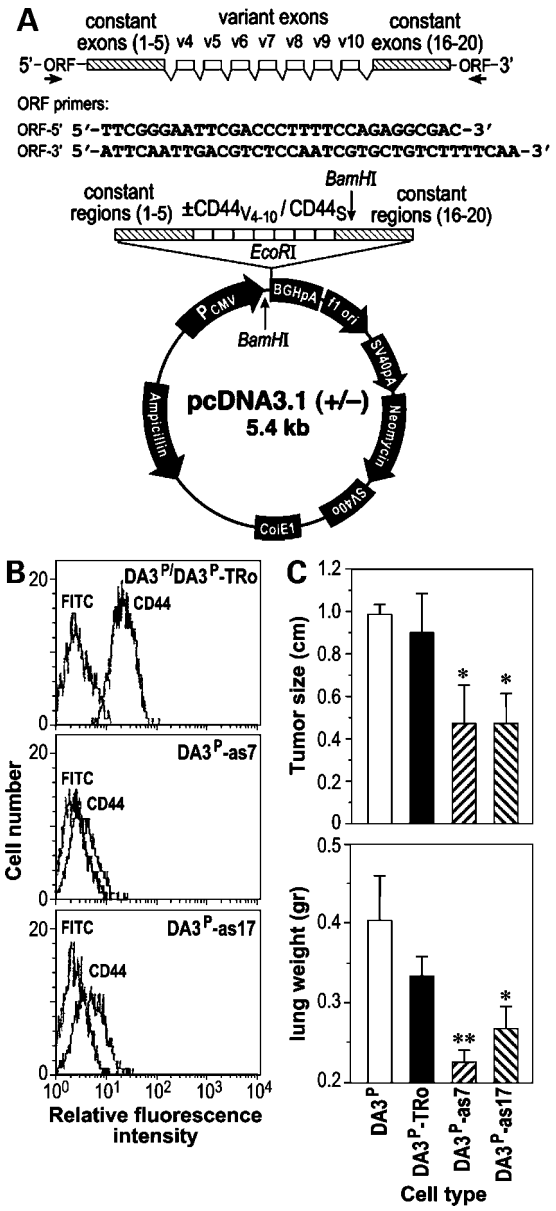

Figure 1. CD44 antisense transfected DA3 parental cells: generation and tumorigenecity. Schematic presentation of the CD44 gene, primers, and plasmid. *Arrowheads,* positions of the 5′ and 3′ ORF primers-sequence. *Hatched columns,* 5′ and 3′ constant exons (excluding introns); *open columns,* variant exons; *lines connecting the exons,* intron sequences. The purified CD44v4-v10 PCR product was digested with *Eco*RI and ligated into the pcDNA3.1 vector further digested with *Bam*HI to assess insert orientation (**A**). Antisense transfected parental DA3 cell clones (as7 and as17) with CD44-specific antisense plasmid reduced the expression of their CD44 receptor when compared with the parental-$DA3^P$-TRo cells as assessed by mean fluorescence intensity (**B**). Decrease of local tumor growth and lung metastases following i.m. inoculation (**C**). Note that the size of the local tumor growth is associated with its metastatic capacity. **, $P < 0.01$; *, $P < 0.05$, Mann-Whitney versus $DA3^P$ cells.

Cell Culture

Mouse adenocarcinoma (DA3) cells generated from DMBA–induced tumors in a BALB/c mouse (22, 23) were kindly provided by Dr. I. Witz (Tel-Aviv University). The cells were maintained in DMEM (Biological Industries) supplemented with 10% heat-inactivated FCS and 2 mmol/L glutamine (Biological Industries). The selective DMEM included 1.5 mg/mL geneticin G418 (Life Technologies). Hybridomas obtained from the American Type Culture Collection were grown in RPMI 1640 (Biological Industries) enriched with 10% FCS and expanded in nude mice. Cell cultures were incubated at 37°C in 5% humidified CO_2. For fluorescence-activated cell sorting analysis, cells were maintained in PBS containing 3% FCS (fluorescence-activated cell sorting buffer).

Construction of Mouse CD44v4-v10, CD44s, and CD44s Antisense Expression Plasmids

CD44v4-v10 cDNA was synthesized from 500 μg polyadenylated RNA prepared from mouse keratinocytes (KLN205 from the American Type Culture Collection) using oligonucleotide primers 3′ and 5′ ORF overlapping CD44 constant regions (Fig. 1A) and cloned into pcDNA3.1 vector (Invitrogen). CD44s cDNA (24) was cloned into *Eco*RI site of pcDNA3.1 in sense and antisense orientations. *Bam*HI restriction enzyme (Fig. 1A) was used to detect insert orientation.

Construction of Human CD44v3-v10 and CD44s Expression Plasmids for DNA Vaccination

Cloning and transfection of human CD44v3-10 and CD44s. This procedure was published previously by us (16). Human CD44v3-v10 cDNA was obtained from total RNA isolated from human keratinocytes (a gift from Dr. Ben Basat, Hadassah University Hospital) using a commercial kit (Promega) followed by reverse transcription-PCR using PTC-100 Programmable Thermal Controller (MJ Research). Primers representing the constant coding regions were used: Ex1-sense: 5′-GAATTCGCCGCCAC-CATGGACAAGTTTTGGTGG-3′ and Ex20 antisense: 5′-TCTAGATTACACCCCAATCTTCATG-3′.

Plasmids were purified using a commercial kit (Promega), subjected to *Eco*RI/*Xba*I double digestion, and cloned into the pcDNA3.1 vector (Invitrogen) in which the gene product was expressed. For cloning of human CD44s, RNA was isolated from the HeLa cervical cancer cell line

Table 1. Cell lines and clones of DA3 mammary tumor used in this study

Cell designation	Description
DA3 or $DA3^{P}$	Parental mammary tumor cell line
$DA3^{hi}$	Parental cell population of highly metastatic DA3 cells
$DA3^{lo}$	Parental cell population of poorly (low) metastatic DA3 cells
$DA3^{P}$-TRo	Parental DA3 cells transfected with empty vector
$DA3^{hi}$-TRo	Parental $DA3^{hi}$ cells transfected with empty vector
$DA3^{lo}$ TRo	Parental $DA3^{lo}$ cells transfected with empty vector
$DA3^{hi}$-asP	Pool of highly metastatic DA3 ($DA3^{hi}$) cells transfected with antisense
$DA3^{P}$-as7, $DA3^{P}$-as17	Clones 7 and 17 of parental DA3 cells transfected with antisense
$DA3^{hi}$as2, $DA3^{hi}$as3, $DA3^{hi}$as 5	Clones 2, 3, and 5 of $DA3^{hi}$ cells transfected with antisense
$DA3^{lo}$-TRvP	Pool of poorly metastatic ($DA3^{lo}$) cells transfected with CD44v4-v10 (CD44v) construct
$DA3^{lo}$-TRv2, $DA3^{lo}$-TRv3	Clones 2 and 3 of $DA3^{lo}$ cells transfected with CD44v construct
$DA3^{lo}$-TRsP	Pool of poorly metastatic ($DA3^{lo}$) cells transfected with CD44s construct
$DA3^{lo}$-TRs2, $DA3^{lo}$-TRs3	Clones 2 and 3 of $DA3^{lo}$ cells transfected with CD44s construct

(obtained from the American Type Culture Collection) according to the above-described protocol.

Stable Transfection of CD44v4-v10 (CD44v) and CD44s cDNA into $DA3^{lo}$ Cells

A mixture of 30 μg polybrene (to anchor the plasmid to the cell membrane) and 10 μg plasmids containing CD44v cDNA, CD44s cDNA, or pcDNA3.1 (control) were added to 5×10^5 $DA3^{lo}$ (low tumorigenic phenotype) cells and suspended in 3 mL medium, incubated (12-24 h), and treated with 30% DMSO for 3 min to allow penetration of the cDNA. Cells were then washed with PBS and resuspended in medium containing 10% FCS. Stable transfectants were generated by continuous selection with neomycin (1.5 mg/mL) following isolation by electronic sorting and by limiting dilution (see Table 1). CD44 expression was confirmed by flow cytometry.

Flow Cytometry

Cell surface expression of CD44 was assessed by indirect immunofluorescence and analyzed by fluorescence-activated cell sorting flow cytometry, as we have described previously (21). Briefly, 0.5×10^6 cells were washed with fluorescence-activated cell sorting medium (3% FCS in PBS) and incubated on ice for 45 min with 100 μL of 10 μg/mL anti-CD44 (KM81) or anti-CD44v6 (9A4) mAb. The cells were then washed with cold fluorescence-activated cell sorting medium and reincubated with 100 μL of 20 μg/mL FITC-conjugated goat anti-rat IgG (H + L chains). After 45 min on ice, the cells were washed and analyzed using a FACStar flow cytometer (Becton Dickinson). Cells incubated with the second antibody alone served as control.

Virtual Lymph Nodes

The virtual lymph node (VLN) was developed by VLN LLC as a novel medical tubular device mimicking the structure and function of a lymph node (see details in ref. 16). BALB/c mice (8-10 weeks old) were anesthetized with Avertin. Two VLN per mouse were inserted under the skin through a 5 cm dorsal midline incision 14 and 1 days before tumor inoculation.

Assessment of Local Tumor Growth and Lung Metastasis

Different transfected cells (Table 1) were injected (0.5×10^6) i.m., close to the hind limb of female BALB/c mice (8 mice per group). Development of the tumor at the injection site was monitored by recording tumor diameter (in cm) over time. Tumor cell invasion into the lungs was evaluated by measuring lung weight (in g) 39 days following inoculation. The results are described as the weight gain of the tumor invaded lung over the normal lung. All the experiments are representative of at least two, and in most cases, three trials showing similar results. To isolate metastatic DA3 cells from lungs, for flow cytometry analysis, the lungs were cultivated in selective medium, thus recovering transfected tumor cells.

Assessment of VLN-cDNA Immunization

Blood serum from immunized mice was used to stain CD44v-expressing cells (Namalwa-TRv), which were further analyzed by flow cytometry. Namalwa cells transfected with CD44v3-v10 cDNA (Namalwa-TRv cells) were kindly provided by Dr. I. Golan (The Hebrew University-Hadassah Medical School). Blood from each immunized mouse was incubated for 12 h at 4°C and then centrifuged for 7 min at 7000 g. A 50 μL volume of the serum was added to 0.5×10^6 Namalwa cells, after which the cells were assayed by flow cytometry using FITC-conjugated secondary antibody, as described above.

Statistical Analysis

Mann-Whitney nonparametric ANOVA was applied to the data. Probability values of <0.05 were considered statistically significant. The correlation between CD44 expression and tumor progression was assessed using the Pearson correlation coefficient.

Results

Induction of resistance to the mammary tumor growth with CD44 cDNA vaccine instead of passive immunization with anti-CD44 mAb (13) requires rational design of the vaccine. Therefore, before engineering the cDNA vaccine, we

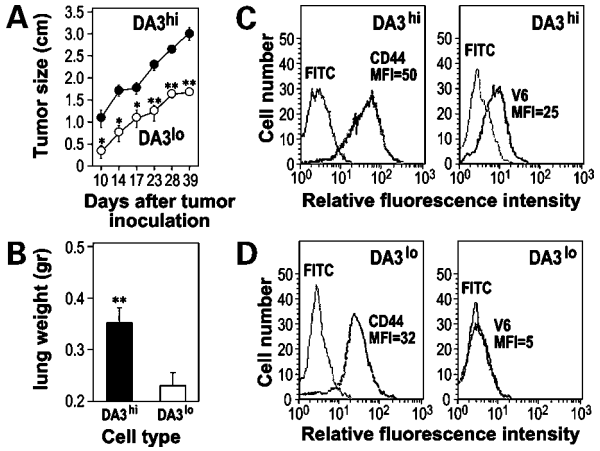

Figure 2. DA3^{hi} cells express CD44 epitopes more intensively than DA3^{lo} cells. BALB/c mice were inoculated with poorly metastatic DA3 cells (DA3^{lo}) or highly metastatic DA3 cells (DA3^{hi}). Local tumor size was measured at different time intervals. The average increase in local tumor growth over time and in the weight of the affected lungs over normal lungs is shown (**A** and **B**, respectively). Flow cytometry analysis revealed more intensive staining of DA3^{hi} cells with KM81 anti-CD44-mAb or anti-CD44v6-mAb (**C**) than that of DA3^{lo} cells (**D**). *FITC*, staining with second antibody only.

verified whether CD44 constant or variant epitopes enhance the tumor progression and accordingly designed a vaccine that induces an immune response against the relevant tumor-supporting epitope(s).

DA3 Mammary Tumor Aggressiveness Is CD44 Dependent

CD44v antisense (full-length CD44v4-v10) and empty plasmids were first transfected into the parental mammary tumor DA3 cell line DA3^{P} [the source of DA3^{lo} and DA3^{hi}, low and high tumorigenic cell lines, respectively (25); see Table 1]. Antisense transfectants, represented by the two clones DA3^{P}-as7 and DA3^{P}-as17 of the parental cells, showed down-regulation of their CD44 receptor when compared with cells transfected with empty vectors (DA3^{P}-TRo) or with the parental DA3^{P} cells (Fig. 1B). Therefore, both CD44s and CD44v were targeted. Furthermore, 30 days after their i.m. inoculation of the antisense transfected cell line local tumor growth and lung metastases were partially, but significantly, inhibited when compared with those derived from DA3^{P} or DA3^{P}-TRo cells (Fig. 1C).

Correlation between High Tumorigenicity of DA3 Cells (DA3^{hi}) and High Levels of Cell Surface CD44

Highly and poorly metastatic sublines were isolated from the DA3 parental cells according to their ability to develop local tumors and lung metastases (see also Fig. 2A and B; ref. 25). We further showed that the highly metastatic line (DA3^{hi}) expresses high levels of CD44, including CD44v, as indicated by staining with anti-CD44v6 mAb (Fig. 2C), whereas the poorly metastatic (DA3^{lo}) cells displayed lower levels of CD44s and no expression of CD44v (Fig. 2D). These results suggest that expression of CD44 in general and CD44v in particular enhances the progression of the mammary tumor.

DA3^{hi} Mammary Tumor Aggressiveness Is CD44 Dependent

We next determined the direct involvement of CD44 in highly mammary tumor-DA3^{hi} aggressiveness using three DA3^{hi} clones expressing different levels of CD44 (as2, as3, and as5; see Fig. 3A, *2-4* compared with *1*). Following i.m. inoculation, their aggressive phenotypes correlated with their CD44 expression. This was reflected by more limited local tumor growth (Fig. 3B, *2-4*) and formation of metastases in the lung (Fig. 3C; results not shown) when compared with that of DA3^{hi} and DA3^{lo} cells transfected with empty vector (DA3^{hi}-TRo and DA3^{lo}-TRo).

We further analyzed the correlation between CD44 expression and the aggressive phenotype of DA3 cells using the Pearson linear correlation test. The intensity of CD44 expression on DA3 cells positively correlated with the enhancement of local tumor size and lung metastases formulation as indicated by an increase in lung weight (Fig. 3D; R = 0.98 and 0.93, respectively).

Expression of the CD44v, but Not of CD44s, Confers an Aggressive Tumorigenic Phenotype on DA3^{lo} Cells

We reconfirmed the prediction that CD44v enhance the progression of tumors in general and assessed this in the mammary tumor in particular. DA3^{lo} cells were transfected with CD44v4-10 cDNA (DA3^{lo}-TRv) as indicated in Table 1. Individual clones were numbered (e.g., DA3^{lo}-TRv2 and DA3^{lo}-TRv3). Reverse transcription-PCR (data not shown) and flow cytometry analysis with anti-CD44v6 mAb (v6 represents the CD44v molecules) revealed that representative clones expressed CD44v6, whereas tumors transfected with empty vector (DA3^{lo}-TRo) do not express the variant eptiope (Fig. 4A). DA3^{lo} cells were also transfected with CD44s cDNA (DA3^{lo}-TRs), including two individual DA3^{lo}-TRs clones (Fig. 4B). Analysis of the tumorigenic potential, assessed by tumor size and lung weight, revealed that local growth and development of metastases of DA3^{lo}-TRv but not DA3^{lo}-TRs cells were similar to those of the DA3^{hi} cells (Fig. 4C and D).

CD44v cDNA Vaccination Induces Resistance to DA3 Tumor Growth

Following our confirmation that also in mammary tumor DA3^{lo}-TRv cells were more aggressive than DA3^{lo}-TRs (Fig. 4), we chose variant epitopes rather than standard epitopes as the target for vaccination. The VLN were loaded with plasmid containing a CMV promoter and human CD44v3-v10 (hCD44v) or human hCD44s cDNA isolated from human keratinocytes by PCR. VLN loaded with an empty vector construct (pcDNA3.1) were used as control. All VLN were transplanted 14 and 1 days before tumor inoculation under the skin of BALB/c mice. Seven of 8 mice transplanted with VLN loaded with empty plasmids (pcDNA3.1) and 5 of 8 mice transplanted with VLN loaded with standard hCD44 cDNA (pcDNA3.1-CD44s) developed local tumors as indicated by measuring growth size as a function of time (Fig. 5A). In contrast, 6 of 8 mice transplanted with VLN loaded with hCD44v plasmids were disease free. The two mice that developed local tumors did so at a significantly lower rate than the mice

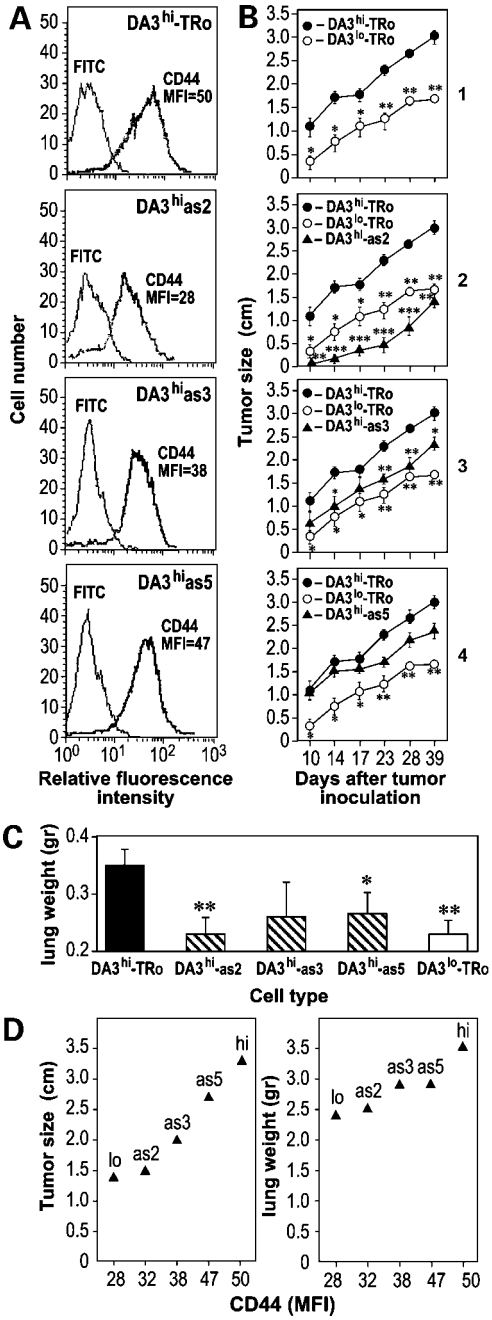

Figure 3. DA3^{hi} cells transfected with CD44-specific antisense plasmid down-regulate their CD44 receptor and display reduced local tumor growth and fewer lung metastases. Flow cytometry analysis of three different DA3^{hi} cloned cells: DA3^{hi}-as2, DA3^{hi}-as3, and DA3^{hi}-as5 (**A,** *2-4*) transfected with CD44-specific antisense plasmids displayed gradually reduced expression of CD44 when their mean fluorescence intensities were compared with those of the parental DA3^{hi} cells (**A,** *1*). Following i.m. inoculation, cloned DA3^{hi} cells containing antisense plasmids showed gradually reduced local tumor growth (**B,** *2-4*) when compared with those of DA3^{hi} cells (**B,** *1*). Lung metastases assessed by measuring lung weight at day 21 (**C**). ***, $P < 0.001$; **, $P < 0.01$; *, $P < 0.05$, Mann-Whitney versus DA3^{hi}-TRo cells. Pearson linear correlation between local tumor growth or metastasis formation (**D**). A positive correlation was observed between CD44 expression and local tumor growth ($R = 0.98$) or metastatic capacity ($R = 0.93$).

inserted with VLN loaded with empty plasmids or CD44s (Fig. 5B). We used human CD44, rather than mouse CD44, to break the self-tolerance to this receptor. In preliminary experiments, we found that VLN loaded with mouse CD44 plasmid do not develop anti-CD44 immunization.

Vaccination with VLN Loaded with CD44v Induces Production of CD44v-Recognizing Antibodies

Blood serum obtained from three mice immunized with hCD44v-cDNA, but not from three mice immunized with hCD44s-cDNA, stained Namalwa cell transfectants expressing hCD44v molecules (Namalwa-TRv) but less those expressing hCD44s molecules (Namalwa-TRs; Fig. 6). The CD44-negative Namalwa cells (Namalwa-TRo) exhibited background staining only. We used human transfectants as indicator cells, rather than mouse transfectants, to avoid nonspecific noise from binding of serum anti-mouse natural antibodies. This finding indicates that human-mouse cross-reactive anti-CD44v antibodies are generated in mice immunized with human CD44v cDNA vaccine. We have shown before, in a different model, that anti-CD44 antibodies can induce resistance to tumor growth (13).

Discussion

We have shown previously that vaccination with VLN loaded with CD44v cDNA (CD44v cDNA) induced a local s.c. inflammatory reaction and significantly ameliorated the clinical and histopathologic manifestations of experimental allergic encephalomyelitis (16) and type 1 diabetes (17). Concomitantly, we also showed that the dissemination of LB T-cell lymphoma in BALB/c mice is CD44 dependent, as lymph node invasion by the s.c. inoculated tumor was markedly reduced after injection of anti-pan CD44 mAb (12, 13). We therefore decided to explore the gene vaccination, using the DA3 mammary tumor, after characterization of its CD44 structure-function relationship, which could allow customization of a more specific vaccine. We have found that the malignant growth of the highly metastatic DA3 mammary tumor is reduced following molecular targeting of its CD44v by a plasmid containing CD44v4-v10 (CD44v) antisense, whereas the progression of poorly metastatic DA3 cells derived from the same parent cell is enhanced by transfection with the same plasmid containing CD44v but not CD44s cDNA both in sense orientation. Note that the expression of even a relatively small quantity of CD44v can influence the development of both local tumor growth and lung metastasis in a correlative manner. Although in many cases growth rate and metastatic spread correlate inversely, there are other examples, documented in our own data (12) as well as in the data of others (26–28), showing as described here a partial or more complete correlation between these two entities. How can even minor expression of CD44v have a substantial effect on tumor cell function? We have found (29), for example, that Namalwa cells (a B-cell lymphoma that does not express endogenous CD44), transfected with CD44v cDNA, which includes the

v3 exon product, but not Namalwa cells transfected with CD44s cDNA, can concentrate soluble fibroblast growth factor-2 on their cell surface CD44 and to orient it toward cell surface fibroblast growth factor receptors to induce cell growth. It was found earlier that only the v3 exon products of CD44v bind and concentrate growth factors and then focus them onto cells expressing the relevant receptors (9). Also, CD44v displays an edge over CD44s in support of cell migration (12). In a metastatic breast cancer cell line (SP1), a signaling association between activated CD44v3 and Tiam-1 (a guanine exchange factor of Rac-1) was detected. This signaling generates cytoskeleton-mediated tumor cell migration (ref. 30; for more details on CD44 downstream pathways, see ref. 9). However, almost complete down-regulation of CD44 (constant-variant) did not entirely reduce DA3 tumorigenicity, indicating that although CD44 enhances the tumor growth, it was not the only factor that supported its development. This finding suggests that the cell surface CD44v of DA3 cells could be an important target for immunologic attack. Indeed, DNA vaccination with VLN loaded with CD44v plasmid, but not with CD44s plasmid, induced significant resistance to the growth of the highly metastatic DA3 mammary tumor. There are conflicting reports in the literature regarding the type of cell surface CD44 isoform that supports tumor progression. On the one hand, it was shown that lymph node metastasis of a pancreatic adenocarcinoma cell line (31, 32), as well as those of an LB T-cell lymphoma (12) and colon carcinoma (33), is CD44v dependent. On the other hand, it was shown that the progression of human melanomas (34) and B-cell lymphomas (35, 36) in immunodeficient mice is dependent on CD44s. Interestingly, CD44v, rather than CD44s, is the functional dominant isoform of many, but not all, human malignant cells, especially those detected in more advanced stages (8). Therefore, it is difficult to decide whether the metastasis of human tumors in immunodeficient mice reflects a real CD44s dependency or is the result of a selective pressure exerted by the artificial environment of the murine species. Using CD44v sense constructs, we reconfirm and present direct evidence that the enhanced progression of mouse mammary tumor is influenced by CD44v.

It should be stressed, however, that, the reported analyses of the association between breast cancer progression and CD44 expression has yielded conflicting findings (9, 37), which claim that the presence of CD44 predicts an unfavorable prognosis (38), a favorable prognosis (39, 40), or its failure to predict prognosis (41, 42). The contradictory

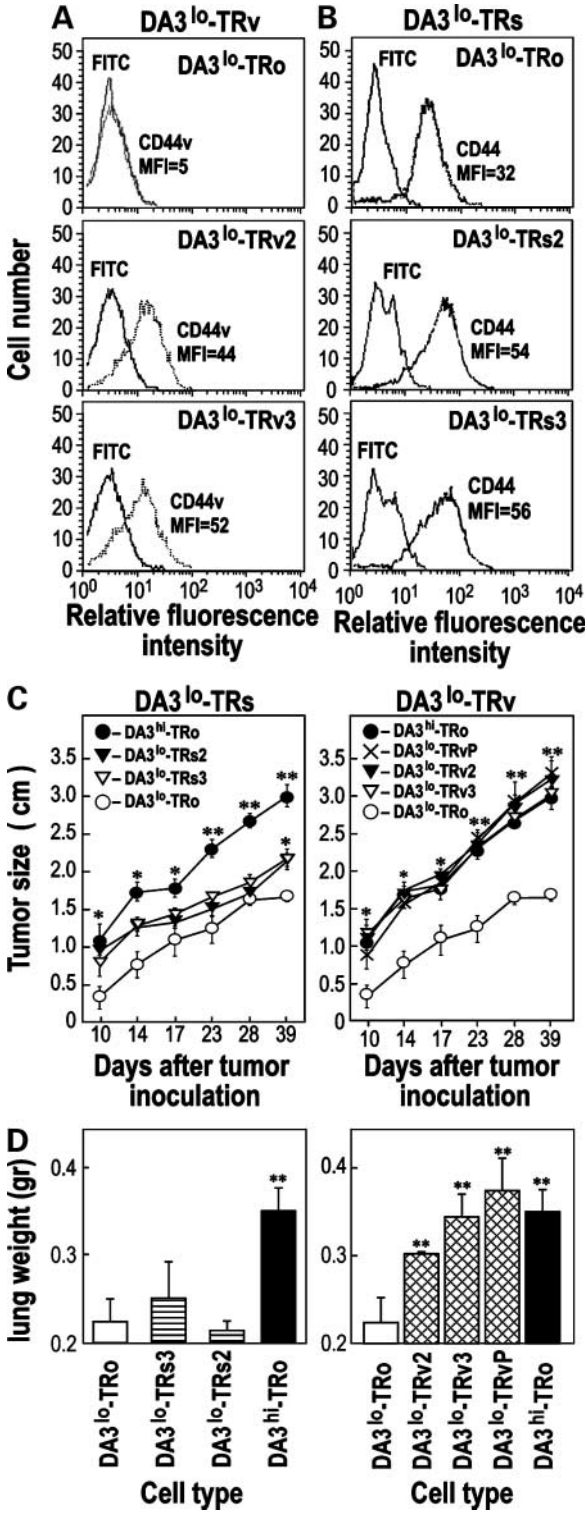

Figure 4. Enhanced local tumor growth and metastasis of DA3^{lo} following transfection with CD44v4-v10-cDNA but not CD44s-cDNA. Poorly metastatic DA3 cells (DA3^{lo}) were transfected with CD44v4-v10 (DA3^{lo}-TRv)-cDNA or CD44s (DA3^{lo}-TRs)-cDNA, cultured in selective medium, and cloned. **A** and **B,** flow cytometry analysis, using anti-CD44v6 or KM81 anti-CD44s mAb, of cells from the tumor cell line (data not shown) and two representative clones (DA3^{lo}-TRv2 and DA3^{lo}-TRv3 or DA3^{lo}-TRs2 and DA3^{lo}-TRs3) as well as DA3^{lo} cells transfected with empty vector (DA3^{lo}-TRo), respectively. The different clones and the cell line (TRvP) were injected i.m. into BALB/c mice and local tumor growth and metastases were assessed. The CD44v, but not the CD44s, transfected cell clones showed enhanced local tumor growth (**C**) and lung metastasis (**D**) when compared with DA3^{lo}-TRo cells. **, $P > 0.01$; *, $P < 0.05$, Mann-Whitney versus with DA3^{lo}-TRo cells.

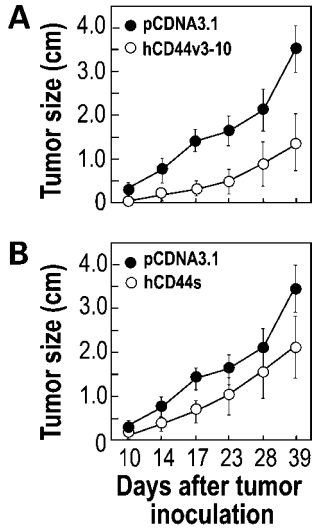

Figure 5. DNA antitumor vaccination by VLN containing hCD44v3-v10 plasmids. VLN containing hCD44v3-v10 plasmid or empty plasmids (**A**) and VLN containing hCD44s plasmids or empty plasmids (**B**) were transplanted under the skin of BALB/c mice 14 and 1 days before inoculation of highly tumorigenic CD44v-transfected DA3 cells. The vaccination with CD44v cDNA (**A**), but not CD44s cDNA (**B**), markedly reduced local tumor growth as indicated by tumor diameter (cm) measured at different intervals after inoculation. *, $P < 0.05$, Mann-Whitney.

data can be explained by the variability of cell surface CD44 affinity to its natural substrate (e.g., hyaluronan). We suggest that too strong an affinity of cell surface CD44 to the matrix substrate locks the tumor in its original primary site, thereby interfering with its metastatic capacity. Too weak an affinity of cell surface CD44 to the matrix substrate interferes with the establishment of metastatic colonies in the extravascular tissue. On the other hand, an intermediate affinity, represented perhaps by some CD44v like the one reported here, generates the balance required for mammary tumor spread. Using different experimental variables in various laboratories, for example, analysis of different subtypes of the same disease or variations in the criteria for a patient's inclusion in a study may lead to examination of tumors with different affinities. Furthermore, variability in cell surface CD44 affinity in breast cancer cells of different patients may produce conflicting findings even in the same study, leading perhaps to the unjustified conclusion that CD44 does not influence the tumor's fate. Technical variability in CD44 analysis, such as using antibodies recognizing distinct CD44 epitopes or arbitrary definition of the cutoff point, separating between high and low CD44 expressers (e.g., in immunochemistry analysis), may further contribute to this confusion.

The enhanced progression of the mammary tumor and the establishment of lung metastases could result from tumor proliferation inside the lung or from accelerated cell migration into the target organ. We showed in a parallel study of a different tumor that the mechanism of enhanced cell migration is far dominant over intraorgan cell proliferation (12). This finding implies that *in vitro* cell proliferation assay of organ infiltrating cells cannot always be used as an indication of metastatic activity or lack of such activity.

The advantage of the VLN is related to its ability to mimic the structure and function of a lymph node. Insertion of the VLN under the skin creates a site of inflammation leading to antigen-presenting cell involvement, including CD44 cDNA expression and presentation. This novel DNA vaccination strategy against mammary tumor has proved the superiority of the CD44v over the CD44s vaccine as indicated by the former's enhanced capacity to reduce local mammary tumor growth. Using VLN-containing CD44 plasmids, we also induced resistance against inflammatory cells involved in the destruction of pancreatic insulin-producing β cells of nonobese diabetic mice, resulting in amelioration of type 1 diabetes in these animals (17). A similar effect was obtained with anti-CD44 mAbs (43). In addition, the VLN-containing CD44 plasmids also induced resistance against inflammatory cells involved in the destruction of myelin in the central nervous system of SJL mice with experimental allergic encephalomyelitis (16). As in the mammary cancer model, the anti-encephalogenic effect of the CD44v cDNA vaccine was stronger than that of the CD44s cDNA vaccine (16).

Immunization of animals with plasmid DNA induced both humoral and cellular immunity to viral and tumor antigens (44–46). Although VLN-containing CD44v cDNA induced antibody production (Fig. 6), we have shown in an independent study that concomitant anti-CD44 cell immunity is not generated by the same vaccine. This vaccination did not also influence cellular immunity to mitogens and

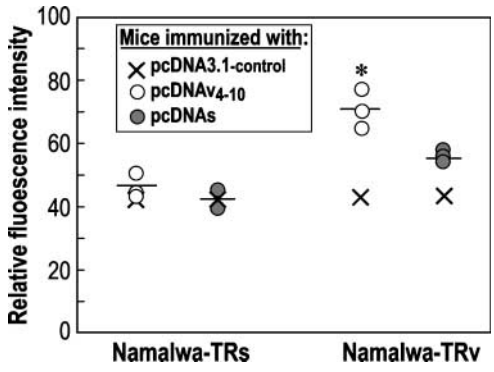

Figure 6. Namalwa cells expressing human CD44 molecules interact with blood serum obtained from mice immunized with VLN containing hCD44v3-v10 plasmids. Serum obtained from mice immunized with VLN loaded with hCD44v-cDNA (pcDNAv4-10), but not with hCD44s-cDNA (pcDNAs) or empty vector (pcDNA3.1), enabled flow cytometry detection, over background, of hCD44v-transfected Namalwa cells but not of the hCD44s-transfected Namalwa cells, indicating the presence of anti-human CD44v antibodies in these mice. Immunization with VLN loaded with CD44s cDNA generated lower level of antibodies detected with Namalwa cells transfected with CD44v, but not with CD44s, cDNA, suggesting better exposure of constant epitope(s) on the former cells. Background staining of the transfected Namalwa cells after immunization with VLN containing an empty vector (pcDNA3.1) is marked by *X*. The relative fluorescence intensity of the stained cells following their interaction with the fluorescein-conjugated second antibody is shown. * $P < 0.05$, Mann-Whitney.

recall antigens (16). Furthermore, mixing splenocytes from CD44 cDNA vaccinated recipients with syngeneic inflammatory spleen cells did not influence the ability of the effector cells to transfer the inflammation to naive recipients (17), ruling out the possibility that anti-CD44 cell-mediated immunity is induced by the vaccine.

We showed the *ex vivo* presence of anti-human CD44 antibodies (which were able to interact with human CD44v-expressing Namalwa cells) in CD44v cDNA-immunized mice. We suggest that the CD44 receptor of the mouse mammary tumor is similarly targeted by the same antibodies *in vivo*, resulting in tumor suppression, as shown earlier by us (13) in a different neoplastic model. This suggestion is based on the well-known cross-homology (60%) between mouse and human CD44 (47, 48), which allows generation of mouse antibody after immunization with human CD44 and binding of anti-human CD44 antibody to mouse CD44 and vice versa as frequently shown in our laboratory. The stronger antibody response to CD44v over CD44s (Fig. 6) can be attributed to a DNA vaccination-related configurational change that generates a superior CD44v immunogen and/or better accessibility of the CD44v epitope than the CD44s epitope on the Namalwa cells.

We showed in earlier publications that injection of anti-CD44 mAb (IgG2b) into mice bearing LB T-cell lymphoma markedly reduced the metastatic lymph node dissemination of the s.c. growing tumor (12, 13). Interestingly, we also found that the same anti-CD44 mAb (mouse IgG2b and human IgG2a) was able to reduce the pathologic activities in collagen-induced arthritis (49) and spontaneous type 1 diabetes (43) in animal models. We have also shown recently that anti-CD44 mAb either passively injected (50) or actively generated by the CD44 cDNA vaccination (16) induces apoptosis in CD44-expressing inflammatory cells of synovial fluid from rheumatoid arthritis patients (50) or encephalomyelogenic mice (16). It is possible that cell surface CD44 targeting by vaccination-induced anti-CD44 antibodies (Fig. 6) delivers apoptotic signals into the tumor cells as well. Indeed, cell surface CD44 is an apoptosis transmitter molecule (9) and anti-CD44 antibodies can deliver agonistic apoptotic signals during encounter with this receptor (50). As the DNA vaccination with CD44v3-v10 cDNA produces anti-CD44 antibodies, it is possible that cell surface CD44 targeting by vaccination-induced anti-CD44 antibodies accelerates apoptosis as discussed previously by us in detail (16). However, the possibility that a minor compartment of cell-mediated immunity is involved in the induction of tumor resistance by CD44 targeting cannot be ruled out.

VLN containing mouse CD44 cDNA rather than human CD44 cDNA were unable to induce resistance to the mammary tumor growth (data not shown). This observation is hardly surprising because the mouse is tolerant to its own CD44. Therefore, we used human CD44v to break the tolerance. The CD44 of mouse and man share some epitopes, while diverging in others, allowing breaking the tolerance to self-CD44 in the mouse by human CD44. The nonhomologous epitopes of human CD44 are immunogenic in mouse, allowing activation of helper T cells recognizing these epitopes. Linked recognition is then generated between helper T cells recognizing the foreign epitopes of human CD44 and B cells recognizing the shared human-mouse epitopes of the same molecule, resulting in breaking of tolerance and synthesis of anti-mouse CD44 antibodies. This concept is well established and is used to explain breaking of tolerance to self-antigens, leading to autoimmunity.

In summary, targeting a CD44v epitope expressed on cells involved in pathologic activities, but not on cells engaged in normal physiologic functions, with relevant variant-recognizing anti-CD44 antibody or relevant CD44v cDNA vaccine, should be less harmful to the patient. Therefore, the targeting of CD44v, expressed on the DA3 mammary tumor with the CD44v cDNA vaccine, may be used as a model for a selective therapy of large platform of CD44-dependent cancer and inflammatory diseases.

Disclosure of Potential Conflicts of Interest

C. Hand: ownership of patent applications covering VLN technology. The other authors reported no potential conflicts of interest.

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