Structural Variability of CD44v Molecules and Reliability of Immunodetection of CD44 Isoforms Using mAbs Specific for CD44 Variant Exon Products

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CD44 can be considered structurally and functionally one of the most variable surface molecules. Alternative splicing of variant exons as well as posttranslational modifications of the molecule (differences in glycosylation) generate a rich repertoire of CD44 isoforms (CD44v), some of which seem to play a key role in tumor growth and progression. Immunodetection of CD44 isoforms *in vivo*, using mAbs specific for CD44 variant exon products, is largely used to identify those CD44 molecules involved in tumor growth and progression and to interfere with CD44-mediated processes. In the present work we demonstrate that the immunoreactivity of some mAbs directed to CD44 exon-specific epitopes can be impaired by the structural variability of the molecule. Our findings demonstrate that (1) specific exon assortment and/or posttranslational modifications of CD44v molecules can mask CD44 exon-specific epitopes; (2) glycosaminoglycan side chains, carried by some CD44v isoforms of high molecular weight, may play a critical role in determining the exact conformation of the molecule, which is necessary for the detection of CD44 variant epitopes by specific mAbs; and (3) in a panel of stable transfectants expressing CD44 N-glycosylation site-specific mutants, generated in the constant region of CD44 extracellular domain, asparagine-isoleucine substitution is sufficient per se to impair the immunoreactivity of several mAbs to pan-CD44. Thus, conformational changes due to the alternative splicing of CD44 variant exons and/or posttranslational modifications of the molecule (different degree of glycosylation), which are cell type-specific, are likely to generate CD44 variants that elude immunodetection. These findings strongly suggest that immunohistochemical analysis of CD44 expression *in vitro* and *in vivo*, using mAbs specific for CD44 variant exon epitopes, can potentially be impaired by a large num-

ber of false negative results. (Am J Pathol 1999, 154:291-300)

CD44 is a transmembrane glycoprotein broadly distributed on the cell surface of several normal and neoplastic cells that is implicated in lymphocyte activation, cell-cell and cell-extracellular matrix interactions, and tumor growth and progression.¹⁻³ CD44 is structurally and functionally polymorphic. Its gene is composed of at least 20 exons, 12 of which can be alternatively spliced. Protein heterogeneity arises predominantly from the variable splicing of 10 exons encoding an extracellular region located between the invariant NH₂-terminal hyaluronic acid-binding domain and the membrane proximal extracellular domain. Alternative splicing of exons encoding CD44 cytoplasmic domain has also been reported.⁴ The most widely expressed CD44 receptor is the 85- to 90-kd glycoprotein that represents the standard CD44 molecule, as it does not contain the products of variant spliced exons. This molecule, commonly referred to as CD44s or CD44H, has been shown to be the major cell surface receptor for hyaluronic acid.⁵ Alternative splicing of variant exons may generate CD44 isoforms of molecular weight ranging from 90 to > 220 kd with a different capability to bind hyaluronic acid⁶ (Figure 1). Additional structural and functional heterogeneity of the CD44 molecules may also be generated by cell type-specific glycosylation. In fact, the extracellular region of CD44s contains seven potential N-linked glycosylation sites and the membrane proximal domain contains four Ser-Gly motifs (serine and glycine residues) that constitute the minimal sequence for the attachment of chondroitin sulfate and other glycosaminoglycan (GAG). Furthermore, several serine and threonine residues, which may provide potential sites for O-linked glycosylation, are available on the molecule (Figure 2).⁷⁻⁹ In CD44 splice variants (CD44v), the degree of glycosylation can be consistently in-

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Figure 1. Organization of the genomic structure of CD44 gene. Numbering of the exons refers to their presence in the standard or variant region of the molecule: exons 1 to 5 encode the 5' standard region; exons 16 to 20 encode the 3' standard region; all of the standard exon products represent CD44s, which can be considered the backbone of all of the CD44 spliced isoforms. The variable portion of CD44 molecules is codified by variant exons v2 to v10. Arrows indicate additional splicing sites. LP, leader peptide; ECR, extracellular region; TM, transmembrane domain; CYT, cytoplasmic domain.

creased because extra N- and O-glycosylation sites are provided by additional inserted exon products. Moreover, isoforms containing exon products v3 and v10 possess additional sites for the attachment of GAG side chains.⁹ Several reports have demonstrated that CD44 hyaluronate-binding ability is tightly regulated and alternative splicing of variant exons, as well as posttranscriptional modifications of the molecule (changes in glycosylation), represent two important regulatory mechanisms of CD44-mediated functions.⁷⁻¹¹

It is clear that the potential to combine at least 13 peptide units in the extracellular region, which are all encoded by one genomic sequence, together with the different degree of glycosylation, makes CD44 one of the most variable surface molecules. On the other hand, such structural variability provides a molecular basis by which the diversity of cellular functions attributed to the CD44 molecular species may be explained. There is increasing evidence in the literature that CD44s and its

splice variants play an important role in tumor growth and progression.^{2,3,12-15}

Following the initial demonstration by Gunthert et al² that a variant of CD44 molecule containing exon product v6 could confer metastatic potential on a rat pancreatic adenocarcinoma cell line, a great deal of scientific interest has focused on attempting to better define the role that these molecules may play in tumor growth and progression, focusing on the possibility of identifying new prognostic tumor markers as well as potential targets for specific therapeutic approaches. Interestingly, an abnormal pattern of CD44 gene activity in terms of qualitative and/or quantitative expression of CD44v has been demonstrated in neoplastic cells compared to their normal counterpart.¹⁴ In several instances soluble CD44v molecules were detectable in serum of patients with neoplastic diseases.^{13,15,16} It seems likely that the possibility of detecting *in vivo* CD44 and its isoforms in both neoplastic tissues and serum specimens could represent a reliable approach to the diagnosis of malignancies and the assessment of their metastatic potential.

To explore this issue, several mAbs to CD44 variant exon products have been generated and are commercially available. These reagents are extensively used in immunohistochemical and immunoenzymatic assays with the aim of correlating the expression of specific CD44v to the biological features of the tumor. There is abundant scientific work in this field which may be of great prognostic value in oncology.¹³

In this study, using gene transfer experiments, reverse transcriptase-polymerase chain reaction (RT-PCR), and immunochemical assays, we demonstrate that the immunoreactivity of several mAbs directed at CD44 variant exon products could be strongly impaired by the structural variability of CD44v molecules. The unpredictable immunoreactivity of these reagents could generate a large series of false negative results, which in turn could affect both the reliability of immunophenotypical studies on CD44v expression *in vivo* and their prognostic value.

Materials and Methods

Cell Lines and Tissue Specimens

Normal and neoplastic tissues from surgical biopsies were obtained from the Department of Surgical Pathology at the Regina Elena Cancer Institute. Tissue samples were snap-frozen in liquid nitrogen and 4-μm cryostat sections were obtained and fixed in absolute acetone for 10 minutes. Fixed sections were used in immunohistochemical assays as previously reported.¹⁷ Cultures of human neoplastic cell lines derived from primary and metastatic tumors were maintained at 37°C in 5% CO₂ according to standard methods. Tissue culture media (RPMI and Dulbecco's modified Eagle medium), L-glutamine, and G418 were purchased from Gibco (Grand Island, NY). Fetal bovine serum was obtained from Irvine Scientific (Santa Ana, CA). Tissue culture plastic ware was acquired from Falcon (Lincoln Park, NJ).

Figure 2. Graphic representation of CD44s molecule decorated with potential N- and O-linked sugar residues. CS, chondroitin sulfate side chains.

Table 1. mAbs Used in This Study

mAb	Clone name	Isotype	Specificity	Concentration	Source
anti-mouse/human pan-CD44	IM7.8.1	rat IgG _{2a}	pan-CD44	10–50 μ g/ml	ATCC
anti-human pan-CD44	A3D8	mouse IgG ₁	pan-CD44	10–50 μ g/ml	Sigma
anti-human CD44 V3	3G5	mouse IgG ₁	v3 specific epitope	5–20 μ g/ml	R & D System
anti-human CD44 V3–V10	polyclonal	rabbit polyclonal antisera	v3–10 epitopes	1:5–1:100	Bender MS
anti-human CD44 V4–5	3D2	mouse IgG ₁	v4/5 spec. epitopes	5–20 μ g/ml	R & D System
anti-human CD44 V5	VFF-8	mouse IgG ₁	v5 specific epitope	10–50 μ g/ml	Bender MS
anti-human CD44 V6	2F10	mouse IgG ₁	v6 specific epitope	5–20 μ g/ml	R & D System
anti-human CD44 V6	VFF-7	mouse IgG ₁	v6 specific epitope	10–50 μ g/ml	Bender MS
anti-human CD44 V7	VFF-9	mouse IgG ₁	v7 specific epitope	10–50 μ g/ml	Bender MS
anti-human CD44 V7–V8	VFF-17	mouse IgG _{2b}	v7–8 spec. epitopes	10–50 μ g/ml	Bender MS
anti-human CD44 V10	VFF-14	mouse IgG ₁	v10 spec. epitope	10–50 μ g/ml	Bender MS

Monoclonal Antibodies and Polyclonal Antisera

All of the commercially available mAbs to CD44 and CD44 variant exon products used in this study are reported in Table 1. These reagents were used according to the manufacturers' instructions. Mouse mAbs anti-human CD44 variants acquired from R&D System (Minneapolis, MN) were derived from a fusion of mouse myeloma cells (SP2/0) with spleen cells from a mouse immunized with a human chimeric affinity-purified fusion protein, CD44v3–10-Fc, containing the full product codified by all of the CD44 variant exons (v3–v10) (Figure 1). The specificity of the mAbs to CD44v was determined (as reported by the data sheet of the reagents) by two independent sets of fluorescence-activated cell sorter (FACS) analyses in which a panel of CD44-transfected COS cells and CD44-transfected neoplastic B cells were used as targets. The cDNA used for such transfections contained various combinations of the variant exons v3–v10, (ie, v3, 8–10; v8–10; v7–10; v6–10, etc.). Several mAbs specific for selected CD44 variant exon products were then obtained after adequate cross-screening. Mouse mAbs specific to CD44 variant exon products and rabbit polyclonal antisera to CD44v3–v10 epitopes, acquired from Bender MedSystem (Vienna, Austria), were also generated using purified CD44 fusion protein as immunogen and were characterized as reported above. mAbs to pan-CD44 named IM-7, A3D8, and 3G5 were commercially obtained (Table 1). These reagents recognize a nonpolymorphic determinant on mouse (IM-7) or human (A3D8 and 3G5) CD44 standard region. mAbs of the BRIC series, named BRIC35, BRIC205, BRIC214, BRIC219, BRIC222, BRIC223, BRIC225, BRIC235, BRIC241, and BRICKZ1, were kindly provided by Dr. Francis Spring (International Blood Group Reference Laboratory, Bristol, UK), and have been previously characterized.¹⁸ All of these reagents, which are considered pan-CD44 monoclonal antibodies, detect epitopes on the invariant CD44 extracellular region. mAb BRIC235, which recognizes an epitope into the NH₂-terminal hyaluronic acid-binding domain of CD44, was used to inhibit CD44-mediated binding to hyaluronate.^{7,18} Fluorescein-labeled goat anti-mouse and goat anti-rat antisera were acquired from Cappel (Malvern, PA).

Production of Stable CD44 Transfectants

cDNA clones encoding amplified segments of human CD44 isoforms containing no variable exons (CD44s), exon v10, exons v6–10, exons v7–10, v3,8–10 and v3–10, were inserted into the *Bgl*/I/*Nar*I cloning site of pRcCMV CD44^{Bgl/Nar} as previously reported.^{6,19} This construct contained full-length CD44 cDNA modified by site-directed mutagenesis to provide a cassette for insertion of alternatively spliced exons at the appropriate site within the CD44 extracellular domain. Development of stable transfectants was performed according to a modified version of previously described protocols.^{6,19} cDNA clones encoding human CD44 standard molecule and isoforms v10, v6–10, v7–10, v3,8–10, and v3–10 were inserted into the pRcCMV expression vector and the construct transfected into Namalwa cells by electroporation using 4-mm cuvettes (750 V/cm, 960 μ F). Transfectants were selected for resistance to geneticin (Gibco) and maintained at a concentration of 2 mg/ml in RPMI (Gibco) supplemented with 10% fetal bovine serum, 2 mmol/L glutamine (Gibco), and gentamicin (15 μ g/ml) (Table 2).

Development of CD44s Mutants in N-Glycosylation Sites

Production and characterization of CD44s N-glycosylation site-specific mutants were previously described in detail.⁷ Briefly, CD44 site-directed mutants were prepared by encoding the desired mutation in overlapping oligonucleotide primers²⁰ and generating the mutations

Table 2. CD44 Phenotype of Namalwa Transfectants

Transfectants	Variant exons expressed	Molecular weight (kd)
Nam CD44s	none	85–90
Nam CD44v10	v10	110
Nam CD44v6–10	v6, v7, v8, v9, v10	170
Nam CD44v7–10	v7, v8, v9, v10	160
Nam CD44v3,8–10	v3, v8, v9, v10	170–200
Nam CD44v3–10	v3, v4, v5, v6, v7, v8, v9, v10	>200

Figure 3. Schematic representation of N-glycosylation site specific mutants in a portion of the invariant CD44s extracellular domain. Amino acids are denoted by their single letter code and mutated N-linked glycosylation sites (NGS) are numbered from the NH₂ terminus. In each mutant one or more asparagine residues (N) are substituted by isoleucine (I). See Materials and Methods section for details.

by PCR using CD44s cDNA in the CDM8 expression vector as a template. Finally, the constructs were stably transfected in CD44-negative MC melanoma cell line using the electroporation method. Transfectants were selected for resistance to geneticin (Gibco) and maintained at a concentration of 1 mg/ml in Dulbecco's modified Eagle medium as described before. Each of these mutants produces a CD44s molecule in which asparagine residues (Asn) in one or more potential N-glycosylation sites are substituted with isoleucine (Ile). The mutants used in this study and the position of the Asn-Ile substitutions are NGS1, Asn in position 25; NGS2, Asn in position 57; NGS5, Asn in position 120; NGS2-5, Asn in positions 57 and 120; NGS3-5, Asn in positions 100 and 120; and NGS3-4, Asn in positions 100 and 110, all substituted by Ile residues (Figure 3). The position numbering refers to the sequence present in the CD44 file in SWISS-PROT Protein Data Bank, accession number P16070.

Immunofluorescence and Immunoperoxidase

Expression of CD44 and its variants on cell lines and transfectants was evaluated immunochemically by incubating the target cells with primary mAbs for 45 minutes at 4°C. Cells were then washed in phosphate-buffered saline (PBS), incubated with a fluorescein-labeled affinity-purified goat-anti-mouse or anti-rat antisera (Cappel) for 1 hour, washed twice, resuspended in PBS, and finally analyzed by flow cytometry (Becton Dickinson, Mountain View, CA). Maintenance of CD44 cell surface expression by the stable transfectants was tested periodically by flow cytometry.

Immunohistochemistry was performed using an indirect avidin-biotin complex (ABC) immunoperoxidase method, with Vectastain ABC Kit (Vector Laboratories, Burlingame, CA). Slides were incubated overnight with selected mAbs at 4°C in a moist chamber. The enzymatic activity was developed using 3-amino-9-ethyl-carbazole as previously reported.¹⁷

Cell Labeling and Immunoprecipitation

For metabolic labeling, cell lines and transfectants were washed with methionine-free RPMI 1640 (Gibco) and starved in the same medium supplemented with 10% dialyzed, heat-inactivated fetal bovine serum for 2 hours. Cells were then labeled with 250 μ Ci/ml ³⁵S methionine (Amersham International, Buckinghamshire, UK) for 12 hours, washed in PBS, and lysed at 4°C for 1 hour in a lysis buffer containing 1% Triton X-100 (Sigma, St Louis, MO), 10 μ g/ml leupeptin (Sigma), 100U/ml aprotinin (Sigma), and 10 μ M phenylmethylsulfonyl fluoride (BRL, Bethesda, MD). Nuclei were removed by centrifugation and lysates precleared by a 2-hour incubation with Protein A-sepharose CL4B beads (Pharmacia, Uppsala, Sweden) coated with rabbit anti-mouse IgG (Cappel). After preclearing, lysates were incubated with protein A-Sepharose CL4B beads previously conjugated with an anti-CD44 mAb for 1 hour at 4°C. Protein A-sepharose beads were then washed and immunoprecipitates eluted by boiling. Finally, precipitated proteins were subjected to sodium dodecyl sulfate/7.5% polyacrylamide gel electrophoresis and the gels were fixed, dried, and analyzed after exposure for autoradiography.

RT-PCR

Total RNA was obtained from cell lines, transfectants, and frozen tissue specimens by guanidine isothiocyanate method as previously described.⁸ cDNAs for PCR were prepared by a oligo-p(dT) method. Total RNA (5 μ g) previously treated with DNaseI RNase-free (all commercial preparations in this paragraph obtained from Boehringer Mannheim) was incubated with 0.1 mol/L oligo-p(dT) (18 bases) for 10 minutes at 65°C and placed in ice for 5 minutes. Then 5 μ l of 5 $\times$ RT buffer, 40 units of RNase-Inhibitor (Boehringer Mannheim), 1 mmol/L dNTP, 25 units of M-MuLV Reverse Transcriptase, and water to a total volume of 25 μ l were added together and the mix was incubated for 90 minutes at 37°C. Three microliters of the reaction volume were used for each PCR reaction. The PCR reactions were carried out in a total volume of 100 μ l with the following reagents added together: 3 μ l of cDNA; 8 μ l of dNTP 1 mmol/L; 0.2 mmol/L of each oligonucleotide; 10 μ l of 10 $\times$ buffer containing 100 mmol/L Tris-HCl, 15 mmol/L MgCl₂, 500 mmol/L KCl, pH 8.3, and 2.5 units of *Taq* DNA polymerase.

The oligonucleotides used as primers in this study were: C4F, CCAATGCCTTTGATGGACCA and C16R, CTGGAATTTGGGGTGTCT, complementary to the constant sequence respectively 5' upstream (standard exon 4) and 3' downstream (standard exon 16) of the variable portion of CD44 molecule (variant exons v3-v10). Exon-specific primers were designed for each CD44 variant exon as follows: V3F2, GGCTGGAGCCAAATGAAG; V3R, GGTGCTGGAGATAAAATC; V4F, TCAACCACACCACGGGCT; V4R, AGTCATCCTTGTGGTTGT; V5F, GTAGACAGAAATGGCACC; V5R, TGTCGTTGTAGAATGTGG; V6F, CAGGCAACTCCTAGTAGT; V6R, AGGTGTCCGTGTTGTCGA; V7F, GCCTCAGCTCATACCAGT; V7R2, AT-

GGGGTGTGAGATTGGG; V8F, ATGGACTCCAGTCAT-AGT; V8R, CGTTGTCATTGAAAGAGG; V9F, GCTTGA-TGTCAGAGTAGA; V9R2, ATCTTCCTTCCAAGCCTTC; V10F2, AGGAATGATGTCACAGGT; V10R, TGATAAG-GAACGATTGAC. To minimize the possibility of amplifi-cation of genomic DNA, upstream and downstream prim-ers were used in each PCR reaction on variant and standard exons respectively. Reaction products were ob-tained in a GeneAmp 9600 thermal cycler (Perkin Elmer, Norwalk, CT) with an initial denaturation step (94°C for 5 minutes) and a total of 30 cycles of denaturation (94°C for 1 minute), annealing (56°C for 1 minute), and extension (72°C for 1 minute), followed by a final elongation (7 minutes at 72°C). A nucleic acid sample (total RNA ex-tract) without addition of reverse transcriptase was used as negative control for each PCR experiment. PCR prod-ucts were separated on a 2% agarose (Boehringer Mann-heim) and gels were stained with ethidium bromide (Sigma).

Treatment of Cells with Inhibitors of N- and O-Glycosylation

To inhibit N- and O-glycosylation, cells were cultured in standard conditions with the addition of the following inhibitors of glycosylation: 5–10 µg/ml tunicamycin (Cal-biochem-Novabiochem, La Jolla, CA) and/or 2 mM phe-nyl-N-acetyl-α-D-Galactosaminide (pNAcGal) (Sigma). Both drugs were used according to the manufacturers' instructions and the cells were incubated at 37°C over-night.

Treatment of the cells with β-D-xyloside (4-methyl-um-belliferil-β-D-xyloside) to inhibit GAG side chain attach-ment on large CD44v molecules was performed by incu-bating each cell line with 1 mmol/L β-D-xyloside at 37°C for 16 hours before performing both FACS analysis and CD44 immunoprecipitation.

Results

Production and Characterization of Transfectants and Cell Lines Expressing CD44 Isoforms

Using CD44-negative Burkitt's lymphoma cell line (Na-malwa), we created a panel of stable transfectants ex-pressing CD44s and some related isoforms, namely CD44v10, CD44v6–10, CD44v7–10, CD44v3,8–10, and CD44v3–10, representative of CD44 molecules contain-ing only constitutive exon products and variable exon products v10, v6–10, v7–10, v3,8–10, and v3–10 respec-tively (Table 2). These transfectants have been exten-sively characterized both phenotypically and functionally using flow cytometry, immunoprecipitation, and RT-PCR and we previously demonstrated that all of them ex-pressed the expected CD44 receptor.⁶ A series of neo-plastic cell lines were also characterized for CD44 ex-pression by flow cytometry, immunoprecipitation (data not shown) and RT-PCR, using CD44 exon-specific prim-

Table 3. Presence of CD44 Variant Exon Transcripts in Different Neoplastic Cell Lines as Evaluated by RT-PCR

Cell lines and origin	Exons							
	v3	v4	v5	v6	v7	v8	v9	v10
Sab (Breast carcinoma)	+	–	–	+	+	+	+	+
Med (Lung carcinoma)	–	–	–	+	+	–	+	+
San (Lung carcinoma)	–	–	–	+	+	–	+	+
DeMa (Lung carcinoma)	+	–	–	+	+	–	+	+
Pag (Lung carcinoma)	–	–	–	+	–	–	–	+
LoVo (Colon carcinoma)	–	–	–	–	–	–	+	+
H 69 (Small cell lung carcinoma)	–	–	–	+	–	+	–	+

ers (Table 3). Cell lines and transfectants were used as targets to test a panel of mAbs directed to CD44 variant exon products as reported below.

Immunoreactivity of mAbs to CD44 Variant Exon Products on Cell Lines and Transfectants

Namalwa transfectants and selected CD44v-expressing neoplastic cell lines were used as targets to study the immunoreactivity of mAbs to CD44 variant exon prod-ucts. Although pan-CD44 mAb IM-7, which recognizes an epitope carried by the constant region of CD44, in-variably detected CD44 molecules on both transfectants and neoplastic cell lines, mAbs directed at specific CD44 variant exon products failed in several instances to detect the respective CD44 isoforms. In fact, among the re-agents used in this study, only anti-CD44v3 and anti-CD44v6 mAbs demonstrated a congruous immunoreac-tivity on all of the target cells carrying the respective epitopes.

A detailed selection of the most intriguing results re-garding the immunoreactivity of these reagents, as eval-uated in FACS analysis, is reported in Figure 4. mAb to CD44v5 was nonreactive on transfectant Nam v3–10, expressing a high molecular weight CD44 isoform (>200 kd) representative of the full variant region v3 to v10. Furthermore, mAb to CD44v6 showed a variable reactiv-ity on both transfectant Nam v3–10 and DeMa cell line (constitutively expressing CD44v3, 6–7, and 9–10) com-pared to Nam v6–10, in which CD44v6 epitope was consistently detected. mAb to CD44v7–8, directed against an epitope encoded by both v7 and v8 variant exons, showed variable staining on Nam transfectant CD44v3–10 but failed to detect the respective target on transfectants CD44v6–10 and CD44v7–10 and breast carcinoma cell line Sab (expressing CD44v3 and 6–10). Finally, mAb to CD44v7 failed to detect the respective exon product on several cell lines, including transfectants Nam CD44v7–10, CD44v6–10, CD44v3–10 (Figure 4A) and neoplastic cell lines Med and DeMa (Figure 4B), despite the presence of CD44 isoforms carrying specific exon product v7, as demonstrated in FACS analysis, RT-PCR (Tables 2 and 3), and immunoprecipitation assay (Figure 5 and see below). Surprisingly, the same mAb was reactive with neoplastic cell line Sab, constitutively expressing a CD44 isoform containing the exon product

Figure 4. Immunoreactivity of mAbs to CD44 variant exon products on selected Namalwa transfectants (A) and neoplastic cell lines (B), as detected by FACS analysis. The top of each panel shows both the cell lines and their CD44 phenotype; the specific mAbs used for immunostaining are reported on the left. Incoherent immunoreactivity is shown by ▲.

v7 (Figure 4B). This finding strongly suggests that the failure to immunodetect specific targets on the above reported cell lines and transfectants is not mAb-dependent but is probably due to the different exon assortment in each CD44v molecule. mAb to CD44v10 was always nonreactive on both Namalwa transfectants and neoplastic cell lines carrying the specific epitopes.

A comparative immunoprecipitation of CD44 molecules expressed on Sab cell line and Nam CD44v7–10 transfectant is shown in Figure 5. The difference in CD44 variant exon assortment in the two cell lines (see Tables 2 and 3) was confirmed by the different molecular weight of CD44 immunoprecipitates.

In fact, CD44 immunoprecipitated from Sab cell line showed a molecular weight of ~200 kd (depending on the presence of v3 and v6 additional exon products), compared to CD44v7–10 which had a molecular weight of ~170 kd.

In Figure 6, using specific primers for exon v7 and v8, we definitely demonstrated with RT-PCR the presence of related transcripts in both breast carcinoma cell line Sab and Nam CD44v7–10 transfectant, despite the restricted reactivity of mAb to CD44v7 epitope on the Sab cell line. Furthermore, mAb to CD44v7–8 failed to detect the specific epitope in both of them.

Immunoreactivity of mAbs to CD44 Variant Exon Products on Normal and Neoplastic Human Tissues

To characterize the expression of CD44 and its isoforms in normal and neoplastic human tissues, we used specific mAbs extensively in indirect immunofluorescence and immunoperoxidase. Altogether we evaluated about 200 tissue specimens from human breast, thyroid, colon,

Figure 5. Comparative CD44 immunoprecipitation from cell lysates of Sab cell line and Namalwa CD44v7-10 transfectant, using a pan-CD44 mAb BRIC 235. **Lane 1:** Sab cell line. **Lane 2:** Nam CD44v7-10. **Lane 3:** Nam CD44s transfectant as positive control. **Lane 4:** Nam CD44v7-10 lysate immunoprecipitated with unrelated antibody as negative control. **Lane 5:** molecular weight standard.

lung, and skin. A panel of experimental human tumors derived from well characterized neoplastic cell lines xenografted in nude mice was also immunohistochemically evaluated. In our experience the mAbs to CD44 variant exon products showed a variable (staining 50–70% of the cells) or heterogeneous (staining <10% of the cells) reactivity with the majority of the neoplastic epithelial tissues, demonstrating the presence of specific CD44 isoforms. In several instances the expected immunoreactivity of some mAbs to CD44 variant exon products was not observed, despite the presence of specific CD44v transcripts in the target tissues. This inconsistent reactivity was negligible for mAbs to CD44v6 and CD44v3. For all other reagents, the occurrence of potential false negative results during immunohistochemical procedures was estimated as summarized in Table 4. However, the presence of specific CD44v transcripts, demonstrated in RT-PCR, do not per se guarantee the expression of CD44 isoforms at detectable protein levels, so the percentage reported in Table 4 could be overestimated.

In any event, the *in vitro* findings reported above demonstrate that the occurrence of false negative results in immunohistochemical studies on CD44v expression *in vivo* using mAbs to CD44 variant exon products is a real possibility. Because many of the mAbs directed at CD44

variant exon epitopes used in this study were raised against nonglycosylated fusion proteins produced by bacteria, it seems likely that some of these reagents could not recognize native antigens. To investigate this hypothesis, mAbs to CD44v5, v7–8, and v10 and polyclonal antisera to CD44v3–10 were used in Western blotting to detect specific CD44 isoforms after denaturation of the corresponding cell lysates from Namalwa transfectants and selected cell lines. In our hands, these reagents did not show any immunoreactivity in either reducing or nonreducing conditions (data not shown).

Differences in Glycosylation Can Affect Immunoreactivity of mAbs to CD44s

The possibility that posttranscriptional modifications of CD44 may affect the immunoreactivity of specific mAbs is supported by the fact that CD44s N-glycosylation site-specific mutants were not always recognized by mAbs to pan-CD44. In fact, as demonstrated in FACS analysis (Table 5), immunodetection of CD44s is severely impaired in MC melanoma transfectants expressing CD44s molecules carrying specific mutations in N-glycosylation sites.

It is remarkable that N-linked sugar residues placed in positions NGS 1, NGS 2, NGS 2–5, and NGS 3–5 (see Figure 3) are critical for immunodetection of CD44 molecules by the majority of the mAbs to pan-CD44 used in this study. As reported in Table 5, substitution of the Asp residues in positions 25 (NGS1), 57 (NGS2), 57–120 (NGS2–5), and 100–120 (NGS3–5) strongly impairs the immunoreactivity of the BRIC mAbs, except for BRIC 214 and KZI, which seem to recognize glycosylation-independent epitopes.¹⁸

As reported above, several mAbs to CD44 variant exon products used in this study were raised by nonglycosylated fusion proteins and the possibility that glycosylation could result in altered tertiary structure of CD44v molecules, masking specific epitopes, has been investigated. Treating cell lines and Namalwa transfectants with tunicamycin and/or phenyl-N-acetyl- α -D-galactosaminide to inhibit N- and O-glycosylation respectively, we were unable to restore the immunoreactivity of those mAbs which failed to detect specific epitopes, but a consistent modulation of the staining was observed in FACS analysis for all other reagents, indicating that differences in the glycosylation status of CD44v, which is cell type-specific, can really impair the immunotargeting of CD44 exon-specific mAbs (unpublished results).

Removal of GAG Side Chains Inserted on Large CD44v Isoforms Can Change the Immunoreactivity of Some mAbs to CD44 Variant Exon Products

To investigate whether the presence of GAG side chains may alter the immunoreactivity of mAbs to CD44 variant exon products, we performed the experiment shown in Figure 7. Breast carcinoma cell line Sab (constitutively

Figure 6. Comparative RT-PCR and FACS analysis on Sab cell line and Namalwa CD44v7-10 transfectant, using CD44 exon-specific primers (V7F and V8F) and specific mAbs to CD44v7 and CD44v7.8 epitopes, respectively. **Lanes 1 and 2:** PCR panel shows v7 and v8 transcripts derived from Sab cell line. **Lanes 3 and 4:** The same transcripts derived from Nam CD44v7-10. **Lanes 5 and 6:** Negative controls in which the absence of genomic DNA amplification was confirmed using nonretrotranscribed nucleic acid samples (total RNA) as PCR templates. **Lane M:** Molecular weight standard. On the bottom is shown the PCR amplification scheme in which each forward primer (V7F and V8F) is complementary to the sequence of CD44 variant exon v7 and v8, and the reverse primer (C16R) complementary to the sequence of the constant exon C16. The FACS panel shows a comparative immunoreactivity on both Sab cell line and NamCD44v7-10 transfectant. **Column 1:** Fitc staining as negative control. **Column 2:** staining with mAb to CD44v7. **Column 3:** staining with mAb to CD44v7.8.

expressing CD44 exon products v3 and v6-10) was stained with mAb to CD44v7 epitope when cultured in basal conditions. However, after treatment with 4-methylumbelliferil- β -D-xyloside, which inhibits the attachment of GAG side chains on CD44v molecules, this immunoreactivity was lost. A parallel decrease in CD44 molecular weight due to inhibition of the attachment of GAG side chains on the CD44v3,6-10 molecules was observed in immunoprecipitation assay (Figure 8). This experiment demonstrates that in particular, CD44 exon assortments (ie, Sab cell line v3,6-10) the presence of GAG side chains is necessary to expose v7 epitope, probably generating an open configuration of the molecule regarding the exon product v7. On the contrary, the immunoreac-

tivity of mAb anti-CD44v7 was not modulated in xyloside-treated Namalwa transfectants CD44v7-10, v6-10, and v3-10, demonstrating the importance of both the exon assortment and the molecular conformation compared to the presence of the GAG side chains (data not shown).

Discussion

CD44 is a ubiquitous molecule expressed mainly on leukocytes but also on fibroblasts and cells of mesodermal

Table 4. Percentage of Potential False Negative Results Using mAbs to CD44 Variant Exon Product in Immunohistochemical Assay Compared to RT-PCR

mAbs	Percentage*
anti-CD44v6	3.3
anti-CD44v3	7
anti-CD44v4-5	57
anti-CD44v5	43
anti-CD44v7	20
anti-CD44v7-8	13
anti-CD44v10	80
polyclonal to CD44v3-10	100

Each mAb was tested on a panel of 30 tissue samples including normal and neoplastic tissue from breast, colon, lung, thyroid, skin, and lymph nodes, previously selected for the presence of specific CD44v transcripts in RT-PCR.

*Percentage calculated as follows: Number of positive cases in immunohistochemistry/Number of positive cases in RT-PCR $\times$ 100.

Figure 7. Immunostaining of CD44 variant isoform v3,6-10 expressed on Sab cell line, using mAb specific to CD44v7 epitope in basal condition and after inhibition of GAG side chain attachment, as demonstrated in FACS analysis. This analysis shows that immunoreactivity of mAb to CD44v7 epitope is lost after β -xyloside treatment. Fitc, negative control; IM-7, positive control of CD44 expression on Sab cell line.

Table 5. Reactivity of a Panel of mAbs to CD44s on MC-melanoma Cell Lines Stably Transfected with CD44s N-linked Glycosylation Site-specific Mutants (FACS Analysis)

mAbs	BRIC 35	BRIC 205	BRIC 214	BRIC 219	BRIC 222	BRIC 223	BRIC 225	BRIC 235	BRIC 241	BRIC KZ1	J173	IM7
Cell lines												
MC-CD44s no mutated sites	100% (175)	100% (828)	100% (687)	100% (752)	100% (658)	100% (698)	100% (409)	100% (752)	100% (721)	100% (567)	100% (167)	100% (357)
MC-CD44 NGS 3-4 mutated sites 3 and 4*	100% (176)	100% (812)	100% (610)	100% (713)	100% (669)	100% (633)	100% (209)	100% (721)	100% (637)	100% (499)	99% (77)	100% (299)
MC-CD44 NGS 1 mutated site 1	11% (7)	25% (12)	97% (341)	22% (19)	26% (29)	16% (12)	6% (6)	21% (17)	24% (20)	98% (200)	11% (9)	22% (10)
MC-CD44 NGS 2 mutated site 2	18% (10)	34% (16)	100% (347)	17% (12)	26% (17)	14% (10)	9% (5)	23% (14)	20% (13)	100% (248)	9% (7)	32% (13)
MC-CD44 NGS 2-5 mutated sites 2 and 5	17% (10)	40% (25)	90% (40)	23% (14)	27% (17)	20% (11)	5% (4)	34% (16)	26% (13)	98% (49)	12% (8)	31% (15)
MC-CD44 NGS 5 mutated site 5	29% (23)	88% (65)	95% (97)	80% (38)	79% (52)	73% (32)	41% (16)	85% (38)	92% (41)	97% (88)	59% (26)	71% (25)
MC-CD44 NGS 3-5 mutated sites 3 and 5	4% (4)	9% (4)	87% (51)	6% (5)	8% (6)	4% (4)	1% (3)	16% (9)	8% (6)	75% (21)	3% (4)	41% (10)

The percentage of positive cells and the values of the mean (in parentheses) are reported.

*The schematic representation of mutated N-glycosylation sites is shown in Figure 3.

and neuroectodermal origin. This receptor can be structurally and functionally considered one of the most variable surface molecules; in fact, alternative splicing of variant exons as well as posttranscriptional modifications (ie, glycosylation) enrich the CD44 repertoire, which in turn may increase the optional functions of the molecule. The theoretical insertion of the whole variable region (amino acids 224–604) of the mature human CD44 sequence generates an extra stretch of 381 amino acids which can produce CD44 isoforms with molecular weight > 220 kd. In comparison, the molecular weight of CD44s standard receptor, which does not include variant exon products, is 85–90 kd. Furthermore, the whole CD44 variable region codified by exon v2 to v10 (reported in Figure 1) possesses four additional potential N-glycosylation sites and a large number of extra O-glycosylation sites.^{7,9} Consequently, CD44v isoforms are likely to be highly glycosylated.

CD44v molecules are preferentially expressed on epithelial malignant lesions and some of them have recently been regarded as promising tools for the improvement of diagnostic accuracy and for predicting the unfavorable outcome of several neoplastic diseases.¹³ Furthermore, the possibility of interfering with specific functions mediated by these molecules has been also proposed as the basis for a new therapeutic approach to inhibiting tumor progression.²

With the aim of identifying *in vivo* the expression of CD44 variants on human tumors, mAbs directed against specific CD44 variant exon epitopes have been developed and are widely used in flow cytometry and immunohistochemical analysis. Although the immunohistochemical use of these reagents may still be informative in large and well-designed studies on human tissues, our findings demonstrate that such assays provide partial information about the real pattern of CD44v expression *in vitro* and *in vivo*.

It is important to remember that these reagents do not allow the identification of the specific exon assortment in CD44v molecules, but rather define exclusive epitopes on a specific exon product, which can sometimes be inaccessible for the complex structural variability of CD44v molecules. In fact, exon-specific epitopes can be included in a short CD44v molecule as well as in larger and highly glycosylated isoforms representative of a more complex exon assortment.

In this study we demonstrate that (1) in a model of well characterized neoplastic cell lines and CD44v transfectants, specific exon assortments and/or posttranscriptional modifications of CD44 variant molecules can mask exon-specific epitopes; 2) GAG side chains, carried by high molecular weight CD44v isoforms, may be part of the epitopes recognized by mAbs to CD44 variant exon products or, more likely, may play a critical role in determining the exact conformation of the molecule necessary to expose exon specific epitopes; (3) in a panel of transfectants expressing CD44 N-glycosylation site-specific mutants generated in the constant region of CD44 extracellular domain, asparagine-isoleucine substitution is sufficient per se to impair the immunoreactivity of several mAbs to pan-CD44.

These data strongly support the hypothesis that some sugar residues, when placed in the invariant CD44s extracellular domain, can be part of the epitopes recognized by specific CD44 mAbs. The finding that the N-glycosylation status of CD44 molecules, which is cell type-specific, can potentially affect the immunoreactivity of several pan-CD44 mAbs helps to explain the difficulties encountered in trying to epitope-map a number of CD44 mAbs over the past 10 years. At the same time, it may explain the failure of some exon-specific mAbs to detect their respective epitopes on highly N- and O-glycosylated CD44v molecules.²¹ In fact, it is noteworthy that both flanking sequences contributed by the alterna-

Figure 8. Comparative immunoprecipitation of CD44v from cell lysates of Sab cell line in basal condition and after treatment with β -xyloside, using mAb BRIC 235. The decrease in molecular weight and the reduction of the smear band observed after β -xyloside treatment are consistent with the inhibition of GAG side chains attachment on CD44 molecules. **Lane 1:** CD44 molecular species immunoprecipitated in basal condition; **Lane 2:** Immunoprecipitation after treatment of the cell line with β -xyloside. **Lane 3:** Immunoprecipitation of the same cell lysate with unrelated mAb used as negative control. **Lane 4:** Molecular weight standard.

tive splicing of CD44v exons, as well as the different degree of glycosylation of CD44 isoforms, are potentially able to modify the conformation of a given epitope and hence render it available for antibody recognition.

In conclusion, we have shown that the reactivity of some mAbs directed at the CD44 variant exon epitopes can be impaired by the structural variability of CD44. These data suggest the potential for a large number of false negative results deriving from immunohistochemical studies focusing on CD44v expression *in vivo*, in particular on neoplastic epithelial tissues in which the glycosylation machinery is qualitatively and quantitatively altered. The possibility of this occurrence should be always considered during the immunohistochemical evaluation of CD44 expression *in vivo*.

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References

1. Arch R, Wirth K, Hofmann M, Ponta H, Matzku S, Herrlich P, Zoller M: Participation in normal immune responses of a metastasis-inducing splice variant of CD44. *Science* 1992, 257:682-685
2. Günthert U, Hofmann M, Rudy W, Reber SZ, Zoller M, Haussmann I, Matzku S, Wenzel A, Ponta H, Herrlich P: A new variant of glycoprotein CD44 confers metastatic potential to rat carcinoma cells. *Cell* 1991, 65:13-24
3. Bartolazzi A, Peach R, Aruffo A, Stamenkovic I: Interaction between CD44 hyaluronate is directly implicated in the regulation of tumor development. *J. Exp Med* 1994, 180:53-66
4. Srean GR, Bell MV, Jackson DG, Cornelis RB, Gerth U, Bell JL: Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons. *Proc Natl Acad Sci USA* 1992, 89:12160-12164
5. Aruffo A, Stamenkovic I, Melnik M, Underhill CB, Seed B: CD44 is the principal cell surface receptor for hyaluronate. *Cell* 1990, 61:1303-1313
6. Bartolazzi A, Jackson DG, Bennett K, Aruffo A, Dickinson R, Shields J, Whittle N, Stamenkovic I: Regulation of growth and dissemination of a human lymphoma by CD44 splice variants. *J Cell Sci* 1995, 108:1723-1733
7. Bartolazzi A, Nocks A, Aruffo A, Spring F, Stamenkovic I: Glycosylation of CD44 is implicated in CD44-mediated cell adhesion to hyaluronate. *J Cell Biol* 1996, 132:1199-1208
8. Bennett KL, Modrell B, Greenfield B, Bartolazzi A, Stamenkovic I, Peach R, Jackson DJ, Spring F, Aruffo A: Relation of CD44 binding to hyaluronan by glycosylation of variably spliced exons. *J Cell Biol* 1995, 131:1623-1633
9. Günthert U. CD44 in malignant disorders: *Curr Top Microbiol Immunol* 1996, 213:271-285
10. Lesley J, Hyman R: Variant cell lines selected for alterations in the function of the hyaluronan receptor CD44 show differences in glycosylation. *J Exp Med* 1995, 182:431-437
11. Skelton TP, Zeng C, Nocks A, Stamenkovic I: Glycosylation provides both stimulatory and inhibitory effects on cell surface and soluble CD44 binding to hyaluronan. *J Cell Biol* 1998, 140:431-446
12. Koopman G, Heider KH, Horst E, Adolf GR, van den Berg F, Ponta H, Herrlich P, Pals ST: Activated human lymphocytes and aggressive non-Hodgkin's lymphomas express a homologue of the rat metastasis-associated variant of CD44. *J Exp Med* 1993, 177:897-8904
13. Naor D, Vogt Sionov R, Ish-Shalom D: CD44: structure, function, and association with the malignant process: *Adv Cancer Res* 1997, 71:241-319
14. Matsumura Y, Tarin D: Significance of CD44 gene products for cancer diagnosis and disease evaluation. *Lancet* 1992, 340:1053-1058
15. Sy MS, Guo YJ, Stamenkovic I: Inhibition of tumor growth *in vivo* with a soluble CD44-immunoglobulin fusion protein. *J Exp Med* 1992, 176:623-627
16. Katoh S, McCarthy JB, Kincade PW: Characterization of soluble CD44 in the circulation of mice. Levels are affected by immune activity and tumor growth. *J Immunol* 1994, 153:3440-3449
17. Natali PG, Nicotra MR, Bartolazzi A, Cavaliere R, Bigotti A: Integrin expression in cutaneous malignant melanoma: association of the $\alpha 3 \beta 1$ heterodimer with tumor progression. *Int J Cancer* 1993, 54:68-72
18. Antsee DJ, Gardner B, Spring FA, Holmes CH, Simpson KL, Parsons SF, Mallison G, Yousaf SM, Judson PA: New monoclonal antibodies in CD44 and CD58: their use to quantify CD44 and CD58 on normal human erythrocytes and to compare the distribution of CD44 and CD58 in human tissues. *Immunology* 1991, 74:197-205
19. Jackson DG, Buckley J, Bell JL: Multiple variants of the human lymphocyte homing receptor CD44 generated by insertions at a single site in the extracellular domain. *J Biol Chem* 1992, 267:4732-4739
20. Ho SN, Hunt HD, Horton RM, Pullen JK, Pease LR: Site-directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene* 1989, 77:51-59
21. Schlossman SF, Boumsell L, Gilks W, Harlan JM, Kishimoto T, Morimoto C, Ritz J, Shaw S, Silverstein R, Springer T, Tedder TF, Todd RF: *Leucocyte Typing V*. Oxford, Oxford University Press, 1995, pp 1713-1719