CD44 Isoform Expression Follows Two Alternative Splicing Pathways in Breast Tissue

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The repertoire of distinct CD44 protein isoforms is generated by means of alternative pre-mRNA splicing of 10 variable exons located in the central region of the CD44 gene. We have used human breast ductal carcinoma as a model to identify two alternative splicing pathways of the CD44 pre-mRNA variable region that account for the generation of all of the CD44 isoforms described in breast tissue. An alternative splicing pathway that reflects inclusion of variable exons in a gradual 3'-to-5' fashion is evidenced in breast ductal carcinoma and its lymph node metastases. This pathway is compatible with a mechanism that generates the standard form of CD44 (devoid of variable exons) and is distinguishable from an alternative splicing pathway that involves exclusively variant exon 3 and is observable in both normal and carcinoma breast tissue. We show that both pathways are detectable in the same cell type in the breast and provide a speculative model by which these splicing routes could take place. (*Am J Pathol* 1998, 153:183-190)

Regulation of cell-cell and cell-matrix interactions via cell adhesion molecules is a critical step during the metastatic spread of tumors.^{1,2} CD44, a family of cell-surface glycoproteins, has been reported to play a role in cell adhesion and tumor dissemination, but the precise nature of the involvement of CD44 in these processes is still under investigation. The CD44 gene consists of 20 exons from which at least 12 can be alternatively spliced from the pre-mRNA.^{3,4} CD44 exons are compartmentalized in the gene according to splicing behavior into four regions (Figure 1): two constant regions consisting of exons 1 to 5 and exons 15 to 17, which are subject to general constitutive splicing; a region composed of exons 18 and 19, which show an alternate use of a short or long cytoplasmic tail, respectively; and a central region that spans exons 6a to 14, also known as variable exons (v-exons)

v1 to v10. Complex alternative splicing applies to this central region, which is responsible for the incorporation of the variable domains into the extracellular portion of the final CD44 protein product. The category of complex alternative splicing has been arbitrarily applied to a variety of genes that have multiple v-exons that appear to be included in or excluded from the processed mRNA in a complex manner to produce multiple mRNA isoforms from the same gene (for review see Ref. 5). Complex alternatively spliced CD44 messages are being described as the result of a cellular response to particular stimuli⁶⁻¹². These stimuli are themselves effectors of physiologically or developmentally regulated phenomena, which suggests that these complex alternative splicing mechanisms are subject to specific factors acting in *trans* together with the spliceosome and/or in *cis* for, supposedly, every possible variable exon. The role of specific alternative splice *trans*-factors that drive the choice of 5' splice sites has been the subject of recent studies.^{11,13} Interestingly, a positive *trans*-acting factor(s) has been suggested to recruit variant exon sequences in a 3' to 5' direction during CD44 splicing, and other dominant *trans*-factors are thought to select distinct CD44 variable exons for inclusion in a cell-type-specific manner.¹⁴ The expression of certain, such generated CD44 isoforms has been implicated in important physiological and pathological conditions, including lymphocyte activation, leukocyte-endothelial transmigration, lymphopoiesis, embryonal development, and the metastatic spread of tumors (for review see Refs. 15-18).

Several functions have been ascribed to some CD44 isoforms. The standard form of CD44 (CD44H), devoid of variable exons, acts as a receptor that binds the extracellular matrix, mostly via hyaluronic acid,¹⁹⁻²⁴ with an activity dependent on post-translational modifications, such as keratan sulfate substitution and O-linked glycosylation.^{25,26} The epithelial form of CD44 (CD44E), which includes v8 to v10, is involved in recognition of a common determinant in CD44H and CD44E promoting homotypic

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Figure 1. Schematic representation of the CD44 gene structure showing the central variable region. The variable exon nomenclature used in this work is shown above each variable exon (shaded boxes). TM, transmembrane domain. Human exon v1 is crossed out to indicate that it contains a stop codon and has not been found to be expressed in human tissues.⁴⁰

cellular aggregation.²⁷ The protein domain coded by exon v3 has been shown to bind growth factors via its heparan sulfate modifications,^{28,29} and a metastatic activity has been attributed to v6-containing isoforms after transfection studies in rat tumors.³⁰ The expression of particular CD44 isoforms is well established in neoplasms such as non-Hodgkin's lymphomas, some gastric carcinomas, colorectal carcinomas, and sarcomas as well as in some cell line models.^{31–35} Human breast cancer has been shown to express CD44 isoforms, but the definition and significance of this expression has been controversial.^{36–39} We have used different techniques, including reverse transcription polymerase chain reaction (RT-PCR), immunohistochemistry, PCR library cloning, and DNA sequencing, to study the nature of the CD44 isoforms expressed in normal breast tissue, breast ductal carcinoma, and axillary lymph node metastases. The CD44 isoforms characterized in this study reveal two pathways of alternative splicing of CD44 v-exons in breast tissue. These pathways are not mutually exclusive and can be easily integrated into a single hypothetical model of CD44 complex alternative splicing. The description of such alternative splicing routes will help in the eventual identification of their underlying alternative splicing mechanisms and, consequently, in our understanding of the role of CD44 in physiological and pathological processes.

Materials and Methods

Materials

Cryopreserved (–80°C) human tissue from normal breast and invasive ductal carcinoma of the breast (including axillary lymph nodes when these were involved and available) from 43 females, diagnosed between 1993 and 1995, were randomly selected from the frozen tissue bank of the Department of Pathology at the Hospital Universitari Germans Trias i Pujol, Badalona, Spain.

CD44 RT-PCR

RT-PCR was performed from 5 µg of total RNA extracted from cryopreserved breast biopsy samples with the Ultraspec RNA solution (Biotecx Laboratories, Houston, TX). The first-strand reaction was performed with the first-strand synthesis kit (Pharmacia, Uppsala, Sweden) followed by PCR using oligonucleotide primers FHCD44 in exon 5 (5'-CCTGAAGAGATCTACCCAGCAACCCT-ACTG3') and RHCD44 in exon 19 (5'-TGGTGC GGCCGT-TACACCCAATCTTCATGTCC3'). PCR cycling condi-

tions were 94°C for 1 minute, 55°C for 1 minute, and 72°C for 2.5 minutes for 30 cycles using *Taq* polymerase Eurobio (Eurobio, Les Ulis, France). Starting amounts of cDNA template were adjusted in all samples by PCR amplification of β -actin as an internal control (data not shown). CD44-specific PCR products were separated by electrophoresis in a 1% agarose gel, visualized by ethidium bromide staining, denatured in 0.5 N NaOH, 1.5 mol/L NaCl, and transferred overnight in the same solution to a nylon membrane (Schleicher & Schuell, Dassel, Germany) by standard methods. Filters were prehybridized in 7% SDS, 0.5 mol/L phosphate buffer, 1% bovine serum albumin and hybridized overnight in the same solution at 42°C with 5 pmol of digoxigenin-labeled oligonucleotide, processed, and stripped using the DIG-oligonucleotide labeling and luminescent detection kit (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol. The oligonucleotide probes used were (5'–3') CD44STD probe in exon 15: CATCTGATTCAGATCCATGAGT and the following CD44 variable exon probes: CD44V2, CAGCCATTTGTGTTGT-TGTGTGAAG; CD44V3, TGGTGCTGGAGATAAAATCT-TCAT; CD44V4, CAGTCATCCTTGTGGTTGCTGAAGT; CD44V5, TTGTGCTTGTAGAATGTGGGGTCTCT; CD44V6, CAGCTGTCCCTGTTGTCAATGGGA; CD44V7, CCATC-CTTCTTCCTGCTTGATGAC; CD44V8, GCGTTGTCATT-GAAAGAGGTC; CD44V9, TGCTTGATGTCAGAGTAGAAG-TTGTG; and CD44V10, CTGATAAGGAACGATTGACATT-AGAGT.

CD44v PCR Libraries

Variable CD44 isoform PCR products were purified using the GeneClean kit (Bio101, La Jolla, CA) from 1% TAE/agarose gels and cloned by blunt-end ligation into pUC18 vector with the SureClone ligation kit (Pharmacia, Uppsala, Sweden). Resulting positive colonies were replica plated onto nylon filters and hybridized as explained above. Plasmid minipreps were prepared by standard methods, and the insert size of each clone was determined by an *EcoRI*-*Bam*HI digestion (New England Biolabs, Beverly, MA) before DNA sequencing with the T7 sequencing kit (Pharmacia).

CD44 Immunohistochemistry

Immunohistochemical detection of CD44 was performed with anti-CD44s, clone 2C5; anti-CD44v3, clone 3G5; and anti-CD44v6, clone 2F10 (all from R&D Systems, Abingdon, UK). Five-micron-thick sections were cut from paraffin-embedded biopsy samples, deparaffinized, hydrated, heated in buffered citrate (citric acid and sodium citrate, pH 6) in a microwave oven twice for 3 minutes with a 2-minute interval, and incubated for 30 minutes with rabbit serum. Incubations with the anti-CD44 monoclonal antibodies (MAbs) were carried out at a 1:1000 dilution for 22 hours at room temperature. Slides were washed and incubated with biotinylated rabbit anti-mouse Ig antibodies at a 1:700 dilution and then incubated in PBS/6% hydrogen peroxide for 15 minutes at

Figure 2. CD44 RT-PCR patterns obtained from duct carcinoma (C), lymph node (L), and normal (N) breast tissue, visualized by hybridization of the RT-PCR product with probe CD44STD. Examples of the CD44 isoform pattern complexity were obtained from biopsies 43, 42, and 41. The histogram shows the summary of CD44 isoform pattern complexity found by RT-PCR in biopsy samples from 43 patients. Pattern complexity was arbitrarily established as high when detection of a CD44v2-v10 (1730 bp) was detected, medium when a CD44v6-v10 (1244 bp) was detected as the top band of a particular pattern, and low when only CD44E (983 bp) and CD44H (587 bp) were detected.

room temperature before the avidin-biotin-peroxidase complex addition (Dakopatts, Glostrup, Denmark). The chromogen 3,3'-diaminobenzidine tetrachloride (Serva, Heidelberg, Germany) was used, and counterstaining was performed with Harris hematoxylin. A nonimmune mouse serum was used as a negative control in this protocol.

Results

Complex CD44 Isoform Expression Patterns in Breast Ductal Carcinoma Samples

To establish the extent and the complexity of the CD44 isoform pattern expressed by normal breast tissue and breast ductal carcinoma we have used a protocol consisting of RT-PCR followed by Southern blot hybridization with the CD44STD probe (in CD44 constant exon 5), which hybridizes to all of the possible CD44 isoforms described to date. The type of CD44 pattern complexity obtained is exemplified in Figure 2. Eighty-one percent (34 of 42) of the carcinoma samples studied showed complex patterns that included detection of CD44v2-10 (as summarized in the histogram shown in Figure 2).

The Complexity of the CD44 Isoform Expression Pattern in Breast Carcinomas Follows a Directional 3' to 5' Preference for Variable Exon Inclusion

The precise exonic content of each of the patterns obtained above was characterized by serial hybridization using variable exon-specific probes from CD44v10 to CD44v2 (lanes v10 to v2 in Figure 3). Three representative samples from breast ductal carcinoma, its lymph node metastases, and normal breast tissue are shown in Figure 3. The hybridization patterns of the carcinoma and lymph node metastases are very similar, and both present a step-wise inclusion of variable exons, starting with the 3'-most variable exons (eg, v10 and v9) in low M_r isoforms and gradually moving toward 5' variable exons (eg, toward v2) with a concomitant increase in isoform M_r . A band at 1730 bp represents the CD44v2-v10 isoform and the band below it is compatible by M_r with CD44v3-v10. Lanes v2 in the carcinoma and lymph node metastases show that the relative signal intensity between bands CD44v2-v10 and CD44v3-v10 is inverted when compared with all of the other lanes. The differential signal in these two bands suggests that the CD44v3-v10

Figure 3. CD44 RT-PCR patterns visualized by hybridization of the RT-PCR product with probe CD44STD and variable exon-specific serial Southern hybridization of breast ductal carcinoma (C), lymph node metastasis (L), and normal breast tissue (N). The probes used appear at the bottom of each lane. Lanes labeled v10 to v2 correspond to the pattern observed in lanes STD after rehybridization with each variable exon probe. The exonic content of the CD44 isoforms derived from this serial hybridization appears on the right. *1 corresponds to the highest band-doublet in complex CD44 isoform patterns that is not compatible by M_r with known CD44 isoforms (see text).

band also results from different combinations of isoforms, including all but one variable exon that would form a polyisoform band at approximately 1600 bp, reactive to all v-exon probes. The two highest M_r bands shown in Figure 3 (*1) hybridize with all CD44 probes used and may correspond to inclusion of intron or other v-exon sequences not as yet identified. The nature of these bands is currently under investigation.

Variable Exon-3 Can Be Alternatively Spliced through a Different Pathway That Is Functional in Normal and Carcinoma Tissue

Exon v3 is subject to the 3'-to-5' exon inclusion preference described above but is also clearly detectable by itself in a low M_r isoform that corresponds to CD44v3 alone (lanes v3, Figure 3). This strict v3-cassette splicing route becomes differentiated from the 3'-to-5' v-exon inclusion preference as the former is unaltered between normal breast tissue and breast ductal carcinoma or its lymph node metastases.

CD44 cDNA Isoform Cloning from Breast Carcinoma Reveals Atypical or Minor Isoforms

A CD44-variable isoform RT-PCR library constructed after gel purification omitting CD44H from the carcinoma sample shown in Figure 3 allowed screening of up to 150 CD44 variable clones by v-exon-specific colony hybridization and DNA sequencing. We can infer the relative levels of expression of each isoform characterized by classifying these into two categories: major isoforms that are reproducibly detected in most samples by hybridization and rare isoforms that have been cloned and characterized from breast carcinoma but are not detectable by hybridization. All major isoforms have been cloned and follow the two alternative splicing trends described, with the exception of the previously described CD44v6,³⁵ which we detect by hybridization although its resulting band is clearly detectable only in the case shown on lane v6 of the lymph node metastases in Figure 3. Other exceptions include the expression of rare isoforms obtained from the CD44v RT-PCR library, such as CD44v9 and CD44v6/v8-v10. These isoforms may represent inaccurate splicing by either of the two pathways proposed or may be the result of other underlying alternative splicing mechanisms (a likely case for CD44v6) of low activity in the breast tissue analyzed. However, the low profile of these minor isoforms does not mask the conditioned v-exon position effect seen in the samples studied.

The CD44v3 exon is reproducibly seen by itself in a CD44H context as well as in the context of major isoforms such as CD44E and CD44v7v10, yielding CD44v3, CD44v3/v8-v10, and CD44v3/v7-v10, respectively. This combination of v3-containing isoforms would be expected from either an overlap or an integration of the two alternative splicing pathways proposed.

Both Alternative Splicing Routes Co-Localize to the Same Cell Type in Breast Tissue

To find out whether these two alternative routes are differentially used by certain cells in breast tissue, we have analyzed 5- μ m-thick tissue sections of the breast samples represented above by immunohistochemistry using MAbs anti-CD44H, anti-CD44v3, and anti-CD44v6 (respectively, clone 2C5, clone 3G5, and clone 2F10 from R&D Systems, Abingdon, UK; Figure 4). A positive immunoreaction for all three MAbs is present in carcinoma cells, whereas normal tissue was unreactive to anti-CD44v6, as expected from the variable exon-specific Southern hybridizations. Infiltrating lymphocytes, negative for v3 and v6 but reactive to anti-CD44H, were used as internal controls (see arrows on Figure 4). CD44H expression in normal breast tissue is restricted to the cell membranes of the myoepithelial cell layer, located on the duct basal portion next to the basal lamina. Immunostaining with anti-v3 presented the same pattern in normal tissue, suggesting that the same pool of cells is capable of expressing CD44H- and CD44v3-containing isoforms and that our RT-PCR analyses were not affected by the presence of inflammatory or stromal cells, which are CD44v negative. Likewise, parallel staining on defined nests of infiltrating carcinoma cells showed expression of CD44H-, CD44v3-, and CD44v6-containing isoforms in all cells. These results, compatible with previously published data³⁶ in that expression of CD44H and CD44v isoforms is restricted to the same cell type, suggest that both splicing pathways can coexist in the same cell.

Discussion

The 3'-to-5' v-exon inclusion preference observed during the expression of CD44 in the breast carcinoma tissues studied appears to respond to a mechanism that ascribes a positional value to each variable exon. This means that variable exon inclusion starts with the 3'-most variable exons and gradually moves toward 5' variable exons with a concomitant increase in isoform M_r . The existence of such a mechanism had been previously suggested,⁴⁰ and a positive *trans*-acting factor responsible for directional v-exon inclusion has recently been postulated after a dominant effect was observed in cell fusion experiments.¹⁴ The variable exon composition of the major isoforms reported herein reveals an inclusion gradient driven by the 3'-most v-exon that is included in the message, such that if a given isoform includes v9, the probability that the same isoform has v10 is 100%. In the same manner, inclusion of v9 is always seen in isoforms containing v8. This trend is true for all variable exons with the exception of v3. Exon v3 can escape this inclusion trend as only 50% of all of the different v3-containing isoforms described also include v4. By parsimony, and as an alternative to the idea of one positive *trans*-acting factor that actively recruits v-exons,¹⁴ we speculate that in breast carcinoma exon v3 in the CD44 pre-mRNA can be somehow tagged for inclusion before undergoing the 3'-to-5' v-exon inclusion preference route. This route

Figure 4. Representative immunohistochemical findings (X400) of normal breast tissue (N) and invasive ductal carcinoma of the breast (C) using MAbs anti-CD44H, anti-CD44v3, and anti-CD44v6. Infiltrating lymphocytes, which stain with anti-CD44H but are nonreactive to anti-CD44v3 and anti-CD44v6, are used as internal controls (**arrows**). Positivity in normal breast tissue is restricted to the cell membrane of myoepithelial cells. Invasive carcinoma nests, reactive to all three MAbs, show positivity mainly on their cell membranes.

would then be aimed at effectively hiding the untagged v-exons on the CD44 pre-mRNA variable region from the active constitutive spliceosome, as sketched in Figure 5. The directional v-exon inclusion would result from enabling gradual splicing of the variable region by the spliceosome in a 3'-to-5' needle-pulling-thread action. This would ensure production of CD44H and/or CD44v3 when exon-v3 was tagged, and subsequently, gradual directional v-exon inclusion would account for the scope of CD44 isoforms expressed by a single cell. Furthermore, this pathway might well be altered by pathological processes such as tumorigenesis or by physiological signals. In both cases, however, the broadening of the CD44 isoform pattern may be induced or reversed by changes in environmental or intracellular signals. A situation that can be envisaged without a need for genetic

mutations causing impairment of the alternative splicing of CD44.

This observation, plus the fact that total impairment of CD44H production is not seen in carcinoma cells, implies that the different degree of the 3'-to-5' v-exon inclusion preference pathway is not the result of a clonal somatic change in the cell. Instead, this difference may respond to an epigenetic alteration brought about by the new environment or different architecture of the carcinoma tissue. Interestingly, expression of at least CD44H and CD44E has been involved in different forms of cellular aggregation.²⁷ Consequently, the question of whether newly developing neoplastic architectural arrangements can signal changes to the basal CD44 splicing patterns of the tissue of origin becomes an important one. In these terms, CD44 isoform expression cannot be categorically

Figure 5. A hypothetical model to represent the concept of integration of the 3'-to-5' v-exon inclusion preference and the strict v3-cassette inclusion splicing routes found in breast carcinoma tissue. The CD44 pre-mRNA is shown at the top. Constant exons and CD44 variable exons are represented by black and white boxes, respectively. Gradual directional inclusion is represented by occlusion of the v-exons in a hypothetical loop or trans-acting factor enclosed within a box to illustrate the needle-pulling-thread effect during the unmasking of the variable region to the active spliceosome. Resulting CD44 isoforms are shown next to each hypothetical pre-mRNA splicing intermediate. The extent of the variability of the CD44 isoforms observed in normal and carcinoma breast tissue is exemplified by the two vertical arrows on the right. We postulate that this 3'-to-5' v-exon inclusion preference is aimed at generating CD44H. This splicing route appears to be rigidly controlled in normal tissue, where expression of CD44v9-v10 and CD44E probably represents legitimate leakage while splicing out the complete variable region. Gradual relaxation of the stringency of this control and its metastases would enable the orderly expression of a much broader set of variable isoforms ranging from low to high M_r .

considered as a tumorigenic or metastatic marker, as it is not distinguishable from either a marker of cell clonality or from a marker of a particular form of tissue architecture. This point will need clarification, as previous works have addressed the prognostic value of CD44 isoform expression in breast carcinoma with conflicting conclusions. Expression of CD44 variant exon 6 epitope and overexpression of CD44H and CD44E have been claimed to be markers of poor prognosis in breast cancers.³⁷⁻³⁹ Contrary to this claim, another report³⁶ has shown that expression of the above mentioned CD44 forms can be detected in normal breast epithelium, thus invalidating their potential as indicators of malignant disease. We have not observed significant correlation between the expression of the CD44 isoforms detected in the samples studied and any particular disease outcome (data not shown). Instead, the molecular dissection of the CD44 expression patterns detected in 81% of the ductal carcinomas studied has pointed to two active pathways of alternative splicing. Understanding the mechanisms underlying these splicing routes will inevitably help to answer the question of whether certain CD44 isoforms act as bona fide metastogenes or whether CD44 isoform expression provides only a window to the deregulation of alternative splicing processes that affect pleiotropically

the splicing of other genes that can act synergistically or independently of CD44 to promote the dissemination of this type of tumor.

The finding of a strict v3-cassette alternative splicing route gains particular interest in the light of the recently described role for v3-containing CD44 isoforms. These have been shown to act as presenting molecules for some growth factors, particularly the family of heparan-binding growth factors, such as basic fibroblast growth factor and heparan-binding epidermal growth factor.^{28,29} CD44 expression in normal breast tissue is confined to the myoepithelial cell layer, located on the duct basal portion next to the basal lamina (Figure 3). As these are mature cells that regulate breast duct contraction, it will be interesting to study whether the v3-containing CD44 isoforms ensured by the strict v3-cassette inclusion splicing play an active role in this process. Previous observations on the regulation of the growth state and contractile phenotype of blood-vessel-lining cells by heparan sulfate proteoglycans set a precedent for a role for CD44v3-containing isoforms in this type of function. The contractile status of smooth muscle fibers results from a balance between the internalization and external degradation of the heparan sulfate proteoglycans of the basal lamina.⁴¹ These proteoglycans are known to alter muscle cell

shape and cytoskeletal organization leading to cell growth inhibition.⁴² Furthermore, mutations in the *Caenorhabditis elegans* equivalent of perlecan, the mammalian basement membrane heparan sulfate proteoglycan, affect the organization of all contractile tissues in the developing nematode.⁴³ Thus, given the confined expression of CD44v3 isoforms found in the breast, it seems likely that modification (via down-regulation, degradation, or internalization) of CD44v3 isoforms associated with heparan sulfate glycosaminoglycans may contribute to regulate breast duct contraction during the secretory phase of lactation.

In brief, our observations reveal the presence of two CD44 alternative splicing pathways within the myoepithelial cell layer of the breast duct and in carcinoma derived from ductal epithelial cells. Whether these mechanisms are also used by other tissues, or whether there are other preferred routes of alternative splicing that result in the expression of particular CD44 isoforms not found in the breast, remains to be seen. Better understanding of the mechanisms used by CD44 to generate the spectrum of isoforms described so far will help to explain the functional involvement of CD44 in physiological and pathological processes, as well as reveal the routes and maneuvers used by a cell to handle very large pre-mRNAs in a purposeful manner.

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