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CD44 in inflammation and metastasis

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CD44 is a major cell surface receptor for the glycosaminoglycan, hyaluronan (HA). CD44 binds HA specifically, although certain chondroitin-sulfate containing proteoglycans may also be recognized. CD44 binding of HA is regulated by the cells in which it is expressed. Thus, CD44 expression alone does not correlate with HA binding activity. CD44 is subject to a wide array of post-translational carbohydrate modifications, including N-linked, O-linked and glycosaminoglycan side chain additions. These modifications, which differ in different cell types and cell activation states, can have profound effects on HA binding function and are the main mechanism of regulating CD44 function that has been described to date. Some glycosaminoglycan modifications also affect ligand binding specificity, allowing CD44 to interact with proteins of the extracellular matrix, such as fibronectin and collagen, and to sequester heparin binding growth factors. It is not yet established whether the HA binding function of CD44 is responsible for its proposed involvement in inflammation. It has been shown, however, that CD44/HA interactions can mediate leukocyte rolling on endothelial and tissue substrates and that CD44-mediated recognition of HA can contribute to leukocyte activation. Changes in CD44 expression (mainly up-regulation, occasionally down-regulation, and frequently alteration in the pattern of isoforms expressed) are associated with a wide variety of cancers and the degree to which they spread; however, in other cancers, the CD44 pattern remains unchanged. Increased expression of CD44 is associated with increased binding to HA and increased metastatic potential in some experimental tumor systems; however, in other systems increased HA binding and metastatic potential are not correlated. CD44 may contribute to malignancy through changes in the regulation of HA recognition, the recognition of new ligands and/or other new biological functions of CD44 that remain to be discovered.

***Keywords*: CD44, cell adhesion, hyaluronan, inflammation metastasis**

***Abbreviations*: aa, amino acid(s); CS, chondroitin sulfate; CSPG, chondroitin sulfate containing proteoglycan; CD44H, 'hematopoietic', also called 'standard', isoform of CD44 which contains none of the alternatively spliced variant exons; CD44-Rg, CD44 receptor globulin, a secreted chimaeric protein composed of the external domain of the adhesion receptor CD44 and the hinge, CH2 and CH3 regions of human immunoglobulin-G heavy chain; ECM, extracellular matrix; GAG, glycosaminoglycan; HA, hyaluronan; HS, heparan sulfate; KS, keratan sulfate; PB, peripheral blood; PBL, peripheral blood lymphocytes**

Introduction

CD44 is a type I transmembrane cell surface glycoprotein consisting of a single polypeptide chain. It is encoded by a single gene, with alternative splicing providing the potential for multiple isoforms. CD44 is expressed throughout development on many different cell types including hematopoeitic cells, fibroblasts, some epithelial and endothelial cells, and cells in the central nervous system. Changes in CD44 expression, including up-regulation, down-regulation and changes in the isoforms expressed, have been associated with a variety of malignancies. Proposed functions of cell surface CD44 include participation in cell adhesion and migration, hematopoiesis, lymphocyte activation, assembly of extracellular matrix (ECM), and metastatic behavior of tumor cells. In many cases, these activities can be attributed to the ability of CD44 to function as a receptor for components of the ECM, especially the glycosaminoglycan (GAG) hyaluronan (HA) (see reviews [1–4]).

The most common isoform of CD44, termed CD44H (for hematopoietic) or CD44s (for standard), is made up of a 248 amino acid (aa) external domain (for mature human CD44H), and highly conserved transmembrane (21 aa) and cytoplasmic (72 aa) domains [5]. This isoform has a $\sim$37 kDa core protein, but usually has a M_r of $\sim$80–90 kDa due to extensive glycosylation. It contains none of the 10 alternative (variant) exons which occupy the middle of the gene. Alternative splicing that includes one or more of the variant exons, generates higher M_r isoforms of CD44 by insertion of additional aa sequence in the

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membrane proximal region of the external domain [6, 7]. The amino-terminal ~170 aa sequence is highly conserved among mammalian species. It contains six cysteines that are thought to form three disulfide bonds, five potential N-linked glycosylation sites, and includes ~100 aa with sequence homology to other HA binding proteins, including link protein and aggregating proteoglycan. This conserved sequence is shared by all CD44 isoforms, regardless of the presence or absence of variant exon splicing.

Since both CD44 and its principal ligand HA are broadly distributed in the body, the specificity of cell–cell and cell-matrix activities in which they participate must depend upon regulation of the receptor-ligand interaction. Thus a major issue in the study of CD44 function, as in the study of other molecules involved in cell adhesion and migration such as integrins and selectins, concerns the regulation of ligand binding activity. While alternative splicing represents one mechanism of altering the function of CD44, cell type and activation-state differences in the post-translational modification of CD44 also influence function. CD44 undergoes extensive post-translational modification, including phosphorylation of serines and palmitoylation in the cytoplasmic domain, and in the external domain, N- and O-linked glycosylation and the addition of glycosaminoglycan side chains. Insertion of alternative exons in the membrane proximal region of the external domain provides additional potential glycosylation sites and further extends the range of the possible carbohydrate modifications that can be added to the polypeptide backbone in a cell-type specific manner. A number of recent studies show that cell specific carbohydrate modification can have profound effects on CD44 function.

This review will discuss two aspects of CD44 function which involve 'carbohydrate interactions': (1) CD44 recognition of carbohydrate ligands, principally HA, including the sequence requirements for this recognition and the importance of this recognition in inflammatory responses and in tumor metastasis; and (2) the regulation of CD44 function by differential glycosylation.

CD44 recognizes HA

Cloning of CD44 cDNAs revealed ~100 aa of the external domain near the amino terminus that show homology with a sequence common to HA binding proteins. Blocking of HA binding with CD44-specific mAb and the introduction of HA binding function by transfection of CD44 cDNA into CD44-negative cell lines proved that CD44 can indeed mediate binding to HA [8, 4]. The aa sequence requirements for this binding were analyzed by Peach *et al.* [9] using a chimaeric protein (CD44-Rg) consisting of the amino terminal two-thirds of the CD44 external domain and the hinge, CH2 and CH3 regions of human IgG heavy chain. This study defined two clusters of basic aa that were involved in HA binding (one within the HA-binding homology region and the other just outside it), both within the conserved amino-terminal portion of the extracellular domain [9]. Mutation of multiple basic residues in the second basic cluster (aa 151–163) reduced HA binding, but alteration of a single arginine (Arg-41) to alanine in the first cluster completely abrogated HA binding (aa numbering according to [5]). Yang *et al.* [10] proposed that a BX_7B motif (where B represents arginine or lysine and X_7 indicates a stretch of any 7 aa, but including one basic residue and no acidic residues) found in all known HA-binding proteins was capable of HA recognition. This idea was based on the results from Western blots of fusion proteins probed with biotinylated-HA. However, Telen *et al.* [11] found that the *CD44* gene expressed in cells with the In^a (a +b–) blood group polymorphism lacked the last basic residue of the first BX_7B motif of CD44 (the Arg 46 is a proline in In^a) but was still able to bind HA upon transfection of the cDNA into $CD44^-$ cell lines, suggesting that the full BX_7B motif was not required.

Some CD44-specific mAbs can influence the function of cell surface CD44 by inhibiting or inducing HA binding. Epitope mapping of these mAbs indicated that regions of the amino-terminal half of CD44 that are not a part of the two basic residue motifs may contribute to HA binding by CD44, since binding of CD44-specific mAbs that inhibit or enhance HA recognition is dependent on aa residues that are distant from the proposed HA-recognition motifs in several distinct regions of the amino-terminal disulfide bonded loop [12, 13].

Presence of the relevant (HA-binding) peptide sequence in the amino-terminal portion of CD44, however, does not ensure that cells expressing CD44 will bind HA. Indeed, most CD44 expressing cells taken from normal animals, as well as many $CD44^+$ cell lines, do not bind HA. As is discussed later, many other factors, that vary in different cell types and different states of cell activation, influence the HA-binding function of CD44 at the cell surface.

Early studies of the HA receptor of fibroblasts (CD44) indicated that it recognized a minimal sequence of three disaccharides of HA, and that its specificity for HA depended upon CD44 being resident in the cell membrane [14, 15]. While detergent solubilized CD44 bound chondroitin sulfate (CS) and heparan sulfate (HS) as well as HA, and a liposome aggregated form bound both CS and HA, CD44 on the cell surface bound HA specifically. Cell surface CD44 is, however, capable of recognizing certain specific CS chains. Toyama-Sorimachi and Miyasaka [16] identified a 600 kDa glycoprotein (gp600) ligand of CD44 secreted by CTLL cells (a cytotoxic T cell line) that consists of a small protein core (18–22 kDa) and extensive chondroitin 4-sulfate (C-4-S) side chains. This 'gp600' was later shown to be serglycin [17]. Binding of serglycin is inhibited by the same mAbs which inhibit HA binding and by HA itself and induced by the same mAb that induces HA-binding, suggesting that the same binding site or one in close proximity is

being used by HA and serglycin. Serglycin binding is not blocked by commercial preparations of CS (C-4-S, C-6-S, CS-A, -B, -C, -D, -E, or -K) suggesting that the chemical form of CS on this proteoglycan may be uniquely specific for CD44 and raising the possibility that other similarly modified CS may be CD44 ligands. On the other hand, the core protein of serglycin or other CD44-binding CS-containing proteoglycans (CSPG) may contribute to the specificity of the interaction with CD44 [17]. A specific CS modification of MHC class II associated invariant (Ii) chain that is exceptional in being expressed on the cell surface, is also recognized by CD44 and may participate in T cell stimulation [18]. The possibility of CD44 binding to cell surface CSPGs that are integral membrane components presents a potential mechanism for CD44-mediated cell–cell interactions involving reciprocal signal transduction.

Non-carbohydrate ligands of CD44

CD44 from specific cell types has been shown to bind a number of non-carbohydrate ligands, but the basis of this binding is less well studied than that of HA. CD44 from cell lysates bound to affinity columns of collagen I and VI, but did not mediate cell adhesion to collagen [19]. Binding of a subpopulation of CD44 molecules from peripheral blood lymphocytes (PBL) to fibronectin was found to depend on a high M_r form of CD44 that is modified by CS [20]. CD44-mediated recognition of collagen I and IV by melanoma cell lines was also dependent on CS modification of CD44 and was required for melanoma cell invasion of collagen matrices [21, 22]. Though CD44-specific mAbs did not inhibit adhesion to collagen (presumably because it was mediated by integrins) they did inhibit the cells' migration on and invasion of collagen gels. Similarly, CD44-related CSPGs mediated microvascular endothelial cell migration and invasion (but not adhesion) on fibrin/fibrinogen gels that was dependent on CS [23]. These studies suggest that CD44 may cooperate with integrin receptors for ECM proteins to mediate cell migration.

CD44 has been reported to bind osteopontin (also termed Eta-1 for early T lymphocyte activation-1), a cytokine with chemotactic activity secreted by activated T cells, osteoblasts, macrophages and other cell types. Osteopontin binding competed with HA binding, implying use of the same binding site [24]. Heparin binding growth factors are able to bind to CD44 by virtue of heparan sulfate (HS) side chain modification of a subset of CD44 molecules that may be specific for certain CD44 isoforms [25, 26].

The possibility that CD44 can bind non-carbohydrate ligands raises a number of questions: (1) What isoforms of CD44 are involved?; (2) Does the binding involve the HA binding site or is it mediated by other regions of the molecule?; (3) What is the role of GAG modification of CD44? Is it cell-type specific and/or isoform specific? As with carbohydrate ligands, appropriate cell function requires strict regulation of the potentially diverse ligand binding activities of CD44 in accordance with cell-type, cell activation state, and the cellular environment. Failure of normal regulatory mechanisms may account for the frequent observations of CD44 involvement in metastatic processes.

Regulation of function by carbohydrate modifications of CD44

CD44 is heterogeneously glycosylated. It appears as a diffuse band on SDS-PAGE and the average apparent M_r may differ among different cell types expressing the same (CD44H) isoform. It contains both N- and O-linked carbohydrate and may have GAG sidechains of CS, HS or keratan sulfate (KS).

Differences in *N*-glycosylation of CD44 that correlate with differences in cell activation have been observed in B cells and peritoneal macrophages. Camp *et al.* [27] found a size difference in CD44, that was largely due to *N*-glycosylation, between resident peritoneal macrophages and macrophages elicited by intraperitoneal thioglycolate injection (an inflammatory stimulus). B cells stimulated to bind HA by culture in IL-5 showed a reduction in *N*-glycosylation compared to resting or LPS stimulated B cells, which did not bind HA [28]. It is not known whether these differences in *N*-glycosylation result from differential use of potential glycosylation sites or differential processing of N-linked carbohydrate chains.

Could differences in glycosylation account for differences between cells in CD44 function? Several studies on glycosylation and CD44 function in cell lines suggest that this is the case. Using fluorescein-conjugated soluble HA to assay for HA binding and a CD44-specific mAb that induces HA binding, cell lines and normal lymphocytes can be classified into (at least) three functional states [4]. 'Active' cells express CD44 that is able to bind HA constitutively. 'Inducible' cells do not bind constitutively, but are able to bind HA in the presence of an inducing CD44-specific mAb. 'Inactive' cells do not bind HA even in the presence of the inducing mAb. Lesley *et al.* [29] used fluorescence activated cell sorting to select sets of variant cell lines in all three activation states. A decrease in *N*-glycosylation of CD44 was observed in 'inducible' variants as compared to their inactive parent, and there was a further decrease in cells that were 'active', compared to 'inducible' cells. Culture in tunicamycin, an inhibitor of an early step in *N*-glycosylation, could convert 'inactive' and 'inducible' cells to a more 'active' state. Treatment with BZαGalNAc (benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside), which inhibited O-linked glycosylation, or with deoxymannojirimycin, an inhibitor of processing of N-linked sugars, did not change the HA binding phenotype of 'inactive' or 'inducible' cell lines [29, 30]. Katoh *et al.* [31] also isolated cell lines that bound HA constitutively and observed reduced *N*-glycosylation in these variants compared to inactive cells

from which they were derived. They were able to convert less active cells to HA-binding cells by culture in tunicamycin and, in some cases, treatment with neuraminidase, which removes terminal sialic acid residues from cell surface carbohydrates. Zheng *et al.* [30] assayed a series of murine cell lines and found that many, but not all, showed an increased ability to bind HA upon culture in tunicamycin and/or after neuraminidase treatment. CD44-Rg receptor globulin secreted by active and inducible cells and bound to beads had binding activity similar to the respective cells from which it was derived [31]. Bead-bound CD44-Rg from inducible cells showed enhanced HA binding upon exposure to inducing mAb and after neuraminidase treatment, again mimicking the behavior of cell surface CD44, and indicating that the cells' binding phenotype was a function of the CD44 molecules themselves and not entirely dependent on the cellular environment.

We have made mutant murine CD44 constructs in which the two most amino-terminal asparagine residues, which result in N-linked sugar additions flanking the first BX_7B motif, have been changed to serines (these mutant constructs are termed N1 and N2). Function of these constructs was tested in a $CD44^-$ cell line which was 'inducible' for HA binding when transfected with wild-type CD44 (Figure 1). Elimination of the N1 glycosylation site resulted in enhanced HA binding such that many cells bound HA constitutively. Elimination of the N2 site had no effect on the 'inducible' phenotype of these cells. Both of these sites were modified by carbohydrate addition in cells which constitutively bound HA when transfected with wild type CD44, as shown by changes in the apparent M_r of the N1 and N2 mutant molecules compared to wild-type. Mutation of either N1 or N2 did not alter the HA binding phenotype of these 'active' cells (data not shown). This result indicates that the 'active' phenotype does not depend on the complete absence of glycosylation at site N1.

Dasgupta *et al.* [32] found that culture of colon carcinoma cell lines transfected with CD44H in phenyl-α-GalNAc (phenyl-*N*-acetyl-α-D-galactosaminide), inhibited O-linked glycosylation and enhanced CD44H-mediated adhesion to HA. In these cells, tunicamycin treatment only slightly increased HA binding. These and the results discussed above indicate that cell-type specific modification of both *N*-glycosylation sites and *O*-glycosylation sites can differentially influence the HA binding function of CD44.

Other studies have indicated that at least some glycosylation steps may be required for achieving a CD44 structure capable of ligand recognition. Lokeshwar and Bourguignon [33] found that solubilized, incompletely glycosylated

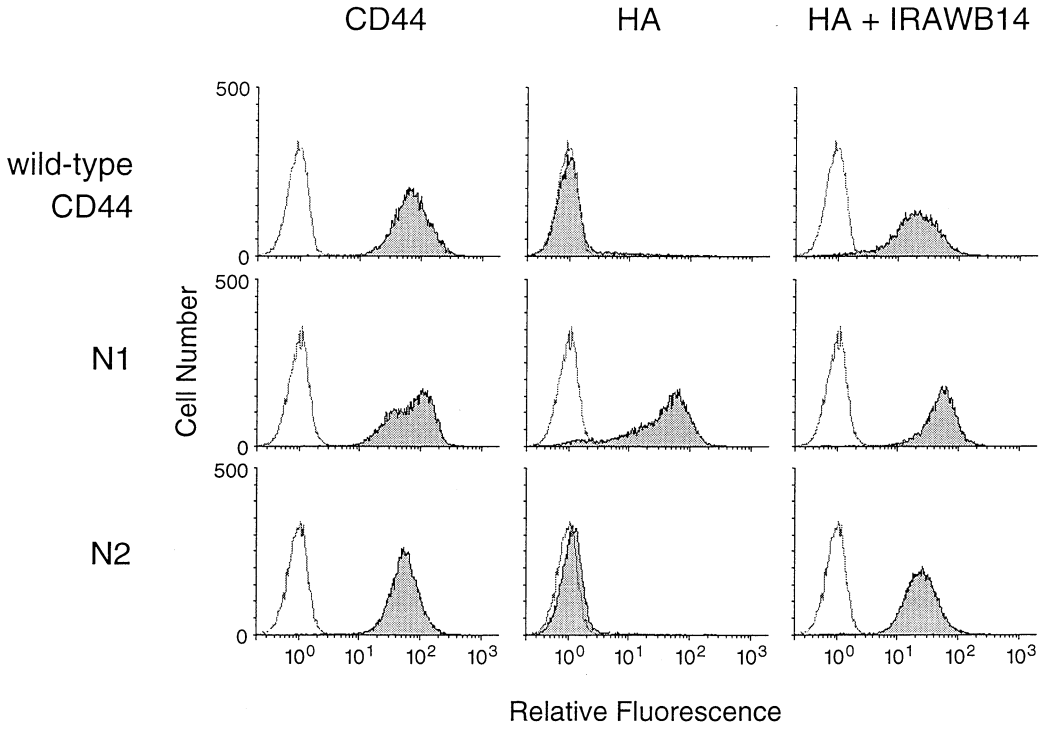

Figure 1. Flow cytometric analysis of the HA binding phenotype of 'inducible' cells transfected with wild type and mutant CD44. Wild type and mutant CD44 constructs in which the two most amino-terminal asparagines were mutated to alanines (N1 and N2 respectively) were expressed in a CD44-negative variant of a pre-B cell line able to bind HA in the presence of the inducing CD44-specific mAb (IRAWB14) [30]. Cells were stained with fluorescein conjugated CD44-specific mAb (first column), fluorescein conjugated HA (second column), or fluoresceinated HA in the presence of inducing mAb (third column).

CD44 did not bind to HA blotted onto nitrocellulose, while the fully glycosylated form did bind. Bartolazzi *et al.* [34] mutated each of the five conserved *N*-glycosylation sites in human CD44H and assayed the mutant constructs for HA binding function upon transfection into two $CD44^-$ cell lines. Their results showed that mutation of any one of these *N*-glycosylation sites resulted in loss of HA binding activity. This would suggest that glycosylation at each of the five sites is necessary for the integrity of the HA-binding structure in the amino-terminal half of CD44. These authors also found that culture in tunicamycin resulted in a loss of HA binding activity in a number of $CD44^+$ human cell lines that constitutively bound HA [34]. This result is in sharp contrast to the results described above, showing that tunicamycin treatment [29–31] and deletion of an *N*-glycosylation site (Figure 1) could enhance HA binding in cells that were inactive or inducible, and indicating that cell specific *N*-glycosylation often exerted a negative influence on CD44 function.

A number of studies indicate that carbohydrate modifications other than those at sites of *N*-glycosylation can have a negative influence on HA binding function. KS modification of CD44H in a highly metastatic variant of a colon carcinoma cell line resulted in greatly reduced HA binding activity compared to a less heavily KS substituted line, which was less metastatic [35]. Keratinase treatment of the heavily substituted cells, and of CD44-Rg from those cells enhanced CD44-mediated adhesion to HA. Lesley *et al.* [29] found that inhibition of CS addition or treatment with chondroitinase converted $CD44^+$ 'inactive' cell lines to the 'inducible' phenotype that was able to bind HA in the presence of inducing mAb. An isoform of CD44 containing variably spliced exons v8–v10, termed CD44E or CD44R1, shows reduced binding of HA when expressed in some cell lines [36], but not in others [37]. These exons encode aa sequences rich in serine and threonine residues which are potential sites of O-linked glycosylation. Inhibition of O-linked glycosylation in cells expressing this isoform of CD44 or in a soluble CD44E-Rg resulted in increased HA binding function, as did replacement of the sequence encoded by the variable exons with a sequence from ICAM-1, which is not heavily *O*-glycosylated. In contrast, CD44E in which variable exon sequences were replaced with another sequence rich in potential *O*-glycosylation sites did not bind HA efficiently [36].

Some GAG modifications may confer new functions on CD44. Variable exon v3 specifies potential sites for the addition of HS side chains [38] which can mediate the binding of heparin-binding growth factors [26] and chemokines [25]. CS modification of CD44 is required for the binding of fibronectin [20], for conferring motility of melanoma cells on collagen I and IV substrates [21, 22], and perhaps for motility of endothelial cells on fibrin substrates [23]. It is not yet established what CD44 isoforms are involved in these latter activities, but CD44H has potential sites for CS modification and has been found to express such modifications in a portion of its molecules in some cells [20, 23].

The diverse and sometimes contradictory array of activities reported for CD44 in different cell types is probably, at least in part, due to differences in the way the molecule is modified by N-linked, O-linked and GAG carbohydrate additions in different cells. Glycosylation at some sites may be important for proper folding of CD44 during synthesis, at least in some cells. But the nature of further modifications may influence whether or not CD44 is able to bind HA. The nature of the modifications may depend on the cell's complement of glycosyltransferases and also on the nutritional environment. Kincade [30] found that growing cells in medium depleted of glucose changed the glycosylation pattern of CD44 and enhanced HA binding for many cell lines that did not constitutively bind HA. Furthermore, a cell line, which was 'inactive' *in vitro*, acquired the ability to bind HA when it was grown as a tumor *in vivo*. HA binding activity was lost again upon tranferring the tumor cells into culture. This result brings up the question whether the CD44-mediated HA binding phenotype observed for cells growing in culture is representative of the activity of these cells *in vivo*.

CD44-carbohydrate interactions in inflammation

In most cases where CD44 is implicated in inflammation *in vivo*, it remains to be determined whether HA (or other GAG) recognition is indeed the mechanism of CD44 function. Though CD44 is expressed on most lymphocytes of both T and B lineages and on most myeloid lineage cells, normal resting leukocytes do not exhibit HA binding activity. Lymphocytes and monocytes activated *in vitro* and *in vivo* upregulate CD44 expression, and elevated CD44 expression has proven a useful marker for memory cells in resting T cell populations (for review see [4]). Yet, the function of the increased expression of CD44 in activated cells and in memory cells has not been established. There are many examples in the literature, often using cell lines, of cell stimulation upon ligation of CD44 with mAbs, suggesting a role for CD44 in cell activation [1, 2, 4], but the relationship of these responses to physiological function is uncertain. In this section, only studies implicating CD44 in the immunologic function of normal cells and/or involving CD44 recognition of carbohydrate ligands will be discussed.

Under specific conditions, CD44-dependent HA binding can be elicited in populations of T cells [39–41], B cells [28, 42, 43] and monocytes [45, 46]. Murikami *et al.* [44, 45] showed that a subpopulation of murine B lymphocytes could be induced to bind HA, by culture for several days in IL-5, or by chronic *in vivo* stimulation through a GVH reaction. Spleens from mice undergoing an *in vivo* allogeneic response developed a transient HA binding

population composed primarily of T cells [39]. Human PBL stimulated with anti-CD3 also exhibited a subpopulation of HA binding cells [40]. *In vitro* culture of human peripheral blood monocytes in the presence of human serum induced HA-binding activity [45], and murine bone marrow-derived macrophages cultured for 6–8 days were able to respond to HA in a CD44-dependent manner [44]. In these cases, HA binding was mediated by CD44 as shown by inhibition with CD44-specific antibodies. The role of these HA-binding cells in immunologic responses is yet to be determined. In the case of *in vivo* activated T cells, the HA binding population was shown to include cytotoxic effector cells assayed *in vitro* [39].

Camp *et al.* [46] have demonstrated participation of CD44 in the early phase of contact hypersensitivity by using CD44-specific mAbs to inhibit ear swelling and leukocyte infiltration in sensitized mice. CD44 expression is up-regulated on leukocytes and synovial cells in arthritic joints in humans [47] and mice [48]. Mikecz *et al.* [48] found that CD44-specific mAb treatment of mice with experimentally induced arthritis dramatically reduced joint swelling and infiltration by inflammatory cells. In this model system [48] and that of Camp *et al.* [46], *in vivo* mAb treatment resulted in shedding of CD44 from the cell surface of lymphoid cells. These *in vivo* studies using mAb to inhibit inflammatory processes in mice suggest that CD44 contributes to the migration of activated leukocytes to sites of inflammation.

On the other hand, CD44-specific mAb treatment *in vivo* had no effect on the entry of adoptively-transferred cells into lymphoid tissues or on the total number of cells in lymphoid tissues (unlike treatments with anti-LFA1 [CD11a/CD18]). This argues against a role for CD44 in the normal steady state recirculation ('homing') of lymphoid cells to lymphoid tissues [46, 48], which had been suggested by *in vitro* studies using (CD44-specific) mAb to inhibit binding of human lymphocytes to frozen tissue sections [49].

A possible mechanism for CD44 involvement in directing lymphocytes to sites of inflammation is suggested by the studies of De Grendele *et al.* [41], who demonstrated 'rolling' of $CD44^+$ cell lines and activated lymphocytes on HA-coated substrates and on endothelial cell monolayers that was HA-dependent and inhibited by a CD44-specific mAb. Clark *et al.* [50] observed rolling of tonsillar lymphocytes on cultured human tonsillar stromal cells and ECM synthesized by these stromal cells. This was shown to be mediated by CD44 binding to HA by inhibition with CD44-specific mAb or soluble HA, and by hyaluronidase treatment of the substrate. These results suggest that CD44 binding to HA may contribute to activated lymphocyte attachment to vascular endothelial cell walls leading to extravasation and/or migration through secondary lymphoid organs.

In vitro exposure to HA has been shown to result in cell activation, proliferation, and/or cytokine secretion, and this observation suggests that CD44 ligation may contribute to inflammatory responses by mediating leukocyte activation, as well as adhesion and migration. HA rapidly induces both TNFα and IL-1β mRNA transcripts, and IGF-1 protein synthesis in 6 to 8 day cultures of murine bone marrow-derived macrophages [44]. These activities are inhibited by a CD44-specific mAb that inhibits HA binding. It has been suggested that binding of soluble ECM components generated by degradation at sites of inflammation (such as lower M_r HA fragments) may stimulate macrophage cytokine production and proliferation and thus contribute to the inflammatory cycle [51]. Granzyme A release from CTL clones was stimulated by serglycin binding, in conjunction with anti-CD3, but only when an activating mAb that induces HA (and serglycin) binding was also present [17]. Galandrini *et al.* [40] showed that HA in conjunction with CD3/TCR-mediated stimuli is costimulatory for human peripheral blood T-cell proliferation, IL-2 production by T-helper clones and release of trypsin-like esterase by cytolytic T cell clones. These activities were again inhibited by a CD44 specific mAb that blocked HA binding. CD44-mediated HA binding induced Ca^{2+} flux, receptor patching/capping, and cytoskeletal reorganization in the T cell line BW5147 [52].

These examples show participation of CD44 in certain model inflammatory responses, activation of the HA binding function of CD44 by immunological stimuli, and leukocyte responses to HA mediated through CD44. This is suggestive evidence that CD44-HA interactions may play a role in inflammation *in vivo*.

CD44 and human tumors

The original interest in the role of CD44 in malignancy arose from experiments reported in 1991. A non-metastatic rat pancreatic tumor line was induced to metastasize by the transfection of cDNA encoding an isoform of CD44 that contained the variable exons 4 to 7 [*CD44(v4–7)*], the so called *meta-1* gene [53]. This finding was particularly exciting because it implicated CD44 as a key molecule in metastasis: a role for which there was no previous direct evidence. Since this initial publication there have been numerous studies on CD44 and cancer (see [54, 55] for the early publications). Although it is now clear that *CD44(v4–7)* is not a universal metastatic gene, in certain cases increases in CD44H and its variant forms may be closely associated with malignancy.

Table 1 summarizes the data from some of the studies of CD44 expression in tissue removed from different human cancers. Data are compared between different studies by assessing whether CD44H and/or its variants are increased in the tumor tissue, and whether the expression of any particular variant is correlated with cancer spread. The different forms of CD44 were measured by either assessing the interaction of antibodies to CD44H and/or its variants with normal and cancerous fixed tissue sections, or by

Table 1. The expression of CD44H and its variants in cancer

Tumor site	*Increased CD44H in cancer*	*Increased variant CD44 in cancer*	*Correlation of variant expression with cancer spread*	*References*
Bladder	Yes	Yes	N/A	56, 57
Breast	Yes	Yes	Yes/No	58–61
Cervix	N/A	Yes	Yes	62, 63
Colon	Yes/No	Yes/No	Yes/No	64–68
Kidney	Yes	Yes	Yes	53, 69
Lung	No	No	No	70, 71
Lymphoma	Yes	Yes	Yes	53
Melanoma	Yes	No	N/A	53
Ovary	No	Yes	Yes/No	72–74
Pancreas	Yes	Yes	N/A	75, 76
Skin	No	No	No	77–79
Stomach	Yes	Yes	Yes	53, 80
Vulva	N/A	Yes	Yes	81, 82

'Yes' means that studies support the conclusion stated at the heading of the column; 'No' means that studies do not support the conclusion stated: 'Yes/No' means that there are some studies that support the conclusion stated, but there are others that do not; and 'N/A' in Tables 1 and 2 means that the information is not available. The references given are meant to be representative and not exhaustive.

assaying for the presence of variant CD44 isoforms in these tissues using reverse transcriptase and PCR. Some studies used both approaches. The results from these two techniques are not strictly comparable, but they are grouped together in Table 1 to obtain an overview of the situation. Although the amount of CD44H and/or its variant isoforms often increases in cancer, there are some cancers where this does not occur (lung and skin cancers). For other cancers (breast, colon and ovary), there is disagreement between studies on whether CD44H expression and/or variant expression is correlated with cancer. Furthermore, in a few studies of basal cell carcinoma or squamous cell carcinoma of the skin, CD44v expression is reduced compared to normal skin [78, 79]; a similar finding has also been reported in an investigation of lung carcinoma [70].

The discrepancies in CD44 expression between different studies are probably mainly caused by differences in the antibodies and/or the nature of the tissue specimens used; the latter is particularly important in the case of RT/PCR methods. There are many reports of the presence of CD44H or its variants in normal tissues [83, 84], so amplifying CD44 transcripts from specimens that have a variable and mixed cellular composition could obscure the true picture. The amount and type of CD44 expression could also depend upon local environmental factors in the tissue specimen, such as cytokines or proteolytic enzymes that are produced by stromal components. It has been shown that CD44 isoform expression can be modulated by cytokines [84], and some variant forms of CD44 appear to be more susceptible to trypsin digestion than CD44H (Gardner, Jones and Turner, unpublished observations). Proteolytic digestion of CD44 could also depend upon the way in which the tissue was handled post-collection, as different degrees of cell damage would release varying amounts of lysosomal enzymes.

It is beyond the scope of this review to go into detail on the specific changes in individual CD44 isoforms present in different cancers because the patterns are so variable, but one can conclude from the literature that the changes observed are not just restricted to the appearance of CD44 (v4–7). This situation is further complicated by the fact that the isoforms themselves may have a number of different post-translational modifications. This increases the potential structural variability of the molecule, and may lead to different patterns in cancer spread, even when the CD44 isoform profile is identical.

CD44 and experimental tumor systems

A relationship between CD44 expression and malignancy has been demonstrated in a number of experimental tumor models. In independent studies it was shown that tumor cells expressing higher amounts of CD44 were more tumorigenic and/or metastatic when injected into nude mice. This result was observed even though the increase in CD44 was produced in different ways. In cultured fibroblasts, both elevated CD44H and CD44 variants were induced by transfection with oncogenes [85, 86]; in cultured melanoma cells, lines expressing high levels of CD44H were obtained by selection [87]; and in human prostate cancers, lines were isolated that spontaneously expressed high levels of CD44H [88, 89]. In other studies, in which a human melanoma in SCID mice [90] or a rat pancreatic carcinoma that carried the *CD44(v4–7)* gene [91] were examined, it

was shown that metastasis was inhibited by treatment with CD44-specific antibodies. Finally, the infusion of soluble chimaeric CD44-Rg was reported to block the growth of CD44H expressing melanoma in mice [92].

While in these cases CD44 expression shows a positive correlation with malignancy, there are examples where CD44 expression may show a negative correlation or may not appear important. Reintroduction of CD44H into human colon carcinoma cells in which the CD44 is down-regulated reduced their tumorigenicity [93]. Ablation of CD44 in a mouse lymphosarcoma had no effect on tumor growth and metastasis [94].

Because human metastasis is a very complex process, the relevance of some of these experimental models to human cancer may be questionable, and the results must be interpreted with a degree of caution. However, these experimental studies do generally agree with the studies using clinical specimens; *ie* increases in CD44 and its variants are often associated with malignant change, but are not always. This is not a surprising conclusion. Metastasis is a complex multistep process that depends upon the tumor type, its location and the interactions between tumor and host cells at a number of points in the process. In some cases, the presence of high amounts of CD44 and/or a particular isoform may contribute to successful metastasis, but in other situations it may not.

Interaction of tumor CD44 with hyaluronan

The most likely role for CD44 in tumor spread is in the arrest of disseminated cells in ectopic sites by binding to HA. This process could initiate a series of events that leads to the establishment of tumor in a new site. The CD44/HA interaction could occur on the surface membranes of endothelial cells in the lymphatics, vascular system, or with mesothelial cells covering serous surfaces in the peritoneal and pleural cavities. Most of the work on this interaction has been done by studying the adhesion of tumor cells to HA immobilized to plastic; a few studies have measured the binding of fluorescein-labelled HA. Again the results are discordant. Some studies suggest that binding to HA via CD44 is associated with increased tumor growth and metastasis [87, 88, 91, 95], whereas other studies indicate that this interaction is either detrimental [93] or unimportant [96].

Increased HA binding, therefore, may be important in some metastatic situations but not in others. This conclusion is best illustrated by describing two examples in some detail. Sleeman *et al.* [96] recently reported extensive studies with rat tumor cell lines. A spontaneous line that was transfected with the metastasis-associated variant gene, *CD44(v4–7)*, showed increased HA binding, but did not show increased tumor growth or become metastatic. These authors also tested the ability of a series of lines, with documented metastasizing capacity, to bind soluble HA, and concluded that these two properties were not correlated. Transfection of a highly-metastatic line, which expressed *CD44(v4–7)* and bound HA, with cDNA encoding a membrane-bound hyaluronidase, abolished the ability of the cells to bind HA, but had no effect on metastasis. Finally, they reported that an antibody to exon v6 reduced the growth and metastasis of a pancreatic line transfected with *CD44(v4–7)*, but the antibody did not block the binding of the tumor cells to soluble HA. This suggested that an interaction of CD44v with a ligand other than HA was important for metastasis in this tumor model.

In the other study, Bartolazzi *et al.* [95] investigated HA binding and the tumor growth of CD44-transfected human melanoma growing in SCID mice. A melanoma line transfected with the *CD44H* gene formed large tumors in 35 days and bound strongly to HA-coated surfaces, soluble HA and tissue HA. On the other hand, the same line transfected with the variant *CD44E* gene, (v8–10), failed to grow *in vivo* and bound to HA poorly. A transfectant with a mutant form of CD44, which could not bind HA (Ala substituted for Arg-41), also failed to grow *in vivo*. This suggests that the interaction between CD44 and HA at the primary site is important for vigorous tumor growth in this situation.

Clearly strong CD44/HA interactions could promote certain steps in the metastatic process, while weak CD44/HA interactions could promote other steps. Reduced expression of CD44 in endometrial carcinoma was associated with the release of cells from the primary tumor into the lymph/vascular space [97], and this release would promote metastasis. In other situations, a subpopulation of tumor cells expressing high CD44 may direct some of the cells along a certain metastatic route. Ascites tumor cells present in the peritoneal cavity in ovarian cancer have less CD44 on their surface than normal cells [98]; however, many ovarian tumors and tumor lines express high levels of CD44 [72, 74, 98, 99]. Ovarian tumors metastasize by invading the mesothelial lining of the peritoneal cavity [100], and our studies have shown that tumor cells that express high CD44 adhere strongly to a mesothelium monolayer (discussed in detail below). Therefore, the discrepancy between the expression of CD44 on tumor cells in ascites and tumor tissue could be explained by selection for a subpopulation of cells with high levels of CD44 that bind to the mesothelium.

CD44 and an ovarian tumor model

The role of CD44 in the binding of human metastatic ovarian tumor cell lines to mesothelial cells was studied as a model of the arrest process that occurs in peritoneal metastasis in ovarian cancer [101, 102]. The adhesion of the tumor lines to mesothelial cells correlated with their CD44-mediated binding to immobilized HA [102, 103]. As mesothelial cells synthesize HA as a pericellular coat [102], it was predicted that this was the main mechanism of arrest for metastasizing ovarian tumor cells. However, it was

noted that the level of CD44 expression was not closely correlated with the number of cells adhering to immobilized HA [103] or with the adhesion strength [104]. For example, two lines that expressed similar amounts of CD44 on their cell membrane (see Table 2 lines OVMZ6 and OAW59) had a two fold difference in the number of cells that attached to HA and an approximately 70-fold difference in the strength of this attachment (avidity). The avidity was determined by measuring the binding of the tumor cells to a plastic surface coated with different concentrations of HA [99]. It was measured in this way to exclude possible interactions by other adhesion molecules on the tumor cell surface; the specificity of the CD44/HA interaction being confirmed by blocking the interaction with an anti-CD44 antibody. A loose relationship between total CD44 expression and adhesion to immobilized HA has also been reported in other tumor cell studies [105].

The unpredictable nature of the binding of ovarian tumor cells to HA via CD44 could not be explained by the presence of CD44 splice variants, because no line expressed variants in high enough amounts to account for the observations (Table 2 and unpublished observations). It seemed more likely that CD44 glycosylation was affecting the interaction (general evidence for this is discussed in a previous section). When different ovarian tumor lines were treated with neuraminidase, the HA-adhesion of two lines with the lowest avidity (OVMZ6 and OAW42) increased; whereas the adhesion of two lines with much higher avidity (OVMZ10 and OAW59) hardly changed (see Table 2). Furthermore, when the same lines were treated with tunicamycin, the adhesion of the high-avidity lines was reduced by a third, but the adhesion of the low-avidity lines was not affected. Tunicamycin treatment did not affect the total expression of CD44 on any of the lines (unpublished observation). These findings suggest that for ovarian tumor cells there is a relationship between the nature of the glycosylation of CD44 and the avidity of the tumor cell for HA. This conclusion is similar to that previously discussed in this review for other cell types.

It is well documented that the glycosylation of membrane molecules varies with cell type, animal species, and culture conditions [106]. In this connection, it was recently reported that CD44 isoforms that were transfected into CD44 negative lymphoma and fibroblastoid lines exhibited HA binding properties that depended upon the host cell line used [107]. This finding might be explained by the synthesis of CD44 with different glycosylation profiles in these cell lines, although no direct evidence to support this conclusion was presented in this study.

CD44 on metastatic ovarian tumor cells could act primarily as a molecule that docks cells that are moving over the mesothelial membranes. This mechanism is similar to that used by rolling leukocytes to dock on vascular endothelium in inflammation and involves the interaction of E selectin with a carbohydrate ligand [108]. CD44-binding to HA has also been recently shown to mediate lymphocyte rolling *in vitro* [41, 50]. Both systems use multiple binding sites on the target tissue to capture the cells, and retention is mediated

Table 2. Binding of ovarian tumor cells to immobilized hyaluronan

Cell line	CD44 expression (RMF)	Percent CD44H (PCR)	Maximum cell adhesion to immobilized HA (%)	Avidity (arbitrary units)	Adhesion after NANase (%)		Adhesion after tunicamycin (%)	
					Control	Test	Control	Test
OVMZ6	95	87.0	33.2 ±8.9	4–10	31.5	60.9	47.8	44.1
					32.3	63.6	42.7	37.2
OVMZ10	67	97.2, 100.0	83.3 ±4.8	10–100	85.2	90.0	87.7	59.6
					84.3	87.9	81.4	45.9
OAW41	7	N/A	2.1 ±1.0	N/A	1.3	2.5	N/A	N/A
					0.5	1.7		
OAW42	32	87.9, 99.7	34.5 ±10.2	4–10	35.6	50.2	30.6	39.8
					37.6	51.3	37.7	39.8
OAW59	100	88.6, 99.9	86.7 ±10.2	100–1000	92.9	99.8	79.1	48.2
					88.2	99.4	82.5	62.4
OAW180	2	N/A	2.2 ±2.2	N/A	N/A	N/A	N/A	N/A

RMF is the median fluorescence of cells incubated with anti-CD44 antibody (BU52) divided by the median fluorescence of cells incubated with a control antibody. Cell adhesion was measured by adding fluorescently labeled cells to a multiwell plate that was precoated with HA (1 mg ml^{-1}). After 5 min incubation, unbound cells were removed by washing and the fluorescence of the bound cells was measured. Adhesion values are given as mean ±SD calculated from five experiments Avidity is defined as the reciprocal of the range of HA coating concentrations (1 mg ml^{-1} to 1 μg ml^{-1}) over which the adhesion was approximately 50% of the maximum value. Test cells were treated with 5 mU of neuraminidase (Arthrobacter ureafaciens; Boehringer) for 30 min at 37 °C or 1–10 μg tumicamycin for 16 h at 37 °C prior to measuring cell adhesion (results are given in each case for two experiments). CD44H transcription was determined by RT/PCR and the PCR products were quantitated by laser densitometry. The CD44 expression, the avidity data, and the maximum cell adhesion data are taken from references 99, 104 and 111 respectively. All other data are unpublished observations (Catterall, Jones and Turner).

by weak interactions involving carbohydrate groupings. If ovarian tumor cells are retained in metastatic sites by this mechanism, the attachment could then be strengthened by interactions between other adhesion molecules, such as integrins, on the target cells. This has already been proposed for selectin interactions in the so called 'selectin-integrin' cascade mechanism [109].

That the avidity of tumor cells for HA can be altered by the glycosylation of CD44 may have important consequences for the role of CD44 in metastasis. Many changes in cell surface glycosylation (increased branching, increased chain length, increased sialylation, and increased fucosylation) have previously been observed in cancer and the frequency of these changes increases with tumor progression [110]. More detailed information on the carbohydrate structure of CD44 is required, before the effect of these changes on the adhesion of metastatic cells can be assessed.

Final comments

For most cancers, the precise role of CD44 in tumor spread is still unclear. Metastasis is a complex multistep process that varies from tumor to tumor and site to site, and it seems probable that CD44 can either be a negative or positive influence on this process. Alternatively, in some cancers it may have no role at all. Its interactions with HA and other ECM molecules (the latter were not discussed in the metastasis section, but were described earlier in this review) are obviously relevant to various processes in metastasis, such as adhesion and migration. The appearance and increase of CD44 isoforms associated with progression in many cancers could be an epi-phenomenon, but this seems unlikely in all cases. Finally, in situations where disseminated tumor cells are retained in a secondary site by binding to HA through CD44, the glycosylation of the latter molecule may be very important in affecting the strength of the interaction.

Conclusions

We have discussed here two aspects of CD44-carbohydrate interactions. The first deals with binding of the carbohydrate ligand HA by CD44. This recognition is mediated by the conserved amino-terminal $\sim$170 aa of the external domain that is found in all CD44 isoforms. Many CD44 functions depend on this interaction. However, it is not yet established to what extent binding of HA is responsible for CD44 involvement in inflammatory responses or in metastasis. HA recognition by CD44 is strongly implicated in some tumor model systems but this conclusion is contradicted by the findings in other systems, indicating perhaps that CD44 may have different functions depending upon the tumor system.

The second aspect of carbohydrate interaction with CD44 is the regulation of CD44 function by post-translational carbohydrate modifications of the polypeptide backbone. It is now well documented that cell specific carbohydrate modifications can influence the HA binding activity of CD44. It is also evident that carbohydrate modifications of CD44, especially the addition of GAG sidechains, can confer other functions on the molecule, besides HA binding. It is important to note that different cell types have different carbohydrate synthetic machinery, and therefore, modify CD44 differently. Even within a given cell line, CD44 glycosylation can be heterogeneous.

Clearly the activity of CD44 can be dependent on the way it is post-translationally modified with carbohydrate. Fully understanding CD44 function will require precise knowledge of the composition, structure and position of the added carbohydrate and knowledge of how it interacts with and modifies the structure and reactivity of the CD44 molecule.

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