Keratan Sulfate Modification of CD44 Modulates Adhesion to Hyaluronate*

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CD44 alternative splicing has been implicated in the regulation of CD44 function. CD44 undergoes significant posttranslational modification in all cells, but the functional consequences of these modifications are poorly understood. In the current study, we have demonstrated that keratan sulfate modification of CD44 significantly modulates its ability to bind to hyaluronate. We observed naturally occurring differences in CD44 keratan sulfate substitution between two clonal variants of the KM12 human colon carcinoma cell line. CD44 on the highly metastatic KM12L4 clone is more heavily substituted with keratan sulfate than CD44 on the poorly metastatic KM12C6 clone. Moreover, CD44H on KM12L4 bound to hyaluronate poorly compared to CD44H on KM12C6. Removal of keratan sulfate from CD44 greatly enhanced CD44-mediated cell adhesion to hyaluronate. Removal of keratan sulfate from CD44H-immunoglobulin fusion proteins also enhanced their adhesion to hyaluronate. The influence of glycosaminoglycan substitution on CD44 function was specific to keratan sulfate substitution; treatment to remove chondroitin sulfate, heparan sulfate, or hyaluronate did not affect CD44-mediated adhesion to hyaluronate. Use of site-directed CD44H cDNA mutants with arginine changed to alanine at position 41 indicated that keratan sulfate modification of CD44 modulates hyaluronate adhesion through its B loop domain. These findings suggest that keratan sulfate modification of CD44 may play an important regulatory role in the broad spectrum of biological processes attributed to CD44, including normal development, tumor progression, and lymphocyte function.

CD44 is the principal cell surface receptor for hyaluronate (1–3) and has been implicated in a wide variety of processes, including cell motility (4, 5), growth control (6), tumor metastasis (5, 7–9), and lymphocyte activation (10–12). Much interest has been devoted to the extensive alternative splicing of CD44 mRNA. Several CD44 isoforms arise from mRNA alternative splicing of at least 10 exons encoding a portion of the extracellular domain (13, 14). The predominant CD44 isoform detected in many normal tissues is CD44H, an isoform encoded by a transcript that does not contain any of the central alternatively spliced exons (15). Inclusion of one or more of the alternatively spliced exons generates individual CD44 isoforms.

While differences in CD44 alternative splicing between cells may result in different cell behavior, cell type-specific post-translational modification of CD44 may also alter their phenotype. CD44 undergoes extensive post-translational modification, including N- and O-linked glycosylation and substitution with high molecular weight glycosaminoglycans (16–20). We have recently demonstrated that the same CD44H isoform expressed on two clonal variants of a human colon carcinoma cell line display very different functional characteristics (21). CD44H reintroduced by stable transfection back into the poorly metastatic KM12C6 colon carcinoma clone binds hyaluronate and mediates a reduction in both in vitro and in vivo growth. In contrast, CD44H transfected into the highly metastatic KM12L4 colon carcinoma clone does not bind hyaluronate and does not mediate reduction in either in vitro or in vivo growth. These results indicate that subtle differences exist between the KM12C6 and KM12L4 cells that alter the ability of CD44H expressed on their cell surface to bind hyaluronate and modulate cell growth.

In the report presented herein, we have examined how CD44H glycosaminoglycan substitution influences CD44H function. We report that CD44H is more heavily substituted with keratan sulfate when expressed on KM12L4 cells than on KM12C6 cells. Moreover, this difference in keratan sulfate substitution significantly modulates CD44H function. Removal of keratan sulfate from cell surface CD44 or from CD44H-immunoglobulin fusion proteins (CD44H receptor globulins) greatly enhances their adhesion to hyaluronate. Use of site-directed CD44H mutants that are unable to bind hyaluronate because of an amino acid substitution in the B loop domain indicates that keratan sulfate substitution modulates hyaluronate binding through this domain. The dramatic impact of this regulatory mechanism on CD44 function indicates that it is an additional mechanism, which, together with alternative splicing, regulates the function of CD44.

MATERIALS AND METHODS

Cell Lines—The human colon carcinoma cell lines KM12L4 and KM12C6 were generous gifts from Dr. I. S. and ε-D. Anderson Cancer Center, Houston, TX) and have been described previously (22). Human colon carcinoma cell line HT29 was obtained from the American Type Culture Collection (Rockville, MD). The SW620 human colon carcinoma cell line was a generous gift from Dr. Lee Ellis (M. D. Anderson Cancer Center, Houston, TX). Cells were grown in Dulbecco’s modified Eagle’s medium/Ham’s F-12 supplemented with 8% fetal calf serum.

These cell lines were also transfected with three constructs, and the resulting transfecants have been characterized previously (21). Briefly, cells designated with the suffix 3H were transfected with CD44H full-length cDNA in the pRC/CMV vector (Invitrogen, San Diego, CA), and

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these cells express CD44H in addition to high molecular weight CD44 isoforms. A mutant form of CD44H with arginine 41 changed to alanine, thereby destroying its affinity for hyaluronate, is expressed on the cell surface of transfected designated with the suffix Δ41R/A. Control transfecteds were transfected with the vector only (no insert) and are designated with the suffix Δneo. Transfectants were grown in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) and G418 (Sigma) added to a final concentration of 500 μg/ml for KM12 and HT29 transfectants and 1500 μg/ml for SW620 transfecants.

Antibodies and Receptorglobulins—The mAb1 F10-44-2 (Biodesign International, Kennebunk, ME) is directed against epitopes common to all CD44 isoforms. The mAbs BU52 (Binding Site, Inc., San Diego, CA) also is directed against epitopes common to all CD44 isoforms. The mAb BRIC 205, which effectively blocks CD44 hyaluronate binding, was a kind gift from D. J. Anstee (Bristol, United Kingdom) (23).

The CD44H receptorglobulinwas prepared as described previously (1). Briefly, oligonucleotide-primed amplification of cDNA sequences of CD44H was performed by polymerase chain reaction. The oligonucleotide primers were designed to encode endonuclease restriction sites to facilitate subsequent cloning into Ig vectors digested with the same restriction enzymes. CD44-Ig constructs were introduced into COS cells by the DEAE-dextran method, and supernatants were harvested 5–8 days post-transfection. Receptorglobulins purified on protein A-Sepharose (Pharmacia, Cambridge, MA) were incubated with 0.1% citric acid, pH = 3.0, dialyzed overnight, and purified protein concentration was determined using the BCA assay (Pierce).

Metabolic Labeling and Enzymatic Digestion—Tissue culture cells were starved in sulfate-free minimum essential medium (Life Technologies, Inc.) for 6 h and then incubated with 10% dialyzed fetal calf serum and 200 μCi/ml Na35SO4 (1000 Ci/mmol, DuPont NEN) for 12 h. Cells harvested with 5 mM EDTA in PBS were washed with PBS and lysed in a buffer containing 1% Nonidet P-40, 50 mM Tris (pH = 8.0), 150 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 10 μg/ml leupeptin, 100 units/ml aprotinin, and 100 μg/ml phenylmethylsulfonyl fluoride. Nuclei were removed by centrifugation, and lysates were preincubated with protein G-agarose beads (Oncogene Science, Cambridge, MA). Lysates were added to each well. For some experiments, the number of viable cells was estimated using a colorimetric assay that depends on the reduction by living cells of tetrazolium salt, MTT, to form a blue formazan salt (24). Briefly, the adherent cells were placed in RPMI 1640 without phenol red containing 0.5 mg/ml MTT (Sigma) for 2 h at 37°C. The medium was then removed and formazan crystals were solubilized with 50 μl of Me3SO. After vigorously shaking the plate, the optical density of each well was measured using an automatic plate reader (Anthos HT2) at a 550 nm measurement wavelength and a 650 nm reference wavelength. The percent specific adhesion was determined by calculating the ratio of OD550/650 of adherent cells to the OD550/650 of all of the cells initially seeded. All experiments were performed in triplicate.

An enzyme-linked immunosorbent assay was used to measure the adhesion of receptorglobulins to hyaluronate. 96-well flat-bottomed plates were coated with hyaluronate or BSA in PBS overnight at 4°C. The plates were washed, and nonspecific sites were blocked with BSA. Receptorglobulin (5 μg/ml) was added to the hyaluronate- or BSA-coated plates and incubated at room temperature for 2 h. For some experiments, receptorglobulin was treated with keratanase (1 unit/ml) for 30 min. The plates were washed with PBS containing 0.1% Tween 20 (0.1%) four times. Horseradish peroxidase-conjugated anti-human immunoglobulin Fc (Sigma) was added to the plates and incubated at room temperature for 1 h. The plates were washed with PBS containing Tween 20 four times and incubated with 0-phenylenediamine (0.4 mg/ml) (Sigma) in citrate phosphate buffer containing H3O4 at 37°C. The reaction was stopped by addition of 2 x H2SO4, and the optical density was measured at wavelength 492 nm.

Immunofluorescence—Cells were detached from plates in 5 ml EDTA in PBS, washed with PBS, and then treated with or without keratanase (1 unit/ml) for 30 min. Cells were washed in PBS and then incubated with BUS2 (5 μg/ml) in 1% BSA in PBS at 4°C for 30 min. Cells were washed in PBS and then incubated with fluorescein-labeled anti-mouse mAb (Sigma) for 30 min at 4°C. Cells were washed in PBS, resuspended in PBS, and analyzed on a FACSscan™ (Becton-Dickinson Co., Mountain View, CA).

RESULTS

Differences between KM12 Clones in CD44 Glycosaminoglycan Substitution—Poorly metastatic KM12C6 cells and the highly metastatic KM12L4 cells express predominantly high molecular weight CD44 alternative splice variants and do not express CD44H (21). CD44H expressed on the cell surface of these two cell lines after CD44H cDNA transfection differ in their functional properties. Only CD44H expressed on KM12C6/H transfecants binds to hyaluronate and reduces in vitro and in vivo growth. CD44H expressed on KM12L4/H transfecants binds to hyaluronate poorly and does not reduce in vitro or in vivo growth. Both cell lines substitute CD44 with sulfated glycosaminoglycans, and the molecular mass of CD44H expressed by the two KM12 clones appears similar by immunoprecipitation analysis (Fig. 1A). Treatment with keratanase and chondroitinase resulted in both a shift in mobility and a decrease in the intensity of the band corresponding to CD44H. This is in contrast to treatment with heparitinase, which did not result in any changes in mobility or band intensity in KM12C6/H. Results from quantitation of 35SO4-labeled oligosaccharide released after treatment with keratanase correlated with the degree of mobility shift seen by PAGE analysis (data not shown). These data suggest that both KM12C6/H and KM12L4/H cell lines substitute CD44H with keratan sulfate and chondroitin sulfate.

However, these two cell lines differ in their degree of keratan sulfate substitution.

1 The abbreviations used are: mAb, monoclonal antibody; BSA, bovine serum albumin; PBS, phosphate-buffered saline; PAGE, polyacrylamide gel electrophoresis.
sulfate substitution of CD44. This was determined by labeling
cell surface proteins with biotin. Keratanase-treated CD44H
immunoprecipitates from the KM12L4αH cells demonstrated a
greater shift in molecular mass than keratanase-treated
CD44H immunoprecipitates from the KM12C6αH cells (Fig.
1B). SW620αH is derived from separate colon carcinoma cell
line and does not substitute CD44H with keratan sulfate.
Therefore, this cell line serves as a negative control for keratanase
treatment. The results indicate that KM12L4 cells more
heavily substitute CD44H with keratan sulfate than do the
KM12C6 cells. Because the CD44H core protein backbones are
identical in the two cell lines, CD44H on KM12C6 presumably
has more of its molecular mass made up by other glycosami
noglycan substitutions or N- or O-linked oligosaccharides.

Effect of Keratanase Treatment on CD44 Adhesion to Hyaluronate—We next examined if these specific differences in
CD44H post-translational modification between the two KM12
donal variants resulted in differences in CD44H function. Cells
treated with keratanase were tested for adhesion to either
hyaluronate or BSA. Significantly enhanced hyaluronate bind-
ing was detected after treatment of KM12L4αneo (control
transflectant) and KM12C6αneo cells with keratanase (Fig.
1B). The near complete abrogation of this enhanced hyaluronate
adhesion by anti-CD44 mAb BRIC 205 indicates that the keratanase-mediated enhancement was attributable to hyaluronate
adhesion by CD44, and not another cell surface protein. Because KM12C6αneo and KM12L4αneo express only high mole-
cular weight CD44 isofoms and virtually no detectable
CD44H, these results indicate that removal of keratan sulfate
from the high molecular weight CD44 isoforms significantly
improves their binding to hyaluronate.

Similarly, keratanase treatment of KM12L4αH cells also
dramatically enhanced their binding to hyaluronate. The de-
gree of enhancement suggests that keratanase treatment en-
hanced both CD44H and high molecular weight CD44 isoform
adhesion to hyaluronate. Again, blocking experiments with
mAb BRIC 205 indicated that the keratanase-mediated en-
hancement was attributable to hyaluronate adhesion by CD44,
and not another cell surface protein. Untreated KM12C6αH
cells bind to hyaluronate extremely well, and treatment of
these cells with keratanase did not enhance their binding.
These data reveal that the differences detected in the ability
of CD44H to bind hyaluronate when expressed on KM12C6 cells
compared to KM12L4 cells are partially a result of the inherent
differences in CD44H keratan sulfate substitution noted be-
tween the cells. Nonetheless, the persistent difference in hya-
loronate binding between KM12C6 and KM12L4 transflectants
even after keratanase treatment suggests that additional fac-
tors which influence CD44 function differ between these two
KM12 clones. The significant inhibition of adhesion by mAb
BRIC 205 measured in all of the keratanase-treated cells sug-
gests that keratanase treatment enhances hyaluronate binding
of at least two different CD44 isofoms through a similar
mechanism.

To examine the possibility that keratanase treatment en-
hanced cell adhesion to hyaluronate through an increase in cell
surface CD44, we performed fluorescence-activated cell sorting
analysis with mAb BU52 to measure cell surface CD44 before
and after keratanase treatment (Fig. 3). No immediate changes
in cell surface CD44 were detected, indicating that keratanase
treatment modified pre-existing cell surface CD44 and did not
induce an up-regulation of CD44 expression.

Effect of Removal of Additional Glycosaminoglycans on CD44 Function—We next examined if enhanced hyaluronate adhe-
sion could be detected after treatment to remove chondroitin
sulfate, hyaluronate, or heparan sulfate. CD44H cDNA trans-
flectants and control transflectants were treated with chon-
droitin ABC lyase (chondroitinase), or 0.2 unit/ml heparitinase
in PBS (pH 5.7) at 37°C for 1 h. However, the CD44H core
protein backbones are identical in the two cell lines, CD44H on KM12C6 presum-
ably has more of its molecular mass made up by other glycosami-
noglycan substitutions or N- or O-linked oligosaccharides.
with heparitinase treatment, and a very minimal increase in KM12L4 cell binding was noted after treatment with chondroitin ABC lyase, hyaluronidase, and heparitinase. These slight increases were not nearly as significant in magnitude as the increase in hyaluronate binding detected after removal of keratan sulfate. Clearly, keratan sulfate removal had the most dramatic impact on CD44-mediated adhesion, and this effect was not seen after treatment with chondroitin ABC lyase, hyaluronidase, or heparitinase.

**Effect of Keratanase Treatment on CD44 Receptorglobulin Adhesion to Hyaluronate**—While the enhanced hyaluronate adhesion measured in keratanase-treated intact cells was significantly inhibited by anti-CD44 mAb BRIC 205, it remained possible that the principal effect of keratanase treatment was not on CD44 itself, but rather on other extracellular proteins associated with CD44 that influence its interaction with hyaluronate. Therefore, to further examine the influence of keratan sulfate substitution on CD44 adhesion to hyaluronate, we examined the effect of keratanase treatment on CD44H receptorglobulins. The CD44H receptorglobulin, which is a fusion protein consisting of the CD44H extracellular domain and human IgG1, has been described previously (1, 26). Purified CD44H receptorglobulin was treated with keratanase and then tested for adhesion to hyaluronate. The molecular mass of CD44H receptorglobulin decreased after keratanase treatment (Fig. 5A), and the resulting CD44H receptorglobulin demonstrated enhanced adhesion to hyaluronate (Fig. 5B). These data indicate that treatment modifies CD44 itself and thereby results in enhancement of its binding to hyaluronate. The CD44H receptorglobulin used in this study was produced in COS cells, which posttranslationally modify CD44H differently than the colon carcinoma cell lines used in this study. Nonetheless, data from these experiments clearly support the functional importance of CD44H keratan sulfate substitution, because similar to the colon carcinoma cell lines examined, COS cells substitute CD44H with keratan sulfate.

**Identification of CD44 Domain Responsible for Enhanced Hyaluronate Binding after Keratanase Treatment**—Monoclonal antibody BRIC 205 inhibits CD44H adhesion to hyaluronate. Our finding that mAb BRIC 205 similarly blocks the enhancement in hyaluronate adhesion displayed by keratanase-treated cells suggests that the degree of keratan sulfate substitution on CD44H affects its binding to hyaluronate through modulation of a single hyaluronate binding domain. To test this hypothesis, we have used a site-directed mutant CD44H cDNA. The CD44 domain responsible for its adhesion to hyaluronate resides in its B loop domain (26, 27). Changing amino acid 41 from arginine to alanine in this domain completely abolishes its affinity for hyaluronate (26). If differences in CD44H keratan sulfate substitution influence its interaction with hyaluronate through the B loop domain, then treatment of a mutant CD44H with arginine 41 changed to alanine should not enhance its binding to hyaluronate. Conversely, the finding that keratanase treatment of this mutant CD44H does enhance its hyaluronate binding would suggest that removal of keratan from CD44H is
unmasking a separate and distinct hyaluronate binding domain.

KM12 clones transfected with a site-directed mutant CD44H cDNA to express CD44H with arginine at position 41 changed to alanine have been described previously. As expected, KM12L4Δ41R/A and KM12C6Δ41R/A cells demonstrate the same hyaluronate binding characteristics as do KM12L4Δneo and KM12C6Δneo cells (Fig. 6). These results confirm that the mutated CD44H expressed by the KM12L4Δ41R/A and KM12C6Δ41R/A cells bind to hyaluronate poorly. Treatment of these cells with keratanase did not enhance their binding to hyaluronate to any greater extent than observed in the control cells. In other words, the keratanase treatment enhanced hyaluronate binding of only the high molecular weight CD44 isoforms, and not of the site-directed mutant CD44H. These results strongly suggest that keratan substitution on CD44H modulates its interaction with hyaluronate through its B loop domain.

Effect of CD44 Glycosaminoglycan Substitution in other Human Colon Carcinomas—Our initial impetus for the studies presented herein was to examine naturally occurring differences in CD44H post-translational modification between two KM12 clones that differ in metastatic potential. We then studied one additional human colon carcinoma cell line (HT29) to determine if the apparent effects of CD44H keratan sulfate substitution could be observed in a colon carcinoma from a completely separate origin. Treatment of CD44 immunoprecipitates from HT29 cells to remove glycosaminoglycans revealed a substitution pattern similar to that seen in KM12L4 cells (data not shown). HT29 cells modify CD44 with keratan sulfate. And similar to our findings in the KM12 clones, re-

moval of keratan sulfate from CD44 enhanced hyaluronate binding in HT29 cells (Fig. 7). This enhancement was detected both in mock-transfected HT29Δneo cells, which express only high molecular weight CD44, and in the HT29ΔH cells transfected to express CD44H. The enhancement could be blocked with mAb BRIC 205 (data not shown). Treatment with keratanase did not increase cell surface CD44 expression (data not shown). No enhancement in hyaluronate affinity was detected in the HT29 transfectants after treatment with chondroitin ABC lyase, hyaluronidase, or heparitinase. Therefore, the effects of keratan sulfate modification of CD44H on HT29 colon carcinoma cells are similar to the effects seen with the KM12 colon carcinoma cells. Specifically, keratan sulfate modification of CD44 reduces its adhesion to hyaluronate.

DISCUSSION

The diversity of biological functions attributed to CD44 may result from its role as a cell surface adhesion molecule that binds to hyaluronate. Given the broad tissue distribution of CD44, it is reasonable to assume that CD44 interaction with extracellular matrix is tightly regulated. This hypothesis is supported by our finding that CD44H expressed on two individual clonal variants of a single colon carcinoma cell line drastically differ in their ability to bind hyaluronate. It has also been reported previously that hyaluronate binding by CD44 expressed on murine T cells is transiently activated during an in vivo immune response (28). Potential mechanisms that regulate CD44 interaction with extracellular matrix include: 1) alternative splicing, 2) phosphorylation of residues in the cytoplasmic domain, 3) interaction of the cytoplasmic domain with intracellular proteins, 4) posttranslational modification by glycosylation or glycosaminoglycan substitution, 5) interaction with other cell surface proteins, 6) interaction with extracellular ligands, and 7) masking or shedding of cell surface CD44 (for review, see Ref. 29).

Several tumor types express different CD44 alternative splice isoforms compared to their normal tissue counterparts.
The mechanism by which keratan sulfate modification of CD44 alters its adhesion to hyaluronate remains unclear. CD44 contains a cluster of basic residues in the B loop region that is responsible for the majority of its adhesion to hyaluronate (27), and mutation of arginine 41 drastically reduces its adhesion to hyaluronate (26). Our data using mutant CD44H keratanase treatment. This rapid increase in hyaluronate adhesion suggests that keratanase treatment modified preexisting CD44 molecules, rather than induced new CD44 expression. This conclusion is supported by fluorescence-activated cell sorting data that demonstrates no change in cell surface CD44 after keratanase treatment. This conclusion is also supported by the receptor globulin adhesion data that demonstrate enhanced CD44H receptor globulin adhesion to hyaluronate after treatment with keratanase. The rapid influence of keratanase treatment combined with the receptor globulin data also suggest that new protein synthesis is not required for the enhanced hyaluronate adhesion. This regulatory mechanism is distinct from that of phorbol 12-myristate 13-acetate-inducible binding of lymphocytes to hyaluronate, in which new protein synthesis is essential (43).

In conclusion, we have demonstrated that CD44 function on colon carcinoma cells is modulated by posttranslational modification. Specifically, we have identified differences in keratan sulfate substitution on CD44 that markedly modulate its interaction with hyaluronate through its B loop domain. The impact of keratan sulfate substitution on CD44 function is significant and may be functionally as important as CD44 alternative splicing. Closer examination of this CD44 regulatory mechanism in investigations of development, tumor metastasis, and lymphocyte function will likely reveal it to be an important regulator for these biological processes.

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