Oncostatin M and Transforming Growth Factor-β1 Induce Post-translational Modification and Hyaluronan Binding to CD44 in Lung-derived Epithelial Tumor Cells*

Joanna Cichy‡§ and Ellen Pure´‡¶

From the ‡Wistar Institute, Philadelphia, Pennsylvania 19104, the §Institute of Molecular Biology, Jagiellonian University, 31-120 Krakow, Poland, and the ¶Ludwig Institute for Cancer Research, New York, New York 10158

CD44, a receptor for hyaluronan (HA), has been implicated in tumor growth and metastasis. Most CD44-positive cells fail to exhibit constitutive HA receptor function but CD44-mediated HA binding on hematopoietic cells can be induced by antibody cross-linking of the receptor and by physiologic stimuli, including cytokines. We now demonstrate that oncostatin M (OSM) and transforming growth factor-β1, cytokines known to regulate the growth of tumor cells, stimulate HA binding in lung epithelial-derived tumor cells. In lung epithelial-derived tumor cells, cytokine-induced binding resulted from post-translational modification of the receptor. OSM-induced HA binding was associated with a reduction in N-linked carbohydrate content of CD44. In addition, OSM induced HA binding via a novel mechanism requiring sulfation of chondroitin sulfate chains linked to CD44. The mechanism underlying transforming growth factor-β1 induced HA binding was distinct from the effects of OSM. The data presented indicate that modulation of the glycosylation and sulfation of CD44 by cytokines provides mechanisms for regulating cell adhesion during tumor growth and metastasis.

CD44 is a broadly distributed cell surface glycoprotein that can mediate cell-cell adhesion and cell-matrix interactions (1–3). CD44 is encoded by a single gene, but is expressed as multiple isoforms ranging from 80 to 250 kDa. This structural diversity is generated by alternative RNA splicing as well as differences in glycosylation and the attachment of glycosaminoglycans (GAGs)1 (4). The most common form of CD44, referred to as standard or hematopoetic CD44 (CD44H), does not contain any of the differentially spliced “variant” exons (5). CD44 isoforms expressing variant exons have been demonstrated in epithelia, activated lymphocytes, and tumor cells (4, 5).

CD44 is the principal receptor for hyaluronan (HA), a glycosaminoglycan that is ubiquitously distributed in extracellular spaces (4, 6). The HA-binding domain, located in the N-termin

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1 The abbreviations used are: GAG, glycosaminoglycan; HA, hyaluronan; OSM, oncostatin M; TGF-β1, transforming growth factor-β1; CD44H, standard CD44; FITC, fluorescein isothiocyanate; HRP, horseradish peroxidase; MEM, minimal essential medium; PBS, phosphate-buffered saline; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene difluoride; ELISA, enzyme-linked immunosorbent assay; PE, phycoerythrin.

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18061
Molecular Basis of Cytokine-regulated Hyaluronan Binding

Hermes III, and G44-26 (PharMingen, San Diego, CA). Purified hyaluronan from rooster comb (HA) was obtained from Sigma. Horseradish peroxidase (HRP)-conjugated and alkaline phosphatase-conjugated anti-fluorescein antibodies were obtained from Roche Molecular Biochemicals (Indianapolis, IN). The fluorescent indicator protein was purchased from Molecular Probes Inc. Recombinant N-glycosidase F, O-glycosidase from Diplomoccus neumanni, and neuraminidase from Atherobacter ureafaciens were all obtained from Roche Molecular Biochemicals. Chondroitinase ABC from Proteus vulgaris, chondroitinase AC and heparinase II from Flavobacterium heparinum, keratanase from Pseudomonas, and hyaluronate lyase from Streptomyces hyalurolyticus were purchased from Sigma.

**Cell Culture—**HTBS8 human lung squamous carcinoma cell line, HTB55 human lung adenocarcinoma, and Calu-6 anaplastic carcinoma were obtained from the American Type Culture Collection (Rockville, MD). Cells were cultured in Eagle’s MEM (Biowhittaker, Walkersville, MD) supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamycin (all from Life Technologies, Inc., Grand Island, NY), 10% fetal bovine serum (FBS) (Atlanta Biologicals, Norcross, GA). Cells were plated, allowed to grow to confluence, and treated with the indicated factors.

**Flow Cytometry—**Cells were harvested using 0.2% EDTA or trypsin/EDTA (0.05%, 0.02%) and stained with 5–10 μg/ml PE-conjugated mouse anti-human CD44 antibody (G44–26) or PE-conjugated anti-human CD3 antibody as an isotype-matched negative control. Cells were then fixed with 3.7% formaldehyde and analyzed on a FACScan (Becton Dickinson, Mountain View, CA). Soluble HA binding was assayed using saturating amounts of FITC-HA. Specific binding of FITC-HA to CD44 was determined by comparison of binding in the presence of blocking anti-CD44 mAb 5F12 or an excess of unlabelled HA.

**Cell Adhesion to Immobilized HA—**96-well tissue culture plates were coated with hyaluronan (ICN) at 1 mg/ml. Cells in suspension (1 × 10⁶/ml) were loaded with 5 μg/ml calcein in HEPES-buffered saline for 30 min at 37 °C. After washing in HEPES-buffered saline, labeled cells were resuspended in MEM supplemented with 10% FBS and aliquoted into HA-coated wells. Plates were then incubated for 30 min at 4 °C. Nonadherent cells were removed by washing and adherent cells were quantified using a fluorescence plate reader (Spectrafluor, SLT-Labinstruments Ges. m. b. H., Salzburg, Austria).

**Enzymatic Digestion—**CD44 was immunoprecipitated with Sepharose-conjugated anti-CD44 (Hermes III) mAb. CD44 was then eluted from beads with 100 mM NaCl, 25 mM Tris, pH 7.4, and 0.02% NaN₃, mixed detergent buffer (0.05% Nonidet P-40, 0.1% SDS, 0.3 M NaCl, 10 mM Tris, pH 8.6), and PBS. Amounts of Nα-sulfated labeled cell lysates were immunoprecipitated and washed as for Western blot analysis. CD44 was then treated with enzymes or directly released by boiling in Laemmli sample buffer and resolved on SDS-PAGE. Bands were detected by fluorography.

**RESULTS**

**Cytokines Induce HA Binding in Lung-derived Epithelial Tumor Cells—**Lung carcinomas represent a significant proportion of human tumors. OSM and TGF-β₁ are known to regulate the growth of tumor cells and the function of lung epithelial cells (27–30, 32, 33). We found that OSM and TGF-β₁ induced soluble HA binding to human squamous carcinoma-derived HTBS8 cells (Fig. 1, A and B), and induced CD44-mediated adhesion of HTB58 cells to an HA-coated substrate (Fig. 1C). HA binding was further increased on cells treated with OSM plus TGF-β₁. HA binding was CD44-mediated since it was abrogated by the anti-CD44 mAb 5F12. The increase in HA binding could not be attributed to increased levels of surface CD44 since no significant increase in binding of anti-CD44 to HA was observed (Fig. 1A). Treatment of HTBS8 cells with hyaluronate lyase to remove endogenous HA increased HA binding capacity to a similar extent in control and OSM-stimulated cells and to a lesser extent in TGF-β₁-stimulated cells (data not shown). These data indicate that CD44 may serve as an anchor for endogenous HA but argue against the possibility that exogenously added HA binds to other components in the pericellular matrix that are anchored by CD44. The enhancement of HA binding induced by OSM and TGF-β₁ was evident at 24 h and increased further at 48 h. Therefore, in subsequent experiments cells were treated with cytokines for 48 h.

**Cytokines Regulate the Post-translational Modification of CD44—**Initial studies indicated that HA binding was evident in cytokine-stimulated HTBS8 cells, even if fixed with formaldehyde prior to assaying. Furthermore, soluble CD44 spontaneously released from cytokine-stimulated HTBS8 cells exhibited enhanced HA binding compared with untreated HTBS8-derived soluble CD44 (data not shown). Therefore, we hypothesized that structural modification of the extracellular domain of CD44 was sufficient to mediate cytokine-enhanced HA binding. Cells of epithelial origin express the standard form of CD44, but expression of variant isoforms of CD44 is also a hallmark of epithelial cells (5). Three major CD44 RNA species contain 1 mM CaCl₂ and 0.01% bovine serum albumin. Treatment with chondroitin ABC (2 units/ml) and AC lyase (2 units/ml) as well as keratanase (30 milliunits/ml), heparinase II (5 units/ml), and hyaluronate lyase (50 milliunits/ml) was performed on CD44 bound to the Sepharose-conjugated Hermes III mAb in PBS at pH 7.4 (chondroitin ABC and heparinase II) or PBS at pH 7.0 (heparinase), or in buffer containing 50 mM sodium acetate, pH 5.2, and 125 mM NaCl (hyaluronate lyase), for 2 h at 37 °C.

**Bio-synthetic Labeling, Immunoprecipitation, and Fluorography—**Cells were incubated in methionine-free medium containing 2% FBS and 200 μCi/ml [³⁵S]methionine/cysteine (Trans³⁵S-label), or in sulfate-free MEM supplemented with 2% FBS and 500 μCi/ml Na₂³⁵SO₄ (ICN). Cytokines were added as indicated. Cells were lysed as for Western blot analysis. Aliquots of [³⁵S]methionine/cysteine-labeled cell lysates were precleared with preimmune serum and then precipitated with anti-CD44 mAb (Hermes III) conjugated to Sepharose. Immune complexes were washed with high salt (0.6 M NaCl, 125 mM KPO₄, pH 7.4, 0.02% NaN₃), mixed detergent buffer (0.05% Nonidet P-40, 0.1% SDS, 0.3 M NaCl, 10 mM Tris, pH 8.6), and PBS. Amounts of Nα-sulfated labeled cell lysates were immunoprecipitated and washed as for Western blot analysis. CD44 was then treated with enzymes or directly released by boiling in Laemmli sample buffer and resolved on SDS-PAGE. Bands were detected by fluorography.

**ELISA for HA Binding—**Cells were lysed and extracts were subjected to immunoprecipitation with Sepharose-conjugated anti-CD44 mAb (Hermes III) mAb. CD44 was then eluted from beads with 100 μM glycine, pH 2.5, and after neutralization to pH 7.0 with Tris-HCl buffer, CD44 was quantified by ELISA. Hermes III mAb-coated wells were used to capture CD44 and FITC-labeled G44–26 mAb and FITC-labeled rooster comb HA were used to quantitate total CD44 and HA binding, respectively. After incubation with alkaline phosphatase-conjugated anti-FITC mAb, the reaction was developed with p-nitrophenyl phosphate.

**Northern Blot Analysis—**Total RNA was isolated by SDS-phenol extraction and resolved by electrophoresis in agarose gels containing 2.2% formaldehyde, followed by capillary transfer to Hybond-N membranes (Amer sham Pharmacia Biotech). Filters were hybridized with a ²⁵P-labeled 1.4-kilobase XhoI-XhoI restriction fragment of human CD44 (5). The hybridization was carried out at 65 °C in 0.5 M phosphate buffer, pH 7.0, containing 7% SDS, 1 mM EDTA, and 100 μg/ml herring DNA. Blots were washed at 65 °C in 40 mM phosphate buffer, pH 7.0, containing 1% SDS, 1 mM EDTA and exposed to x-ray film.

**Western Blot Analysis—**Cells were lysed in PBS containing 1% Nonidet P-40, 0.1% sodium deoxycholate, and protease inhibitors (0.2 units/ml aprotinin, h. h., 100 μg/ml leupeptin, and 1 μU methylmethan sulfonyl fluoride). Cell lysates were normalized based on protein concentration as determined using the BCA kit (Pierce, Rockford, IL) and equal amounts of protein were subjected to immunoprecipitation with anti-CD44 mAb (Hermes III) conjugated to Sepharose. Immune complexes were washed once with lysis buffer followed by three washes with PBS and resolved on SDS-7.5% PAGE under nonreducing conditions. CD44 was visualized by enhanced chemiluminescence (ECL) (Amer sham Pharmacia Biotech) after electrophoresis to a PVDF membrane (NEN Life Science Products Inc., Boston, MA) and incubation with mouse anti-CD44 antibodies (G44–26) and donkey anti-mouse IgG antibodies conjugated to HRP. HA binding was analyzed by ECL after incubation of PVDF bound CD44 with FITC-HA followed by HRP-conjugated anti-fluorescein antibodies. CD44 was immunoprecipitated with Sepharose-conjugated anti-CD44 (Hermes III) mAb. CD44 was recovered from beads by boiling for 5 min in the presence of 0.1% of SDS. After addition of Triton X-100 (final concentration of 1%), affinity purified CD44 was treated at 37 °C for 24 h with 40 units/ml N-glycosidase F in 0.2 M sodium phosphate, pH 8.0, and 20 μM EDTA, or for 4 h with 100 milliunits/ml neuraminidase in 50 mM sodium acetate, pH 5.5, containing
of approximately 1.6, 2.2, and 5 kilobases are characteristic for human cells expressing only the standard form of CD44 (5). Transcripts of the same electrophoretic mobility were detected in HTB58 cells. Although we did not observe any significant increase in expression of CD44 at the cell surface, stimulation of HTB58 cells with OSM or TGF-$\beta_1$ did up-regulate the steady state levels of the 1.6-, 2.2-, and 5-kilobase CD44 mRNAs. Importantly, however, cytokine stimulation did not lead to the expression of alternatively spliced mRNAs encoding variant exon(s) (Fig. 2).

Two species of CD44 with average molecular mass of 90 and 180 kDa were immunoprecipitated from detergent lysates of HTB58 cells. Although we did not observe any significant increase in expression of CD44 at the cell surface, stimulation of HTB58 cells with OSM or TGF-$\beta_1$ did up-regulate the steady state levels of the 1.6-, 2.2-, and 5-kilobase CD44 mRNAs. Importantly, however, cytokine stimulation did not lead to the expression of alternatively spliced mRNAs encoding variant exon(s) (Fig. 2A).

Sulfation can facilitate functional interactions between adhesion molecules, including L-selectin, and their ligands (34, 35). Recently, an increase in incorporation of sulfate into a 90-kDa species in response to tumor necrosis factor-$\alpha$ was suggested to regulate the function of CD44 in a human leukemic cell line (19). However, the component of CD44 that was inducibly sulfated was not determined. Both the 90-kDa form and species of >210 kDa were detected in anti-CD44 immunoprecipitates from lysates of sulfate-labeled HTB58 cells (Fig. 2D). The 90-kDa form of CD44 immunoprecipitated from parallel cultures of unlabeled cells detected by immunoblotting and the CD44 immunoprecipitated from $[^{35}S]$sulfate-labeled cells co-migrated. The highly sulfated high molecular weight species exhibited extensive microheterogeneity with an average mass of >210 kDa compared to an average mass of 180 kDa for the high molecular species detected by immunoblotting (Fig. 2) or $[^{35}S]$methionine biosynthetically labeled CD44. The >210-kDa species was readily detectable by $[^{35}S]$sulfate labeling in anti-CD44 immunoprecipitates even following washing under stringent conditions with high salt and mixed detergent buffers and was detectable by immunoblotting when the blot was overexposed (Fig. 2C). These data indicate that the >210-kDa species was less abundant although it was highly sulfated and therefore a major species detected by $[^{35}S]$sulfate labeling. Conversely, the more abundant 180-kDa species of CD44 exhibited negligible sulfation. Treatment with OSM resulted in a marked increase in sulfate incorporation into CD44, particularly of the >210-kDa species while treatment of HTB58 cells...
with TGF-β1 had only a slight effect on incorporation of [35S]sulfate into CD44 (Fig. 2D). These data suggested that changes in sulfation are potentially involved in regulating the HA binding function of CD44 in OSM-stimulated lung-derived epithelial cells.

**HA Binding of Isolated CD44**—Previously described assays detect HA binding to the total repertoire of CD44 species expressed by cells. To establish the biological significance of the differentially modified forms of CD44, we developed a cell-free blot assay for measuring HA binding to distinct species of CD44. Total cell lysates or CD44 immunoprecipitated from detergent lysates were resolved by SDS-PAGE. Proteins were transferred to PVDF membranes that were then incubated with FITC-labeled HA followed by HRP-conjugated anti-FITC antibodies. Reactivity was detected by ECL. To determine if HA binding to isolated CD44 correlated with HA binding to intact cells, we compared CD44-mediated HA binding to intact Calu-6, HTB55, and HTB58 cells by flow cytometry and to CD44 affinity purified from these cell lines and evaluated in the cell-free blot assay. The three cell lines expressed similar levels of CD44 but differed in their capacity to bind HA (Fig. 3A); Calu-6 cells constitutively bind HA, HTB55 cells do not bind HA, and HTB58 cells exhibit an intermediate level of constitutive HA binding. In all three cases, HA binding to isolated CD44 in the cell-free blot assay correlated very well with HA binding to intact cells (Fig. 3, A and B). The anti-CD44 antibody 5F12 and an excess of unlabeled HA ablated the signal in the cell-free HA blot assay (Fig. 3C), demonstrating the specificity of HA binding to isolated CD44. Furthermore, pretreatment of CD44 bound to PVDF membranes with an antibody, F10-44, that enhances HA binding to intact cells (4) (data not shown), also augmented HA binding in the cell-free blot assay (Fig. 3C). Thus HA binding of CD44 assayed in the cell-free system mimicked the HA binding activity of intact cells.

Using the cell-free blot assay we could discriminate binding to the different species of CD44 expressed by HTB58 cells. Analysis of total cell extracts of unstimulated cells did not reveal any reactivity with HA. In contrast, prominent HA binding to a major 90-kDa species that co-migrated with the predominant form of CD44 was evident in analysis of whole cell lysates from OSM-stimulated HTB58 cells (Fig. 3D). When we enriched for CD44 by analyzing anti-CD44 immune complexes, HA binding to both the 90-kDa and 180-kDa species of CD44 isolated from HTB58 cells was detected (Figs. 3 and 4). Furthermore, treatment of HTB58 cells with OSM resulted in markedly increased HA binding to both the low and high molecular weight forms of CD44 (Fig. 4). However, we did not detect any significant difference in affinity of CD44 for HA in the cell-free blot assay following TGF-β1 stimulation suggesting that OSM and TGF-β1 utilized different mechanisms for modulating CD44 receptor function.

**The Role of Glycosylation in CD44-HA Interactions**—To elucidate the role of carbohydrates in cytokine-induced HA binding, HTB58 cells were subjected to metabolic or enzymatic deglycosylation and analyzed for CD44-mediated HA binding by flow cytometry. Disruption of N-linked glycosylation by exposure to tunicamycin was found to inhibit CD44-HA interactions whereas treatment of cells with neuraminidase, to cleave terminal sialic acid residues, markedly enhanced the CD44-mediated constitutive binding of HA to intact HTB58 cells (Table I). Treatment with neuraminidase also enhanced HA binding of cytokine-stimulated cells but to a lesser extent in OSM-treated cells than in cells stimulated with TGF-β1. The effects of tunicamycin and neuraminidase were not due to changes in the density of cell surface CD44 (Table I).

To determine the role of glycosylation in HA binding to isolated receptor, CD44 immunoprecipitated from lysates of HTB58 cells was subjected to digestion with N-glycosidase F or neuraminidase. The apparent molecular mass of both the 90- and 180-kDa species of CD44 was reduced by treatment with N-glycosidase F to 70 and 150 kDa, respectively (Fig. 4A). Furthermore, treatment with N-glycosidase F eliminated the differences in mobility of both species of CD44 isolated from control and OSM-treated cells. Thus, the more rapid migration of CD44 induced by OSM could at least partly be attributed to modification of N-linked carbohydrate moieties. Neuraminidase induced a more modest reduction in the apparent molecular weight of the receptor, but CD44 immunoprecipitated from OSM-stimulated cells still migrated somewhat more rapidly than CD44 from unstimulated cells following treatment with neuraminidase.
Consistent with the results obtained with tunicamycin-treated cells, enzymatic deglycosylation of N-linked carbohydrates from isolated CD44 abrogated HA binding. Also similar to intact cells, hydrolysis of sialic acids augmented CD44-HA interactions of both the 90- and 180-kDa isoforms of CD44 immunoprecipitated from either control or cytokine-stimulated cells (Fig. 4, A and B). The binding of HA to neuraminidase-treated CD44 was blocked by an excess of unlabeled rooster comb or human umbilical cord HA as well as by 5F12 antibody (data not shown). When N-glycosidase F and neuraminidase were given together, the enhancement of HA binding induced by neuraminidase was no longer evident, suggesting that sialic acid residues of N-linked oligosaccharide chains are involved in inhibition of CD44 receptor function.

The Role of Sulfation in Cytokine-stimulated HA Binding Activity of CD44—As described above, sulfated CD44 species of 90 kDa and high molecular mass forms of >210 kDa were detected in HTB58 cells (Fig. 5). The higher molecular weight form was consistently the major sulfated form of CD44, particularly after OSM stimulation. The 90-kDa band from lysates of [35S]sulfate-labeled cells stimulated with OSM was somewhat more intense than in untreated cells, but this most likely resulted from an increase in synthesis of CD44 rather than an increase in the incorporation of sulfate groups on a molar basis.
since the ratio of \[^{35}S\]methionine to \[^{35}S\]sulfate incorporated in OSM-treated cells was similar to that of control cells (Table II). In contrast, OSM given alone or in combination with

![CD44 Immunoblot](image)

**TABLE I**

**Effect of tunicamycin and neuraminidase on HA binding to HTB58 cells**

|       | + Tunicamycin | + Neuraminidase |
|-------|---------------|----------------|
| Control | 24.2          | 6.0            | 74.4           |
| OSM    | 51.4          | 6.2            | 68.8           |
| TGF    | 47.0          | 8.3            | 86.3           |
| OSM + TGF | 60.2      | 7.7            | 65.0           |

|       | CD44 expression (MFI) |
|-------|-----------------------|
| Control | 617.3       | 514.5          | 535.3          |
| OSM    | 474.6       | 473.1          | 477.9          |
| TGF    | 620.0       | 490.0          | 500.3          |
| OSM + TGF | 445.1  | 550.2          | 430.9          |

**TABLE II**

**Comparison of \[^{35}S\]methionine and \[^{35}S\]sulfate incorporation into CD44 in HTB58 cells**

|       | \[^{35}S\]Methionine | \[^{35}S\]Sulfate | \[^{35}S\]Methionine | \[^{35}S\]Sulfate |
|-------|-----------------------|-------------------|-----------------------|-------------------|
| Control | 1                      | 1                 | 1                     | 1                 |
| OSM    | 1.40 ± 0.20            | 1.47 ± 0.15       | 1.30 ± 0.06           | 13.10 ± 7.50      |
| TGF    | 1.37 ± 0.15            | 1.50 ± 0.29       | 1.10 ± 0.08           | 2.00 ± 0.83       |
| OSM + TGF | 1.67 ± 0.06 | 2.40 ± 0.75       | 1.40 ± 0.11           | 13.10 ± 7.50      |

TGF-β1 dramatically increased the ratio of sulfate to methionine incorporated into the >210-kDa form.

To determine whether sulfation was required for the adhesion of CD44 to HA, HTB58 cells were incubated in the presence of sodium chlorate \((\text{NaClO}_3)\). Treatment of cells with \text{NaClO}_3 inhibited the incorporation of sulfate into the 90- and >210-kDa forms of CD44 without affecting the synthesis or cell

![FIG. 5. Chlorate inhibits sulfation of CD44, HTB58 cells were cultured in the absence or presence of 40 mM sodium chloride for 48 h. Cells were then incubated for 48 h in sulfate-free MEM containing 2% FBS, 500 μCi/ml Na\(^{35}\)SO\(_4\), and 40 mM sodium chloride, 50 ng/ml OSM, and 10 ng/ml TGF-β1 as indicated. CD44 was immunoprecipitated with Sepharose-conjugated anti-CD44 mAb. The immune complexes were subjected to anti-CD44 immunoblot analysis (A) and cell-free HA blot analysis (B). The remaining \[^{35}S\]-labeled immune complexes were resolved on SDS-PAGE followed by fluorography (C). Data shown in D represent 10 times longer exposure of the data shown in the right panel of C. Molecular weight markers in kDa are indicated.](image)
in the absence or presence of 40 mM sodium chlorate for 48 h. Cells were then analyzed by flow cytometric analysis of HA binding. HTB58 cells were incubated in the presence of OSM, and 10 ng/ml TGF-β1 as indicated. Cells were stained with FITC-HA in the presence or absence of anti-CD44 5F12 mAb. Percentage of positive cells is shown as the mean ± S.D. from four independent experiments. B, ELISA of HA binding by detergent-solubilized CD44. HTB58 cells treated as in A were lysed and extracts were subjected to immunoprecipitation with Sepharose-conjugated anti-CD44 mAb. CD44 was then eluted from beads and quantified by ELISA. The data are shown as the mean of triplicate wells from three experiments ± S.D. The difference between chlorate-untreated and chlorate-treated cells was statistically significant (Student’s t test p < 0.001) only in samples containing OSM.

surface expression of CD44 (Fig. 5 and data not shown). Chlorate did not decrease the capacity of control or TGF-β1-stimulated cells to bind HA but markedly compromised OSM-induced HA binding (Fig. 6A). These results establish that sulfation of CD44 is an important mechanism underlying OSM-induced HA binding to intact cells. However, chlorate did not affect HA binding to isolated CD44 in the cell-free blot assay (Fig. 5B). Therefore, OSM appears to induce HA binding via two mechanisms, one involving sulfation and the other glycosylation. These results suggest that the OSM-induced HA binding to cell-free CD44 is more likely dependent on the differential glycosylation than on sulfation described above. These results also indicate that the enhancement of HA binding mediated by sulfation is only evident under nondenaturing conditions and/or that association of low and high molecular weight forms of CD44 is required for the sulfation-dependent function of CD44. To test this hypothesis we performed a “functional” ELISA to measure HA binding of detergent-solubilized CD44 (Fig. 6B). The total CD44 immunoprecipitated using anti-CD44 mAb conjugated to Sepharose was eluted from beads and captured on anti-CD44-coated wells. The levels of CD44 and HA binding were quantitated by ELISA (Fig. 6B). Since immunoprecipitated CD44 contains both low and high molecular weight isoforms, the activity of CD44 determined by this assay reflects the binding of the combination of both forms compared with the blotting assay that discriminates between HA binding to each isoform separated by SDS-PAGE. OSM given alone or together with TGF-β1, but not TGF-β1 alone, significantly enhanced HA binding as measured in the ELISA. Furthermore, chlorate partially inhibited the OSM-induced HA binding detected in the ELISA (Fig. 6B), suggesting that both sulfation-dependent and -independent mechanisms are involved in HA binding of cell-free CD44 derived from OSM-stimulated cells.

To characterize the target of sulfation, CD44 immunoprecipitated from [35S]sulfate-labeled cells was subjected to digestion with glycosidases or enzymes that cleave different GAGs. Removal of N-linked carbohydrates by N-glycosidase F and sialic acids by neuraminidase as well as treatment with O-glycosidase after removal of sialic acids had no effect on sulfation of either the 90- or >210-kDa forms (data not shown). Digestion of CD44 with keratanase and hyaluronate lyase did not reduce the sulfation of CD44 and treatment of CD44 with heparinase II which reduced the molecular mass of the 90- and 210-kDa forms did not significantly decrease the degree of sulfation of the receptor (data not shown). However, chondroitin ABC and chondroitin AC lyase digestion of CD44 immunoprecipitated from HTB58 cells reduced the apparent molecular mass of the 90- and 180-kDa forms of CD44 (Fig. 7, B and C) and resulted in the release of sulfate from the high molecular weight species of CD44 (Fig. 7A). Thus the extensive sulfation associated with the high molecular weight form of CD44 is mainly due to modification with chondroitin sulfate chains.

The highly sulfated species of CD44 of >210 kDa were enriched in cells stimulated with OSM (Figs. 5 and 7). Although less prominent following treatment with chlorate, we could still detect the >210-kDa species of CD44 in cells treated with OSM with prolonged exposure to film (Fig. 5, panel D). Therefore, we conclude that although OSM may induce an increase in the length or number of chondroitin sulfate chains attached, OSM also induces an increase in the incorporation of sulfate into the chondroitin sulfate attached to CD44.

discussion

The formation of metastatic deposits requires modulation of the adhesive properties of tumor cells, allowing them to disseminate through the bloodstream and lymphatics from their site of origin to distal sites. The accumulation of HA is often increased around colonies of metastatic cells due to secretion of high levels of HA by tumor or stromal cells (27). The importance of regulated HA binding is evidenced by the fact that CD44 mutants that do not bind HA fail to promote growth and metastasis of some tumors (26). Our data demonstrate that OSM and TGF-β1 augment HA binding in tumor-derived HTB58 cells. Both OSM and TGF-β1 were previously shown to regulate the growth of tumor cells (27–30). Taken together, these data suggest that OSM and TGF-β1 may be important in modulating tumor growth and metastasis by regulating HA binding activity. These studies add OSM and TGF-β1 to a network of soluble factors that regulate CD44-HA interactions (4, 16, 17) and provide the first example of cytokine regulation of the affinity of CD44 on epithelial-derived tumor cells.

Modification of the sugar moieties of CD44 has previously...
been suggested as a mechanism for regulating affinity of the receptor for HA (11–13, 36). Depending on the cell type in which CD44 was expressed or on the experimental approach used, including deglycosylation of surface proteins or hydrolysis of carbohydrates from soluble CD44-immunoglobulin chimeric proteins, N-deglycosylation was demonstrated to either abrogate or augment HA binding to intact cells, whereas removal of sialic acids was found to either augment or have no effect on HA binding. However, these studies did not reveal whether the observed changes in CD44 function were due to deglycosylation of CD44 or deglycosylation of other molecule(s) on the cell surface which may cooperate with CD44 to regulate HA binding function. Likewise, interpretation of previous results obtained using recombinant soluble CD44-immunoglobulin chimeric proteins was complicated by the fact that glycosylation occurs in a cell-specific manner and therefore the structure of oligosaccharides decorating CD44-immunoglobulin fusion proteins is largely dependent on the transfected cells expressing the chimeric transgene. In addition, these approaches could not clarify the contribution of different CD44 isoforms to HA binding in cells that express multiple isoforms of CD44. The cell-free HA blotting assay described herein allowed us to evaluate the HA binding activity of each of multiple species of CD44 expressed on a single cell type. We demonstrated that the 90-kDa as well as the high molecular mass 180-kDa species of CD44 expressed on HTB58 cells bind HA. Furthermore, we established a direct correlation between alterations in the glycosylation of CD44 and its HA binding activity. Thus, in the absence of any cell surface constraints, removal of N-linked carbohydrates abrogated HA binding to both the 90- and 180-kDa forms of CD44, whereas removal of sialic acids augmented their affinity for HA. The fact that HA binding to the highest molecular mass species of >210 kDa was not detectable once the anti-CD44 immune complexes were resolved by SDS-PAGE may indicate a requirement for cooperativity between the multiple forms of the receptor for HA binding to the highest molecular mass form. However, we cannot rule out a low level of HA binding to this form, below the level of detection of the HA blot assay. In addition, our data indicate that CD44 binding induced by OSM was associated with a decrease in N-linked carbohydrate content and optimal OSM-induced HA binding required sulfation of proteoglycan forms of CD44. The mechanism(s) by which TGF-β1 induced HA binding is clearly distinct from those involved with stimulation with OSM. In initial studies of TGF-β1-stimulated HTB58 cells we found that TGF-β1 induced accumulation of a species of CD44 that exhibits altered migration upon isoelectrofocusing similar to that induced by neuraminidase treatment (data not shown). Thus TGF-β1-enhanced HA binding to HTB58 cells may be partially due to decreased sialylation of CD44. The fact that in contrast to the cell-bound receptor, enhanced HA binding was not observed for cell-free CD44 derived from TGF-β1-treated cells may indicate that association with other molecules, facilitated by TGF-β1-mediated desialylation of CD44, is required for TGF-β1-enhanced HA binding function.

Consistent with the role of sulfation of OSM-induced HA binding to epithelial-derived tumor cells established in this study, a recent study of a human leukemic cell line, SR91, also suggested a role for sulfation in regulating the HA binding function of CD44 (19). However, in that case a single species of CD44 with an apparent molecular mass of 90 kDa was detected and tumor necrosis factor-α increased the sulfation of this form. Our data indicate that an increase in sulfation of the higher molecular form of CD44, which was attributed to the presence of chondroitin sulfate chains, mainly accounted for the enhancement in HA binding in OSM-treated HTB58 cells. Furthermore, our data obtained with CD44 isolated from chloride-treated HTB58 cells indicate that an increase in sulfation of CD44 per se rather than other cell surface molecules is likely responsible for the effect of OSM. Interestingly, the apparent discrepancy between the requirement for sulfation in the ELISA and blot assays suggests that the sulfation-dependent HA binding function of CD44 may involve cooperativity of the low and high molecular forms of CD44. Since in OSM-treated HTB58 cells the adhesion of CD44 to HA is partially dependent upon an increase in the extent of sulfation of the chondroitin sulfate chains attached to the receptor and only a small fraction of the total CD44 appears to be decorated by GAG, we propose that highly sulfated chondroitin sulfate chains may
facilitate oligomerization of the receptor and ultimately HA binding. In this regard, it is interesting to note that CD44 has been shown to recognize and bind several molecules modified by chondroitin sulfate chains, including serylgin and the invariant chain of class II MHC molecules (37, 38). More recently, alternatively spliced CD44 isoforms were reported to promote cellular adhesion through the recognition of chondroitin sulfate-modified CD44, which may imply a role of the chondroitin sulfate chains in receptor oligomerization (39).

In summary, our results demonstrate that cytokines regulate the adhesion function of CD44 on epithelial-derived tumor cells. Furthermore, we established that various cytokines utilize different mechanisms to regulate the transition of CD44 from the low to the high affinity state, including differential glycosylation and a novel mechanism involving sulfation of the chondroitin sulfate chains attached to CD44.

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Joanna Cichy and Ellen Puré

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