Cytokines regulate the affinity of soluble CD44 for hyaluronan

Joanna Cichy\textsuperscript{a,b,∗}, Ellen Puré\textsuperscript{b,c}

\textsuperscript{a}Faculty of Biotechnology, Jagiellonian University, Ul. Gronostajowa 7, 30-387 Kraków, Poland
\textsuperscript{b}Wistar Institute, Philadelphia, PA 19104, USA
\textsuperscript{c}Ludwig Institute of Cancer Research, New York, NY 10158, USA

Received 24 October 2003; accepted 11 November 2003

First published online 27 November 2003

Edited by Masayuki Miyasaka

Abstract CD44, a receptor for the extracellular matrix glycosaminoglycan hyaluronan, has been implicated in many adhesion-dependent cellular processes including tumor growth and metastasis. Soluble CD44 has been identified in the serum of normal individuals. Furthermore, tumor progression is often associated with marked increases in plasma levels of soluble CD44. Release of soluble CD44 by proteolytic cleavage (shedding) of membrane-anchored CD44 is likely to alter cellular responses to the environment due to modification of the cell surface and the potential for soluble CD44 to influence CD44-mediated hyaluronan binding to cell surfaces. Cellular activation is typically required to induce hyaluronan binding to cell surface CD44 but the affinity of endogenous soluble CD44 for hyaluronan remains unknown. In this study, we demonstrate that oncostatin M and transforming growth factor β1 (TGF-β1) which stimulate hyaluronan binding to HTB58 lung epithelial-derived tumor cells, also induce the release of soluble CD44. Interestingly, soluble CD44 released by oncostatin M-treated cells retained the ligand-binding properties of the membrane-anchored receptor. In contrast, soluble CD44 released from TGF-β1-treated HTB58 cells differed in its hyaluronan-binding capacity from cell surface CD44 expressed on TGF-β1-stimulated cells. These data indicate that the mechanisms that regulate the generation of soluble CD44 may also govern the binding of the released receptor to hyaluronan and therefore determine the impact on CD44-dependent physiologic and pathologic processes.

© 2003 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Key words: Shedding; Tumor; Adhesion; Oncostatin M; Transforming growth factor

1. Introduction

Cleavage of membrane-anchored proteins (shedding) is associated with modification of cell surfaces and the release of biologically active molecules. As a result, proteolytic release of membrane proteins represents an important mechanism for altering cellular responses to the environment. Accumulating evidence suggests that adhesion molecule expression and activity can be regulated through shedding [1,2]. The inflammatory response, and the formation of tumor metastasis, involves interactions between cell adhesion molecules on leukocytes and tumor cells with endothelium and subendothelial parenchymal cells and matrix at sites of inflammation and tumor dissemination, respectively. Regulated proteolysis has been proposed as a mechanism to rapidly down-regulate the levels of adhesion molecules and thereby disrupt cell–cell and cell–matrix interactions. For example, L-selectin is rapidly down-regulated following activation of leukocytes resulting in alterations in the trafficking patterns of activated compared to naive leukocytes [1,2]. In addition, soluble adhesion receptors may exhibit biologic activity. For example, soluble syndecan-1 ectodomain, a heparan sulfate proteoglycan shed from cell surfaces into wound fluids, is a potent inhibitor of heparin-mediated fibroblast growth factor 2 mitogenicity [3].

CD44 is a widely distributed cell surface glycoprotein implicated in cell–cell adhesion and cell–matrix interaction largely through its affinity for hyaluronan (HA), a ubiquitous extracellular matrix glycosaminoglycan [4–6]. CD44 expression and HA binding has been linked to the metastatic potential of tumors [7] as well as autoimmune and inflammation [8,9]. CD44 derives from a single gene, but is expressed as multiple isoforms ranging in molecular weight from 80 to 250 kDa. This structural diversity arises from utilization of alternatively spliced mRNA templates, which can be further modified posttranslationally by glycosylation or the attachment of glycosaminoglycans. The most common form of CD44 (CD44H) has an apparent molecular weight of 80–100 kDa and does not contain differentially spliced exons [10]. This structural diversity of CD44 translates into a pleiotropic adhesion molecule capable of interacting with multiple molecules in addition to HA, including growth factors, cytokines, and extracellular matrix proteins such as fibronectin and collagen. Importantly, the ability of CD44 on the vast majority of cell types to bind HA with high affinity is not constitutive, rather cellular activation is required to induce HA binding. However, HA-binding ability of cell surface CD44 appears to be required to exert biological effects on HA-related cellular functions, including growth and metastasis of some tumors [11]. A variety of mechanisms have been implicated in the transition from inactive to active forms of CD44, including variant exon usage, receptor oligomerization, glycosylation and sulfation. The ability of cell surface CD44 to bind HA can be modulated by soluble factors, including cytokines [12,13]. In addition, an excess of soluble CD44 (sCD44) may compete with membrane-anchored CD44 for HA binding, thus providing another mechanism for modulation of cell surface CD44-mediated HA interactions.

Significant levels of sCD44 have been detected in serum and lymph from a variety of species. Increased serum levels of CD44 have been documented in association with malignancy...
and with immune activation whereas immunodeficiency is correlated with low concentrations of sCD44 [14], indicating that immunological and malignant activity may promote the release of sCD44. The generation of sCD44 is believed to involve mainly proteolytic cleavage (shedding) of cell surface, and possibly differential splicing of CD44 since a CD44 transcript devoid of the transmembrane and cytoplasmic domains, and thus encoding a soluble truncated CD44 receptor, has been identified in mouse fetal myoblasts CD44 (reviewed in [15]). Although correlation between elevated plasma levels of CD44 and malignant diseases has generated interest in CD44 as a potential biomarker for tumor malignancy, the role of sCD44 remains to be elucidated. To gain insight into the biology of sCD44 it is important to determine its HA-binding ability. Previous reports demonstrated that a chimeric protein consisting of the extracellular domain of CD44 and IgG Fc can block binding of cell surface CD44 to HA. However, little is known about the relative HA-binding capacity of native sCD44 released from cells compared to cell surface CD44.

In this study we demonstrate that osteosarcoma (OSM) and transforming growth factor β1 (TGF-β1) induce the release of sCD44 from lung epithelial-derived tumor cells and that sCD44 released by cytokines from these cells either retains the ligand-binding properties of the membrane-anchored receptor or differs in its HA-binding capacity from cell surface CD44 depending on the stimulant, suggesting that sCD44 may differentially contribute to CD44-mediated processes.

2. Materials and methods

2.1. Materials

Human recombinant OSM and human recombinant TGF-β1 were purchased from R&D Systems (Minneapolis, MN, USA). The following anti-human CD44 monoclonal antibodies (mAbs) were used [10]: 5F12 that blocks HA binding (a generous gift of Dr. B.F. Haynes, Duke University Medical Center, Durham, NC, USA), Hermes III (American Tissue Type Collection (ATCC), Rockville, MD, USA), and G4-26 (PharMingen, San Diego, CA, USA). Purified HA from rooster comb and hyaluronic acid from Streptomyces were purchased from Sigma (St. Louis, MO, USA). Fluorescein-conjugated rooster comb HA (FITC-HA) was prepared as described [16]. FITC-labeled anti-human CD44 antibodies and phycoerythrin (PE)-labeled staphylococcal protein A (SPA) were purchased from PharMingen. Biotinylated hyaluronic acid-binding protein was obtained from Seikagaku (Tokyo, Japan). Alkaline phosphatase-conjugated anti-fluorescein antibodies were obtained from Roche (Indianapolis, IN, USA). CD44-2-Rg vector [4] was a generous gift of Dr. I. Stamenkovic (Harvard Medical School, Cambridge, MA, USA). The bicinechonic acid kit was purchased from Pierce (Rockford, IL, USA), and enhanced chemiluminescence (ECL) system from Amersham Pharmacia Biotech.

2.2. Cell culture

HTB58, a human lung squamous carcinoma cell line, was obtained from ATCC. HTB58 cells were cultured in Eagle’s modified essential medium supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamicin and 10% heat-inactivated fetal bovine serum. Equal numbers of cells were plated, grown to confluency, and sCD44 was quantified by ELISA. Plates (Microlite™ 2. Dynex, Chantilly, VA, USA) were coated with 2 μg/ml of mAb Hermes III in Tris-buffered saline (50 mM Tris–HCl pH 9.5, 150 mM NaCl). The plates were then washed with PBS containing 0.05% Tween 20, and multiple protein-binding sites were blocked with 3% bovine serum albumin (BSA) in PBS. Standards and samples were added and incubated at room temperature for 2 h. Affinity-purified CD44-immunglobulin fusion protein (CD44-2-Rg) containing the complete extracellular domain of CD44 was used as a standard. After washing, FITC-labeled anti-CD44 G44-26 mAb was added to the wells and incubated at 37°C for 30 min. Bound antibody was detected by incubation with alkaline phosphatase-conjugated anti-FITC mAb and developed with p-nitrophenyl phosphate. Absorbances were then read at 405 nm with a plate reader, and after subtraction of background values, the readings were used to construct a standard curve.

2.3. Biosynthetic and sulfate labeling, immunoprecipitation and fluorography

Cells were incubated for 48 h in methionine-free medium containing 200 μCi/ml [35S]methionine/cysteine (Trans [35S]-label; ICN). Conditioned media were collected and centrifuged at 300,000 x g for 10 min followed by 600 x g for 15 min to remove cells and cellular debris. Conditioned media were normalized based on cell number, preincubated with pre-immune serum and then precipitated with G44-26 followed by protein A/G plus agarose. Immune complexes were washed sequentially with high salt (0.6 M NaCl, 125 mM KPO4, pH 7.4, 0.02% NaN3), mixed detergent buffer (0.05% NP-40, 0.1% sodium dodecyl sulfate (SDS), 0.3 M NaCl, 10 mM Tris pH 8.6), and phosphate-buffered saline (PBS). CD44 was then released by boiling in Laemmli sample buffer and resolved by SDS-8% polyacrylamide gel electro-phoresis (PAGE) under non-reducing conditions. Bands were detected by fluorography.

2.4. Flow cytometry

Cells were harvested using 0.2% EDTA, stained for HA binding using HA-FITC, and analyzed on a FACScan. Non-specific reactivity was based on staining with HA-FITC in the presence of blocking anti-CD44 mAb 5F12. To determine the level of HA associated with cell surface, HTB58 cells were stained with biotinylated HA-binding protein (bi-CD44) followed by PE-conjugated streptavidin. Specificity was verified by pretreating the cells with 20 U/ml hyaluronidase at 37°C for 2 h followed by staining with bPG and PE-conjugated streptavidin.

2.5. CD44-specific enzyme-linked immunosorbent assay (ELISA)

sCD44 was quantified by ELISA. Plates (Microlite™ 2. Dynex, Chantilly, VA, USA) were coated with 2 μg/ml of mAb Hermes III in Tris-buffered saline (50 mM Tris–HCl pH 9.5, 150 mM NaCl). The plates were then washed with PBS containing 0.05% Tween 20, and multiple protein-binding sites were blocked with 3% bovine serum albumin (BSA) in PBS. Standards and samples were added and incubated at room temperature for 2 h. Affinity-purified CD44-immunglobulin fusion protein (CD44-2-Rg) containing the complete extracellular domain of CD44 was used as a standard. After washing, FITC-labeled anti-CD44 G44-26 mAb was added to the wells and incubated at 37°C for 30 min. Bound antibody was detected by incubation with alkaline phosphatase-conjugated anti-FITC mAb and developed with p-nitrophenyl phosphate. Absorbances were then read at 405 nm with a plate reader, and after subtraction of background values, the readings were used to construct a standard curve.

2.6. ELISA for HA binding

To quantify CD44 capable of binding HA, plates were coated with Herbs III mAb, washed and incubated with standards and samples as described above. In some instances, conditioned media were concentrated on Centricron YM-10 (Millipore, Bedford, MA, USA), or subjected to immunoprecipitation with Sepharose-conjugated anti-CD44 Hermes III mAb. CD44 was then eluted from the beads with 100 mM glycine, pH 2.5, and after neutralization to pH 7.5 with Tris–HCl. CD44 was used for ELISA. FITC-labeled HA was added to the wells and incubated at 37°C for 30 min followed by incubation with alkaline phosphatase-conjugated anti-FITC mAb and p-nitrophenyl phosphate.

The level of sCD44 occupied by endogenous HA was examined as follows: streptavidin-coated plates (Pierce) were coated with 2.5 μg/ml of bPG or biotinylated anti-CD3 Ab as negative control, in Tris-buffered saline. After blocking of non-specific protein-binding sites with 100 mM glycine, pH 2.5, and after neutralization to pH 7.5 with Tris–HCl, CD44 was used for ELISA. FITC-labeled HA was added to the wells and incubated at 37°C for 30 min followed by incubation with alkaline phosphatase-conjugated anti-FITC mAb and p-nitrophenyl phosphate.

Detection of HA released by cells

In order to determine the HA content in the media we developed a modified ELISA based on competitive binding of endogenous and FITC-labeled exogenous HA to microwell-bound bPG. The conditioned media from cells incubated for 48 h were added to ELISA plates (Pierce) coated with bPG and blocked with 3% BSA. Conditioned media were incubated at room temperature for 2 h to capture endogenous HA. Unlabeled purified HA was used as a standard. Unlabeled and pre-incubated bPG were then detected by incubation with FITC-DA at 37°C for 30 min followed by incubation with alkaline phosphatase-conjugated anti-FITC mAb and p-nitrophenyl phosphate.
3. Results

3.1. OSM and TGF-β1 increase the release of CD44 from tumor cells

Most of the known effects of CD44 on cell adhesion and migration are intimately associated with its capacity to promote cell attachment to HA. We previously demonstrated that OSM and TGF-β1 induce transition of transmembrane CD44 from its low to its high affinity state in lung-derived tumor HTB58 cells [13], but the effects of cytokines on the release of sCD44 and the HA-binding capacity of sCD44 have not been investigated. To determine if cytokines regulate shedding of CD44, release of CD44 from untreated and cytokine-stimulated HTB58 cells was investigated. HTB58 cells were found to spontaneously release CD44 into the conditioned media (Fig. 1A). OSM and TGF-β1 induced comparable, statistically significant increases in levels of sCD44 as determined by ELISA (Fig. 1A). Flow cytometry analysis confirmed that the release of sCD44 into the culture media was associated with 20–30% reduction in cell surface CD44 in HTB58 cells treated with OSM alone or a combination of OSM and TGF-β1 as previously reported [13]. On average, the reduction in membrane-bound receptor following TGF-β1 treatment was less pronounced compared to OSM-treated cells. In fact, the effect of TGF-β1 was variable, ranging from 0% to 20% reduction in cell surface CD44 ([13], and data not shown). These differences may reflect variability in CD44 cell surface staining as a result of accumulation of pericellular matrix in TGF-β1-treated cells.

Immunoprecipitation of CD44 from 48-h conditioned media from metabolically labeled HTB58 cells revealed the release of three major species of sCD44 of approximately 180 kDa, 90 kDa and 60 kDa (Fig. 1B). No difference in the apparent molecular mass of sCD44 was observed between control and cytokine-treated cells.

Importantly, a different profile of CD44 species was detected in conditioned media of [35S]sulfate-labeled HTB58 cells subjected to immunoprecipitation using anti-CD44 mAb. Specifically, although large sulfated species which did not penetrate the gel were recovered from conditioned media of untreated and cytokine-treated cells, only in conditioned media from cells treated with either OSM or OSM plus TGF-β1 were additional sulfated species of 150–210 kDa detected (Fig. 1C).

3.2. Cytokines regulate the affinity of sCD44 for HA

To determine if sCD44 released from HTB58 cells retains function we used an ELISA specific for CD44 capable of binding HA. Total CD44 from conditioned media of control or cytokine-treated cells was first quantified by CD44-specific ELISA. Comparable amounts of CD44 were then captured on anti-CD44 Hermes III-coated ELISA plates. Bound receptor was then incubated with FITC-labeled HA and the bound ligand was detected using alkaline phosphatase-conjugated anti-FITC mAb. Only a small proportion of the total spontaneously released CD44 that accumulated over the 48-h culture period in HTB58 cell conditioned media exhibited affinity for HA. Interestingly, although the sCD44 released in response to treatment with OSM exhibited a significantly higher capacity to bind HA compared to spontaneously released CD44, the sCD44 released in response to TGF-β1 did not (Fig. 2). sCD44 released from OSM+TGF-β1-treated cells bound HA.
to a much lesser extent than from cells stimulated with OSM alone. These data indicate that although OSM and TGF-β1 both induce CD44-mediated HA binding to HTB58 cells and the release of comparable levels of sCD44 (Figs. 2A and 1A, respectively), the sCD44 released exhibits markedly different capacities to bind HA depending on the stimuli. The potential for dichotomy between the affinity of cell surface CD44 and sCD44 in cytokine-stimulated HTB58 cells is further emphasized by the fact that a combination of OSM and TGF-β1 was most potent in inducing the release of sCD44 and inducing HA binding to cell surface CD44 (Fig. 2A and [13], and Fig. 1A, respectively), but induced marginal binding of the sCD44 to HA (Fig. 2B).

We have previously demonstrated that in HTB58 cells HA-binding function of cell surface CD44 is regulated by receptor sulfation induced in response to OSM and OSM plus TGF-β1 [13]. Since sCD44 generated in HTB58 cells was found to be sulfated (Fig. 1C) it raised the question of whether sulfation of sCD44 regulates its interactions with HA. As demonstrated in Fig. 2B, treatment of cells with NaClO3 reduced the ability of sCD44 generated in response to OSM, TGF-β1 and OSM plus TGF-β1 to interact with HA by 71%, 44% and 93% respectively, indicating that sulfation of sCD44 markedly enhances its HA-binding capacity. HA binding to sCD44 spontaneously released from cells treated with chlorate was below the level of detection of the ELISA. Since OSM- and TGF-β1-generated sCD44 species differed in sulfation pattern (Fig. 1C), these differences may at least partially account for the observed differences in HA binding between sCD44 released under various conditions.

**Table 1**

<table>
<thead>
<tr>
<th>Cytokines increase HA levels in HTB58 cells</th>
<th>Reactivity of bPG with the cell surface (MFI)</th>
<th>Level of HA in conditioned medium (μg/ml per 10^6 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>260</td>
<td>0.46</td>
</tr>
<tr>
<td>OSM</td>
<td>600</td>
<td>0.48</td>
</tr>
<tr>
<td>TGF</td>
<td>350</td>
<td>0.22</td>
</tr>
<tr>
<td>OSM+TGF</td>
<td>797</td>
<td>0.56</td>
</tr>
</tbody>
</table>

Cells were cultured for 48 h with 50 ng/ml OSM and/or 10 ng/ml TGF-β1. Surface levels of HA were analyzed by flow cytometry after staining with bPG followed by PE-conjugated streptavidin. Cells treated with hyaluronidase prior to staining with bPG+PE-conjugated streptavidin were used as a negative control. Results are expressed as an average of two independent experiments (left column; MFI, mean fluorescence intensity). Conditioned media from cells incubated for 48 h were added to ELISA plates coated with bPG. Unsaturated bPG-binding sites were then detected by incubation with FITC-labeled HA followed by alkaline phosphatase-conjugated anti-FITC Ab. The data represent HA concentration in conditioned media after subtracting values obtained for culture media and are shown as the mean of quadruplicate wells from two experiments.

Fig. 2. Comparison of HA binding by cell surface CD44 and sCD44. Cells were cultured in the absence or presence of 40 mM sodium chlorate for 48 h. Cells were then treated for an additional 48 h with 40 mM sodium chlorate, 50 μg/ml OSM and/or 10 ng/ml TGF-β1 as indicated. CD44-mediated HA binding in HTB58 cells was determined by flow cytometry after exposure of cells to FITC-HA. Specific binding was calculated by subtracting non-specific binding in the presence of blocking HA-binding anti-CD44 5F12 mAb. Fluorescence intensity is shown from one representative experiment out of three independent experiments (A). Conditioned media were concentrated and subjected to CD44-specific ELISA to estimate the amount of CD44. Aliquots of media containing comparable amounts of CD44 were then analyzed using functional ELISA specific for CD44 capable of binding HA. The data are shown as the mean of triplicate wells from three experiments (B). The difference between control and cytokine-treated cells depicted in A was statistically significant as determined by Student’s t-test (P < 0.05). In B, the difference between control and cytokine-treated cells was statistically significant for OSM- and OSM+TGF-β1-treated cells. The effect of chlorate treatment shown in B was statistically significant.

Fig. 3. sCD44 is associated with HA in cytokine-treated HTB58 cells. Conditioned media from cells incubated for 48 h were split into two groups. The first group was subjected to CD44-specific ELISA. The second group was added to ELISA plates coated with bPG to capture sCD44 associated with HA. After two rounds of incubation with microwell-bound bPG, the conditioned media depleted with CD44-HA complexes were subjected to CD44-specific ELISA. The percentage of sCD44 associated with HA is shown as the mean of duplicate wells from two experiments.
3.3. TGF-β1 induces the release of CD44 complexed to HA

HTB58 cells synthesize HA (Table 1), raising the possibility that the decreased capacity of sCD44 released by TGF-β1 and a combination of TGF-β1 and OSM to bind HA may be due to occupation of the released receptor with endogenous HA. To address this possibility, HA from conditioned media of untreated and cytokine-stimulated HTB58 cells was captured on ELISA plates coated with HA-binding protein (bPG). CD44 associated with HA was then detected by anti-CD44 mAb. This assay revealed that the CD44 released from TGF-β1- or TGF-β1+OSM-treated cells is associated with HA while CD44 released spontaneously and from OSM-treated cells was predominantly free of HA. To quantify the proportion of the sCD44 occupied by endogenous HA, HA-associated CD44 was depleted from conditioned media by incubation with microwell-bound bPG. The depletion was considered to be complete if sCD44 was no longer able to bind to microwell-bound bPG. The level of free CD44 was then determined by CD44-specific ELISA. As demonstrated in Fig. 3, only 1% of sCD44 was found to be complexed with endogenous HA in supernatants from untreated cells, whereas approximately 30% of total sCD44 was associated with endogenous HA in TGF-β1- and OSM+TGF-β1-treated cells and 10% in OSM-stimulated cells. To determine if association of CD44 with HA resulted from higher expression of HA in TGF-β1-treated cells, we examined the levels of HA on the cell surface where HA is synthesized, as well as in conditioned media of HTB58 cells. Flow cytometry revealed that treatment of HTB58 cells with TGF-β1 slightly increased the bPG binding to the cell surface (~35%), whereas stimulation with OSM plus TGF-β1 or OSM alone resulted in profound increases in HA surface levels (207% and 131%, respectively) (Table 1). Similarly, the level of HA released into the conditioned media was highest in OSM plus TGF-β1- and OSM-treated cells (Table 1). Thus, formation of complexes between sCD44 and endogenous HA appears to be preferentially induced by TGF-β1 and does not correlate with levels of endogenous HA detected by the bPG staining. However, it should be noted that the lower levels of HA detected in the conditioned media from TGF-β1-stimulated cells might result from partial depletion of HA as the result of formation of complexes with sCD44.

4. Discussion

A wide variety of cells have been reported to shed CD44, including leukocytes and epithelial cells. Some cells release basal levels of sCD44 constitutively. In addition, the release of sCD44 can be induced experimentally by phorbol 12-myristate 1-ace-tate and ionomycin in vitro [9,17]. Although the physiological inducers of CD44 release are largely unknown, crosslinking of CD44 by specific antibodies leads to CD44 release [18], which raises the possibility that generation of sCD44 is ligand-inducible. The regulated proteolysis of this receptor may play a role in metastasis-associated cell migration since increases in the levels of sCD44 are found during tumor progression [14,19]. This possibility is also consistent with the finding that CD44 cleavage is induced by Ras, an oncprotein involved in cell motility and migration [20]. Interestingly, stable transfection of murine mammary carcinoma cells with sCD44 led to inhibition of tumor cell proliferation. These changes in tumor cell growth were dependent on the ability of sCD44 to bind HA, indicating that sCD44, which has affinity for HA, can act as a competitive inhibitor of HA–protein interactions [21]. However, so far nothing is known about the mechanisms regulating the affinity of sCD44 for HA.

We previously demonstrated that in HTB58 cells, HA binding to the cell surface can be regulated by OSM and TGF-β1, cytokines that also have the potential to regulate tumorigenesis [13]. The data presented in this paper demonstrate the ability of both OSM and TGF-β1 to increase the release of CD44 from HTB58 cells. This observation provided a unique opportunity to study shedding of CD44 in the context of differential HA binding. There are several major findings in this study: (i) shedding of CD44 appeared to correlate with an increased affinity of cell surface CD44 to HA, (ii) cytokines regulate HA-binding activity of both the transmembrane and soluble forms of CD44, (iii) differences in HA-binding capacity of sCD44 are stimulant-dependent, (iv) either membrane-anchored or soluble CD44 can associate with HA. Taken together these results indicate that epithelial cell CD44–HA interactions are regulated by two mechanisms: cytokine-mediated modulation of CD44 adhesion function and release of CD44. To our knowledge this is the first demonstration that shedding of CD44 triggered by physiological stimuli can result in generation of sCD44 which differs in HA binding. This is an important finding since sCD44 is generally assumed to simply antagonize the effect of membrane CD44. Future studies, however, will be required to fully understand the biological effects of sCD44 with different affinity for HA on HA-related cellular functions. Based on the findings presented in this study it can be proposed that sCD44 serves a dual function: as an antagonist of cell surface CD44 or as a decoy receptor or matrix component. Since sCD44 released by OSM is able to bind HA, the physiological effects of the OSM-generated sCD44 may contradict that of its cell surface counterpart through competition for the same ligand. In contrast, sCD44 released by TGF-β1 is unlikely to compete efficiently with membrane-bound CD44 for HA. Instead, TGF-β1 may alter the repertoire of CD44 anchored to the cell surface through release of sCD44 at least partially associated with HA and possibly other matrix components into the extracellular milieu.

It has been demonstrated that the transmembrane form of CD44 plays a role in the assembly of HA-rich pericellular matrices [22,23]. Our data demonstrate that CD44 is also associated with HA after it is proteolytically cleaved from the cell surface and that the level of sCD44–HA complexes is differentially regulated by various cytokines. There are at least two, not mutually exclusive, scenarios by which sCD44–HA complexes may be generated. Transmembrane CD44 may be first proteolytically cleaved and released from the cell surface and then establish physical associations with HA subsequently to release of the receptor from the cell surface. Alternatively, transmembrane CD44 may play a seminal role in the formation of matrix at the cell surface where subsequent proteolysis of the membrane-anchored CD44 would result in release of CD44 pre-assembled into complexes in association with HA. The composition of the extracellular matrix and its organization are the major determinants of environmental signaling that controls cell fate under physiologic and pathologic conditions. Our studies indicate that CD44 released from the cell surface may also be involved in assembly of the matrix surrounding lung epithelial cells.
Acknowledgements: This work was supported by KBN Grant 6P05A14721 to J.C., and ROI AI4583 from PHS, NIH to E.P. The authors are indebted to the Commonwealth Universal Research Enhancement Program, Pennsylvania Department of Health and The Wistar Institute Flow Cytometry Facility for their support.

References