Regulation of the release and function of tumor cell-derived soluble CD44

Joanna Cichy\textsuperscript{a,b,*}, Paulina Kulig\textsuperscript{a}, Ellen Pure\textsuperscript{b,c}

\textsuperscript{a}Faculty of Biotechnology, Jagiellonian University, 30-387 Krakow, Poland
\textsuperscript{b}Wistar Institute, Philadelphia, PA 19104, United States
\textsuperscript{c}Ludwig Institute of Cancer Research, New York, NY 10158, United States

Received 23 August 2004; received in revised form 4 February 2005; accepted 23 February 2005
Available online 8 March 2005

Abstract

CD44, a major receptor for glycosaminoglycan hyaluronan (HA), is a broadly distributed cell surface glycoprotein implicated in multiple functions, including tumor growth and dissemination. The affinity of surface CD44 for HA is subject to regulation at several levels. CD44 is found in multiple phases, including as an integral transmembrane protein and as soluble fragment of the extracellular domain found in the circulation and other body fluids. Transmembrane CD44 and its ability to interact with HA have been a focus of numerous studies in the past, but the function of soluble CD44 remains obscure. Interestingly, malignant diseases are often associated with an increase in the plasma level of CD44. The delineation of the HA binding capacity of tumor-derived soluble CD44 is an important step toward understanding the biological function of this molecule. In this study, we demonstrate that tumor cells activated to bind HA by cytokines rapidly release CD44 upon treatment with phorbol ester (PMA). The affinity for HA of the soluble CD44 released in response to PMA varied depending on the cytokine pretreatment. These results suggest that the function of tumor-derived soluble CD44, like the transmembrane form of the receptor, can be regulated.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Shedding; Tumor; Adhesion; Oncostatin M; TGF; Phorbol ester

1. Introduction

About 22\% of the known proteases are membrane-bound proteins, suggesting an important role for proteolytic processing at the cell surface [1]. The cleavage of the extracellular domains of membrane-anchored proteins (shedding) has important biological consequences. Firstly, the cleavage of the extracellular domain of a given receptor, which is often a consequence of ligand binding or cell activation, may trigger successive cleavage events that release the intracellular region of the receptor from its transmembrane tether. This allows for translocation of the intracellular fragment to the nucleus, where it may play a role in regulating gene transcription [2]. Secondly, shedding may result in the release of extracellular regulatory fragments. There is increasing evidence that functional fragments of the cell adhesion receptor, CD44, can be liberated by extracellular and intramembrane proteolysis (reviewed in [3]). CD44 is a major receptor for hyaluronan (HA), a glycosaminoglycan that is ubiquitously distributed in extracellular spaces and retained on the surface of some cells [4]. In addition, CD44 has been demonstrated to interact with numerous other molecules, including; fibronectin, collagen, growth factors and cytokines, metalloproteinases, as well as VLA-4 [5–8]. Under homeostatic conditions, CD44 appears to be involved in regulating the metabolism of HA. A prominent role for CD44 has been demonstrated in targeting of T cells to inflammatory sites [8], autoimmune and chronic inflammatory diseases [9–11] as well as tumor growth and dissemination [12,13]. Although much remains to be determined regarding the
functional significance of the physical association of CD44 with other molecules, most of the known effects of CD44 on cell adhesion and migration are associated with its capacity to bind HA. Importantly however, the ability of CD44 to bind HA with high affinity is not constitutive, but rather, cellular activation is required in most cases to induce high affinity of CD44 for HA.

Significant levels of soluble CD44 (sCD44) have been detected in several body fluids. Increased serum levels of CD44 have been documented in association with tumor progression [14], indicating that tumor progression and the tumor environment may promote the release of sCD44. Soluble CD44 can potentially be generated through several mechanisms including de novo synthesis of an alternatively spliced form of CD44 lacking the transmembrane and cytoplasmatic domains of the receptor [15], or enzymatic liberation of CD44 from extracellular matrices [16]. However, the major mechanism underlying the production of soluble CD44 is thought to involve shedding of this receptor from the cell surface. The shedding of CD44 can be constitutive and induced by various factors, among which, phorbol esters (PMA), are particularly potent. Soluble CD44 was generally assumed to antagonize the effect of membrane-bound CD44, through competing with cell surface CD44 for HA binding. However, as we recently demonstrated, soluble forms of CD44 generated from lung epithelial-derived tumor cells (HTB58) by treatment with cytokines, oncostatin M (OSM) and TGF-β1 differ in their capacity to bind HA [17]. OSM and TGF-β1 both increased the affinity of membrane CD44 for HA [18]. However, whereas the shedding of CD44 induced by OSM resulted in the generation of sCD44 that retained the high affinity for HA of the membrane-bound CD44, soluble CD44 released from TGF-β1-treated HTB58 cells differed in its HA-binding capacity from cell surface CD44 [17]. These data indicate that the affinity of soluble CD44 for HA may vary depending on the mechanism by which it is released. In this context it is interesting to note that shedding induced by different stimuli may occur with different kinetics. For example, 48-h treatment of HTB58 cells with OSM and TGF-β1 is required in order to accumulate a significant amount of sCD44 in conditioned media, whereas treatment with PMA results in copious shedding of the receptor within minutes, suggesting that different mechanisms mediate the release of CD44 in response to cytokines and PMA.

In the current study we characterize the ability of PMA-released CD44 to bind HA. To activate membrane CD44 to interact with HA, HTB58 cells were stimulated with OSM and TGF-β1 prior to PMA treatment. Tissue levels of both cytokines have been associated with tumor progression [19,20]. Whereas, OSM has the potential to facilitate tumorigenesis through the regulation of tumor growth and modulating tumor microenvironment, TGF-β1 may contribute to this process for example through the inhibition of the protective functions of the immune system against tumor. Taken together, it is likely that both cytokines are involved in regulating the affinity of CD44 for hyaluronan in tumor cells.

Our data demonstrate that PMA induces the release of CD44 from HTB58 cells and that soluble CD44 released by PMA from these cells either retain the ligand-binding properties of the membrane-anchored receptor or differ in its capacity to bind HA compared with the membrane form of CD44, suggesting that soluble CD44 may impact differently on CD44-HA 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). The following anti-human CD44 mAbs were used [21]: 5F12 that blocks HA binding (a generous gift of Dr. B. F. Hayes, Duke University Medical Center, Durham, NC), F10-44, which enhances HA binding, Hermes III (American Tissue Type Collection, Rockville, MD), and G44-26 (PharMingen, San Diego, CA). Purified hyaluronan from rooster comb (HA) and phorbol esters (PMA) were obtained from Sigma (St. Louis, MO). Fluorescein-conjugated rooster comb HA (FITC-HA) was prepared as described [22]. TAPI-1 was obtained from Peptides International (Louisville, KY), FITC-labeled anti-human CD44, PE-labeled anti-human CD44, PE-labeled anti-human CD3 antibodies and PE-labeled streptavidin were purchased from PharMingen. Alkaline phosphatase-conjugated anti-fluorescein antibodies were obtained from Roche (Indianapolis, IN). CD44-2-Rg vector [4] was a generous gift of Dr. I. Stamenkovic (Harvard Medical School, Cambridge, MA). The BCA kit was purchased from Pierce (Rockford, IL).

2.2. Cell culture

HTB58 human lung squamous carcinoma cell line was obtained from the ATCC (Rockville, MD). HTB58 cells were cultured in Eagle’s MEM supplemented with 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 50 μg/ml gentamycin and 10% heat inactivated fetal bovine serum (FBS). Cells were plated, grown to confluency and where indicated treated with 50 ng/ml OSM and/or 10 ng/ml TGF-β1 for 48 h or 5 × 10⁻⁷ M PMA for 90 min–4 h. Optimal doses and kinetics as determined in preliminary experiments were used in this study.

2.3. Biosynthetic 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) or with sulfate-free medium supplemented with 500 μCi/ml [³⁵S]Na₂SO₄ (ICN). Conditioned media were
collected and centrifuged at 300 \( \times g \) for 10 min followed by 600 \( \times g \) for 15 min to remove cells and cellular debris. Serum-free conditioned media were normalized based on protein concentration as determined by BCA assay, and equal amounts of protein were precleared 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% SDS, 0.3 M NaCl, 10 mM Tris pH 8.6), and PBS. CD44 was then released by boiling in Laemmli sample buffer and resolved by SDS-8% PAGE under non-reducing conditions. Bands were detected by fluorography.

2.4. Flow cytometry

Cells were harvested using 0.2% EDTA and stained for CD44 with PE-conjugated mouse anti-human CD44 antibody (G44-26) or PE-conjugated mouse anti-human CD3 antibody as a negative control. Cells were then analyzed on a FACScan. HA binding was assayed using HA-FITC. Negative reactivity was based on staining with HA-FITC in the presence of blocking anti-CD44 mAb 5F12.

2.5. CD44 specific ELISA

Soluble CD44 was quantified by ELISA. Plates (Microplate™ 2, Dynex, Chantilly, VA) were coated with 2 \( \mu \)g/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 nonspecific protein-binding sites were blocked with 3% BSA in PBS. Standards and samples (conditioned media normalized based on protein concentrations using BCA kit) were added and incubated at RT for 2 h. Affinity purified CD44–immunglobulin fusion protein (CD44-2-Rg) containing the complete extracellular domain of CD44H was used as a standard. After washing, FITC-labeled anti-CD44 G44-26 was added to the wells and incubated at 37 \( ^\circ C \) 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 Hermes III mAb, washed and incubated with standards and samples as described above. In some instances, conditioned media were concentrated on Centricon YM-10 (Millipore, Bedford, MA), or subjected to immunoprecipitation with Sepharose-conjugated anti-CD44 Hermes III mAb. CD44 was then eluted from Sepharose 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 \( ^\circ C \) for 30 min followed by incubation with alkaline phosphatase conjugated anti-FITC mAb and \( p \)-nitrophenyl phosphate.

The specificity of the ELISA for HA binding was established as follows. Instead of CD44-specific Hermes III mAb, plates were coated with irrelevant (anti-DNP), isotype-matched mAb, washed and incubated with standards and samples as described above. No detectable FITC-labeled HA binding was observed under these conditions, indicating that captured CD44 was required to bind FITC-HA (data not shown). Moreover, the pretreatment of CD44 bound to Hermes III Ab immobilized on ELISA plates, with an antibody F10-44, that enhances HA binding to intact cells, augmented HA binding in the indicated ELISA. No enhancement was observed when anti-DNP instead of Hermes III Ab was used to coat the ELISA plates. Finally, the anti-CD44 5F12 ablated the signal in the ELISA (data not shown). Taken together, these controls demonstrate the specificity of HA binding to soluble CD44 in the described ELISA.

3. Results and discussion

3.1. PMA regulates the release of CD44 from lung-derived tumor cells through a metalloproteinase-mediated mechanism

We previously demonstrated that OSM and TGF-\( \beta \)1 increase the release of soluble CD44 and augment the capacity of membrane CD44 to bind HA in HTB58 tumor cells [17,18]. CD44 cell surface expression has been shown to be rapidly down-regulated in many cell types by the protein kinase C agonist PMA. PMA, which mediates the activation of endogenous proteases through the activation of PKC [23,24], was found to be a potent inducer of CD44 release in HTB58 cells (Fig. 1). Therefore, we used PMA to induce the release CD44 in order to investigate the regulation of the affinity of rapidly-generated soluble CD44 for HA. Cells were stimulated with OSM and TGF-\( \beta \)1 for 48 h to upregulate the affinity of the cell surface CD44. Cells were then washed to remove excess cytokines and any sCD44 that had accumulated during the initial treatment period, and then stimulated with PMA. FACs analysis demonstrated that treatment with PMA reduced the levels of cell surface CD44 on control and cytokine-treated HTB58 cells (Fig. 1A). The reduction in cell surface CD44 levels was associated with the concomitant release of soluble CD44 into the culture media (Fig. 1B).

To investigate the role of endogenous proteinases in the release of soluble CD44 from PMA stimulated cells we studied the effect of TAPI, a broad specificity metalloproteinase inhibitor on PMA-induced shedding. The PMA-induced generation of soluble CD44 was completely...
blocked by TAPI, thus confirming that endogenous metalloproteinase activity was responsible for the release of CD44 (Fig. 1C).

### 3.2. Profile of PMA-released CD44

CD44 release induced by PMA was rapid and produced soluble CD44 ectodomains with an average molecular mass of 60 kDa (Fig. 2A). The longer exposure of cells to PMA resulted in the detection of additional species immunoprecipitated by anti-CD44 with average molecular mass of 90 kDa, however under these conditions the 60 kDa species was still predominant (Fig. 2B). This profile of sCD44 species was different from the profile of the CD44 species detected in the 48-h conditioned media from metabolically labeled HTB58 cells, which contained three major species of sCD44 of approximately 180 kDa, 90 kDa and 60 kDa [17]. A species with an Mr of ~60 kDa was also detected in [35S]sulfate-labeled PMA-treated cells (Fig. 2C). In addition to the 60 kDa species, conditioned media from [35S]sulfate-labeled HTB58 prestimulated with either OSM or OSM+TGF-β1 and then treated with PMA for 4 h were enriched with a 150–210 kDa species (Fig. 2D). These 150–210 kDa species resembled those observed in the 48-h conditioned media from [35S]sulfate-labeled HTB58 cells stimulated with either OSM or OSM+TGF-β1 [17]. Similarly to the 48-h conditioned media from [35S]sulfate-labeled HTB58 cells, the 4-h media from cells treated with PMA did not reveal any 90 kDa sulfated species (Fig. 2D, respectively). However, in comparison with the 48-h conditioned media from [35S]sulfate-labeled HTB58 cells, the 4-h media from cells treated with PMA contained much more 60 kDa species.
less of the large sulfated species which did not penetrate the gel ([17] and Fig. 2D). Although a potential significance of the various soluble CD44 species is currently unknown, it is tempting to speculate that they might contribute differently to the CD44-mediated processes.

3.3. PMA-released CD44 from untreated and cytokine-stimulated HTB58 cells differs in HA binding capacity

To determine if CD44 released from cytokine-stimulated HTB58 cells upon PMA treatment differs in function, we used an ELISA specific for CD44 capable of binding HA. We took two approaches to address whether the shed CD44 can interact with HA. In the first approach, we used total conditioned media obtained from HTB58 cells and soluble CD44 present in these media was captured on anti-CD44 Hermes III-coated ELISA plates. Bound CD44 was then incubated with FITC-labeled HA and the captured ligand was detected using alkaline phosphatase-conjugated anti-FITC mAb. Since the conditioned media, in addition to shed CD44, may contain other HA binding molecules, in a second approach designed to avoid the potential of interference of the assay by such molecules, soluble CD44 was first immunoprecipitated from conditioned media with Sepharose-conjugated Hermes III mAb. CD44 was released from beads and immobilized on ELISA plates using Hermes III mAb. The bound receptor was then subjected to the ELISA, as described above. Since both approaches gave comparable results (data not shown) suggesting that nothing in the conditioned media interfered with our detection of soluble CD44, in subsequent experiments, we primarily used total conditioned media instead of immunoprecipitated CD44.

Whereas PMA-induced shedding of CD44 was generally accompanied by a modest increase in CD44-mediated HA binding at the cell surface (Fig. 3A), the affinity of soluble CD44 generated by PMA-treated cells differed in capacity to bind HA depending on the cytokine pretreatment (Fig. 3B). Thus, CD44 released by PMA from cells pretreated with OSM or OSM + TGF-β1 exhibited a capacity to bind exogenous HA. In contrast, soluble CD44 generated by PMA in untreated and TGF-β1-treated cells exhibited much lower binding to HA. Interestingly, a comparison of soluble CD44 detected in the 48-h conditioned media [17] with that released by 4-h treatment with PMA, in most cases, revealed similar patterns of HA binding activity, with one significant exception. Whereas soluble CD44 recovered from the conditioned media of cells stimulated with OSM for 48 h exhibited a much higher capacity to bind HA than sCD44 generated by treatment with OSM + TGF-β1, in cells prestimulated with either OSM or OSM + TGF-β1 and then treated with PMA the HA binding capacity was similar ([17], and Fig. 3B, respectively). Since spontaneously released and PMA-generated soluble CD44 species differed in either molecular weight or sulfation pattern, these differences may account for the observed differences in HA binding by soluble CD44 released under these different conditions.

Shedding is largely attributed to two families of metalloproteases localized on the cell surface: the disintegrin-like metalloproteases (ADAMs) and, to a lesser extent, the membrane type metalloproteases (MT-MMPs). These proteases have been linked to the release of a variety of structurally and functionally diverse membrane proteins, including adhesion molecules such as L-selectin and MUC-1 [25,26]. Interestingly, CD44 has been demonstrated to be processed in vitro by members of both families, namely MT1-MMP and ADAM10 [27–29] and more recently ADAM17 [23]. The involvement of different metalloproteases in CD44 shedding may indicate that different stimuli activate different signaling events. Although much remains to be known about the nature of the proteases involved in the proteolytic processing of CD44 in vivo, there is accumulating evidence that the shedding of CD44 plays a role in its functional activation.

A remarkable amount of processed ectodomain of CD44 is detected in tumor tissues [29] and enhanced CD44
processing has been demonstrated in malignant tumors [30]. Moreover, hyaluronan oligosaccharides promote CD44 cleavage, suggesting that the generation of soluble CD44 is inducible by the form of its principle ligand associated with various tumors [31]. Finally, this study indicates that certain forms of rapidly-released CD44 retain functionality and therefore may compete with membrane CD44 for HA. In contrast to CD44 released by PMA from OSM-pretreated cells, sCD44 generated by PMA from TGF-β1-pretreated cells is unlikely to be an effective competitor with membrane CD44 for HA. The release of CD44 which lacks significant HA binding capacity may represent a mechanism to alter the repertoire of membrane CD44.

Acknowledgements

This work was supported by KBN grant 6P05A14721 to JC; PHS grants HL65507, AI25185 and P50-HL676 to EP.

References