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# Myoepithelial-Specific CD44 Shedding Is Mediated by a Putative Chymotrypsin-like Sheddase[1]

Maggie C. Lee,[2] Mary L. Alpaugh,[2] Mai Nguyen, Maria Deato, Lena Dishakjian, and Sanford H. Barsky[3]

*Department of Pathology and Revlon/UCLA Breast Center, University of California Los Angeles School of Medicine, Los Angeles, California 90024*

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**Our previous studies have demonstrated that myoepithelial cells, which surround incipient carcinomas *in situ* of the breast and other organs, exert antiinvasive and antiangiogenic effects *in vitro* through the elaboration of a number of different suppressor molecules among which include the shed membrane CD44. The present study addresses the mechanism of this myoepithelial CD44 shedding. This CD44 shedding is enhanced by PMA pretreatment, is specific for myoepithelial CD44, and inhibited by chymotrypsin-like inhibitors (chymostatin, $\alpha_1$-antichymotrypsin, TPCK, and SCCA-2) but not by trypsin-like inhibitors (TLCK), nor papain-like inhibitors (SCCA-1) nor hydroxamate-based or general metalloproteinase inhibitors (BB2516 (marimastat), 1,10-phenanthroline, and TIMP-1). The effect of PMA can be mimicked by exogenous chymotrypsin but not by other proteases. The CD44 shedding activity cannot be transferred by conditioned media, cell–cell contact, peripheral membrane, or integral membrane fractions. However, cell-free purified integral plasma membrane fractions obtained from myoepithelial cells pretreated with PMA also exhibit CD44 shedding which is inhibited by chymotrypsin-like inhibitors. These findings support the presence and activation of a putative chymotrypsin-like sheddase as the mechanism of CD44 shedding in myoepithelial cells.** © 2000 Academic Press

***Key Words:*** **myoepithelial cells; CD44; chymotrypsin; sheddase; PMA; antiangiogenesis; anti-invasion.**

Abbreviations used: HMS, human matrix-secreting; CM, conditioned media; PAI-1, plasminogen activator inhibitor-1; TIMP-1, tissue inhibitor of metalloproteinases; PMA, phorbol 12-myristate 13-acetate; DCIS, ductal carcinoma *in situ;* HMEC, human mammary epithelial cells; K-SFM, keratinocyte-serum-free medium; Mab, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; DCI, 3,4-dichloroisocoumarin; TLCK, *N* $\alpha$-*p* -tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-*L*-phenylalanine chloromethyl ketone; SCCA, squamous cell carcinoma antigen; MMP, matrix metalloproteinase; TNF-$\alpha$, tumor necrosis factor-$\alpha$; TGF$\alpha$, transforming growth factor-$\alpha$; NP-40, Nonidet P-40; PA, plasminogen activator.

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[2] These authors contributed equally to the work.

[3] To whom correspondence should be addressed. Fax: (310) 441-1248. E-mail: sbarsky@ucla.edu.

Our previous studies have indicated that myoepithelial cells which surround ductal epithelium of glandular organs such as the breast exert multiple paracrine suppressive effects on incipient cancers which arise from this epithelium (1–3). This paracrine suppression may keep the genetic alterations occurring within malignant epithelial cells in check so that the evolving cancer exists for a number of years only as an *in situ* lesion confined within the ductal system (4). Our laboratory has established immortalized myoepithelial cell lines and transplantable xenografts from benign human myoepitheliomas of the salivary gland (HMS-1, HMS-3), breast (HMS-4, HMS-5) and bronchus (HMS-6) (with their respective xenografts designated as HMS-#X) (1–3, 5, 6). These cell lines and xenografts express identical myoepithelial markers as normal myoepithelial cells *in situ* and display an essentially normal diploid karyotype. In previous studies we have demonstrated that our myoepithelial cell lines/xenografts and myoepithelial cells *in situ* constitutively express high amounts of proteinase and angiogenesis inhibitors which include TIMP-1, protease nexin-II, $\alpha$-1 antitrypsin, an unidentified 31-33 kDa trypsin inhibitor, thrombospondin-1, soluble bFGF receptors, and maspin (1–3). Our human myoepithelial cell lines inhibit both ER-positive and ER-negative breast carcinoma cell invasion and endothelial cell migration and proliferation (angiogenesis) *in vitro* (3, 7). Our myoepithelial cell lines also inhibit breast carcinoma proliferation *in vitro* through an induction of breast carcinoma cell $G_2$/M arrest and apoptosis (3), the latter phenomenon of which also occurs *in situ* within DCIS (8). In our previous studies demonstrating that myoepithelial cells inhibited both invasion and

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angiogenesis we noted that treatment with PMA potentiated both types of suppression (1, 7). In examining possible mechanisms for these effects we surveyed our myoepithelial cell lines for PMA-induced changes in expression levels of general categories of molecules that had been implicated in tumor invasion and angiogenesis, namely proteinases/proteinase inhibitors, angiogenic factors/angiogenic inhibitors and adhesion molecules. In the latter category we noted in a recent study (9) that PMA profoundly decreased the levels of surface CD44 on myoepithelial cells through a shedding phenomenon. In this study (9), we demonstrated that myoepithelial cells constitutively express and shed both the 85 kDa standard (CD44s) and the 130 kDa epithelial (CD44v8-10) isoforms and that PMA pretreatment enhances this shedding. Our observations were made by using CD44 isoform-specific antibodies as well as CD44 slice-variant-specific primers (9). The decrease in cell-associated CD44 and concomitant increase in CD44 in conditioned media (CM) was due to PMA-enhanced cell shedding and not altered levels of CD44 synthesis from either alternative splicing or changes in gene transcription, either of which could contribute to cell surface CD44 alterations. Furthermore the increase in CD44 in CM never occurred without a concomitant decrease in cell-associated CD44 suggesting that this was a true shedding and not a secretory phenomenon. Furthermore other possibilities to explain the decrease in cell-surface CD44 immunoreactivity such as PMA-induced conformational alterations or PMA-induced alterations in epitope accessibility within the extracellular domain of CD44 were also excluded by these dual observations. Cell fractionation experiments further revealed that virtually all of the CD44 was plasma membrane associated and it was this membrane-associated CD44 that was decreased with PMA pretreatment. The myoepithelial cell-associated decrease in CD44 was first in evidence by Western blot 12 h following PMA treatment but was detected within 2 h by flow cytometric analysis. The PMA-induced reduction in cell-associated CD44 was observed in all 6 of the myoepithelial cell lines examined but not in any of the nonmyoepithelial lines. Furthermore normal mammary epithelial cells (HMECs) though expressing the same standard and variant (epithelial) isoforms of CD44 as myoepithelial cells did not shed CD44 in response to PMA. Because the CD44 shedding phenomenon seemed to be myoepithelial specific, we decided to study it further and investigate its mechanism in the present study.

## MATERIALS AND METHODS

*Cell lines.* Human myoepithelial cell lines (HMS-1–6) from benign myoepithelial tumors of diverse origins had been previously established in our laboratory (1–3, 5, 6) and maintained in keratinocyte serum-free medium (K-SFM) supplemented with bovine pituitary extract (50 $\mu$g/ml) and recombinant human epidermal growth factor (5 ng/ml). (GIBCO-BRL, Gaithersburg, MD). Other nonmyoepithelial cell lines used included Hs578T (American Type Culture Collection (ATCC), Rockville, MD), and human squamous cell carcinoma lines of salivary gland and vulva, A253 and A431 (ATCC). These latter cell lines were maintained in Eagle MEM supplemented with 10% FBS (GIBCO-BRL) and penicillin–streptomycin antibiotics. Conditioned media from the myoepithelial cell lines untreated and pretreated with PMA (5 $\mu$M) was collected in basal K-SFM and concentrated up to 10-fold with Centriprep-10 concentrators (Amicon, Beverly, MA). Coculture experiments involving cell–cell contact of PMA-pretreated and untreated cell types of various combinations were also conducted.

*Pharmacological manipulations.* Confluent monolayers of myoepithelial cells (HMS-1-6) were also pretreated with PMA (5 $\mu$M) (Sigma Chemical Co., St. Louis, MO) for 20 min followed by harvesting of the cells 1 to 24 h later. The effects of PMA pretreatment were studied in the presence and absence of plasminogen (10 $\mu$g/ml) and in the presence of various proteinase inhibitors. The following specific proteinase inhibitors at a 10- to 1000-fold range of concentrations (the median concentration so designated) were studied: hydroxamate-based and general metalloproteinase inhibitors including 0.05 mM EDTA, 100 $\mu$M TAPI, 3.2 nM TIMP-1, 0.2 mM 1,10 phenanthroline and 100 $\mu$M BB2516 (marimastat); trypsin-like, chymotrypsin-like and general serine protease inhibitors including 10 $\mu$M aprotinin, 0.2 mM leupeptin, 0.2 mM chymostatin, 0.2 mM $\alpha_1$-antichymotrypsin, 10 nM plasminogen activator inhibitor–1, 10 nM $\alpha_2$-antiplasmin, 100 $\mu$M 3,4 DCI, 540 nM TLCK, 100 $\mu$M TPCK and 5 $\mu$M SCCA-2; and papain-like and general cysteine proteinase inhibitors including 1.0 $\mu$M pepstatin A, 10 $\mu$M CA-074, 10 $\mu$M E-64, 10 $\mu$M steffin A, 5 $\mu$M SCCA-1, 0.2 mM leupeptin and 0.2 mM cystatin. All inhibitors were obtained from Sigma Chemical Co. except for TIMP-1 (Calbiochem-Novabiochem Corp., San Diego, CA), CA-074 (Peptides International, Louisville, KY), BB2516 (a gift of Dr. Howard Reber, UCLA, Los Angeles, CA), SCCA-1 and SCCA-2 (gifts of Dr. Gary Silverman, Children's Hospital, Boston, MA) and TAPI (Immunex Corp., Seattle, WA). Myoepithelial cells (HMS-1–6) and nonmyoepithelial cells (Hs578T, A253, and A431) were also treated with different proteinases. The following proteinases at a 10- to 1000-fold range of concentrations (the median concentration so designated) were used: type I collagenase (human MMP-1) (0.05 mU) (Oncogen, Cambridge, MA), chymotrypsin, trypsin, elastase, pronase (1 U each) (Sigma Chemical Co.), hyaluronidase (1 U) (Sigma Chemical Co.) and cathepsins B (1 U), D (1 U), L (0.5 mU), and G (2.0 mU) (all from Calbiochem, La Jolla, CA).

*Antibodies.* Monoclonal antibodies to the standard and epithelial isoforms of CD44 were used. Individual and a combination of MAbs to CD44s ($IgG_1$-clone DF1485, Zymed Laboratories, San Francisco, CA) and CD44v8-10 (Dr. Graeme J. Dougherty, UCLA, Los Angeles, CA) were used in Western blot and flow cytometric studies. Western blots were performed using the appropriate primary antibodies at the manufacturers' recommended dilutions and a 1:50,000 dilution of horseradish peroxidase-conjugated goat anti-mouse as secondary antibody (Amersham Life Sciences, Arlington Heights, IL) followed by development of the reaction with the ECL detection system (Amersham Life Sciences, Arlington Heights, IL). A loading control antibody to $\beta$-actin (a gift of Dr. Howard Reber, UCLA) was used in all immunoblotting experiments to normalize for cell protein. Scion Image software was used for densitometric analysis of bands. Cells were harvested by brief incubation in PBS containing 5 mM EDTA, pelleted and resuspended in lysis buffer A (PBS, 1% (v/v) NP-40, 5 mM EDTA and 10 mM PMSF). Lysates were incubated on ice for 10 min, then microcentrifuged for 5 min to pellet nuclei and other insoluble cellular debris. Supernatants were removed and stored at −20°C. Aliquots were thawed, added to an equal volume of nonreducing sample buffer containing 125 mM Tris, 20% (v/v) glycerol, 4.6% (w/v) SDS, pH 6.8, and incubated at 100°C for 5 min. Total cellular proteins were separated on precast 10% Tris–HCl polyacrylamide electrophoresis gels (Bio-Rad Life Science Products, Hercules,

CA), and transferred electrophoretically to nitrocellulose membranes (Gibco BRL, Gaithersburg, MD) and subjected to Western blot analysis.

*Flow cytometric analysis.* Myoepithelial cells were pretreated with PMA (5 $\mu$M) for 20 min followed by harvesting of the cells 1 to 24 h later. Cells were harvested with PBS containing 5 mM EDTA, and centrifuged. The pellet was then resuspended with FITC-CD44 mAb or control FITC-IgG and incubated on ice for 30 min. Cells were then washed extensively with HBSS containing 0.1% Na azide and 2% fetal bovine serum and subject to flow cytometry using a Becton–Dickinson FACScan.

*Cell fractionations.* PMA-pretreated cells were homogenized in buffer B (20 mM Tris–HCl, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20 $\mu$M leupeptin, 0.15 $\mu$M pepstatin A) at 4°C with a Dounce homogenizer and centrifuged at 13,000*g* for 15 min at 4°C to obtain the particulate fraction. Subcellular fractions of the particulate fraction (P1, nuclear enriched, P2, plasma membrane + mitochondria enriched; and P3, microsomal enriched) were prepared and recommended enzyme markers were followed as described previously (10). Further subfractionation of the mitochondria–plasma membrane fraction was carried out by isopycnic centrifugation in Percoll. In some experiments the sedimented membranes were dissolved in SDS sample buffer and assayed for CD44 by Western blot. In other experiments the sedimented membranes were subsequently extracted in 1 M NaCl/EDTA (peripheral fraction) or detergent 1% NP-40 (integral fraction) and transferred to a layer of untreated HMS-1 cells to study the effect of this transfer on CD44 shedding. In other experiments the sedimented membranes were suspended in ice-cold Tris–HCl (30 mM, pH 7.2) containing various components to be tested. The reaction mixture (20 $\mu$l) was transferred to 37°C and incubated for 1–12 h. The reaction was stopped by the addition of an equal quantity of SDS sample buffer and incubated at 100°C for 5 min. Then aliquots of reaction mixtures were subjected to Western blot analysis using CD44 mAb. The sedimented membranes were also added to 10 volumes of ice-cold 1 M NaCl in 30 mM Tris–HCl (pH 7.2) and kept 15 min on ice. Membranes were collected by centrifugation at 10,000*g* for 15 min at 4°C. Washed membranes were reconstituted in ice-cold Tris–HCl (30 mM, pH 7.2) and incubated and processed as before.

*Statistical analysis.* Results were analyzed with standard tests of statistical significance, including the two-tailed Student's *t* test and a one-way analysis of variance (ANOVA).

## RESULTS

*Mechanism of myoepithelial CD44 shedding.* The myoepithelial cell-associated decrease in CD44 was detected within 2 h by flow cytometric analysis following PMA pretreatment ($P < 0.01$) (Fig. 1A). The PMA-induced myoepithelial CD44 shedding was not affected by either plasminogen addition (10 $\mu$g/ml) or depletion ($P > 0.1$) or $\alpha_2$-antiplasmin (Fig. 1B), indicating that the shedding was not mediated by plasminogen activators (PA) or plasmin. The PMA-induced myoepithelial CD44 shedding could be blocked however by the chymotrypsin-like serine proteinase inhibitors, chymostatin, $\alpha_1$-antichymotrypsin, TPCK and SCCA-2 (Fig. 1C) ($P < 0.01$, $P < 0.01$, $P < 0.01$, $P < 0.05$) but not by trypsin-like serine proteinase inhibitors like plasminogen activator inhibitor (PAI-1) ($P > 0.1$), papain-like cysteine proteinase inhibitors ($P > 0.1$), or hydroxamate-based or general metalloproteinase inhibitors like TIMP-1 (Fig. 1C) ($P > 0.1$) (Table I). With the chymotrypsin-like proteinase inhibitors, e.g., chymostatin, there was a dose response of inhibition (Fig. 1D). The chymotrypsin-like inhibitors alone in the absence of PMA-pretreatment exerted no effect on myoepithelial CD44. These results implicated a putative chymotrypsin-like sheddase. As further indirect proof, purified exogenous chymotrypsin mimicked the effect of PMA. Chymotrypsin cleaved CD44 on myoepithelial cells over a similar time course of 12 h (Fig. 1E). Chymostatin inhibited the cleavage of CD44 by chymotrypsin. The inhibitory effects of chymostatin and other chymotrypsin-like serine proteinase inhibitors like SCCA-2 (but not the papain-like cysteine proteinase inhibitors like SCCA-1 and cystatin) on both exogenous CD44 cleavage and on PMA-induced myoepithelial CD44 shedding were also observed in flow cytometric studies (Fig. 1F).

*Evidence for a specific myoepithelial sheddase.* The CD44 shedding activity could not be transferred by CM (Fig. 2A) nor abolished by the removal of CM (Fig. 2B). The susceptibility of myoepithelial CD44 to exogenous proteolytic cleavage was highly chymotrypsin sensitive and relatively insensitive to other proteases (Fig. 2C). Other nonmyoepithelial CD44 did not exhibit this chymotrypsin sensitivity (Fig. 2D). These other nonmyoepithelial CD44 also did not exhibit PMA-induced shedding (data not shown). Coculture experiments where PMA-pretreated HMS-1 cells were subsequently mixed with untreated HMS-1 cells at dilutions to insure cell–cell contact between each of the populations showed no evidence of CD44 shedding in the untreated cells; furthermore when peripheral membrane fractions or integral membrane fractions of PMA-pretreated HMS-1 cells were transferred to untreated HMS-1 cells there was no evidence of CD44 shedding in the HMS-1 cells (data not shown). However cell-free membrane preparations of PMA-pretreated HMS-1 cells showed a progressive CD44 shedding over 1–8 h (Fig. 2E). This CD44 shedding was equally observed in membranes that had been washed with 1 M NaCl in 30 mM Tris–HCl (pH 7.2) to remove peripheral membrane proteins suggesting that the factor responsible for the CD44 shedding was an integral membrane protein. As before, this CD44 shedding could be inhibited by the chymotrypsin-like serine proteinase inhibitors but not by the trypsin-like serine proteinase inhibitors, the papain-like cysteine proteinase inhibitors or the metalloproteinase inhibitors. CD44 shedding was not observed in membrane preparations obtained from HMS-1 cells that were initially untreated or subsequently treated with proteinase inhibitors alone. The collective findings suggest that the membrane factor responsible for shedding and its substrate, CD44, both have to be present in *cis* orientation for activity and support the presence and activation of a putative

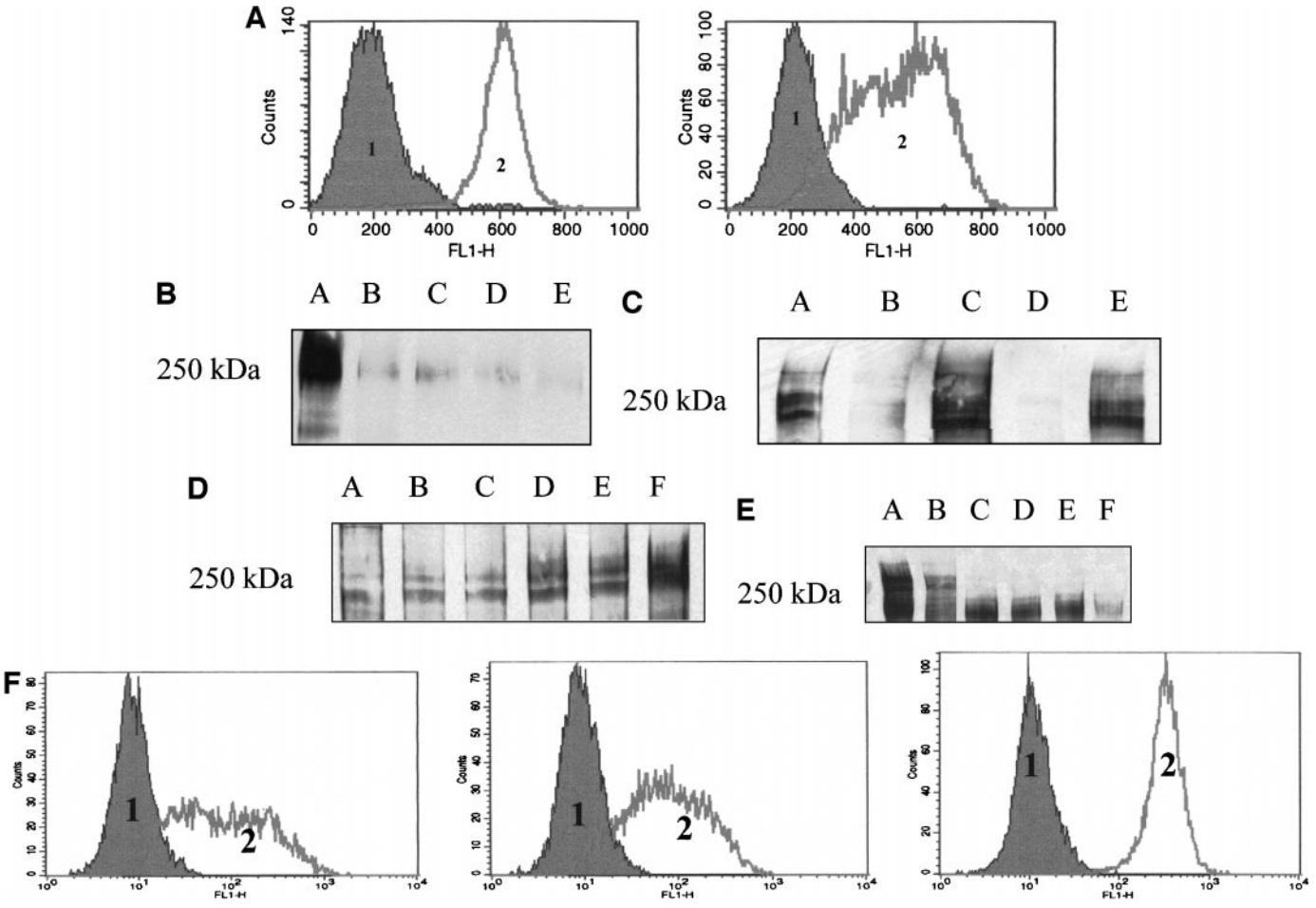


**FIG. 1.** CD44 flow cytometry or Western blot of HMS-1 cells: (A) Flow cytometry of untreated HMS-1 cells depicts cell surface CD44 fluorescence (top). PMA pretreatment results in a decrease in cell surface CD44 at 2 h as demonstrated by an emergence of a cell population with decreased fluorescence (bottom). Peak 1, FITC-IgG; peak 2, FITC-CD44 mAb; top, no pretreatment; bottom, PMA pretreatment. (B) PMA-induced CD44 shedding is not affected by either the presence or absence of plasminogen or the presence of $\alpha_2$ antiplasmin: Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1; lane C, PMA-pretreated HMS-1 cells in the absence of plasminogen; lane D, PMA-pretreated HMS-1 cells in the presence of plasminogen; lane E, PMA-treated cells in the presence of $\alpha_2$ antiplasmin. (C) PMA-induced CD44 shedding is inhibited by chymostatin and $\alpha_1$-antichymotrypsin but not at all by plasminogen activator inhibitor-1 (PAI-1) or TIMP-1. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 in the presence of PAI-1; lane C, PMA-pretreated HMS-1 in the presence of chymostatin; D, PMA-pretreated HMS-1 cells in the presence of TIMP-1; E, PMA-pretreated HMS-1 cells in the presence of $\alpha_1$-antichymotrypsin. Other chymotrypsin-like serine proteinase inhibitors, TPCK, and SCCA-2 (Table I) were effective at inhibition but not inhibitors belonging to other classes. (D) Dose response of increasing concentrations of chymostatin on CD44 shedding inhibition: Lane A, PMA-pretreated HMS-1 cells in the presence of 2 $\mu$M chymostatin; lane B, PMA-pretreated HMS-1 in the presence of 10 $\mu$M chymostatin; lane C, PMA-pretreated HMS-1 in the presence of 20 $\mu$M chymostatin; lane D, PMA-pretreated HMS-1 in the presence of 200 $\mu$M chymostatin; lane E, PMA-pretreated HMS-1 in the presence of 1 mM chymostatin; lane F, PMA-pretreated HMS-1 in the presence of 2 mM chymostatin. Other chymotrypsin-like inhibitors showed a similar dose response. (E) Time course of exogenous chymotrypsin (1 unit) cleavage of HMS-1 CD44. Lane A, untreated HMS-1 cells; lane B, HMS-1 cells treated with chymotrypsin (1 unit) for 1 h; lane C, HMS-1 cells treated with chymotrypsin (1 unit) for 2 h; lane D, HMS-1 cells treated with chymotrypsin (1 unit) for 4 h; lane E, HMS-1 cells treated with chymotrypsin (1 unit) for 8 h; lane F, HMS-1 cells treated with chymotrypsin (1 unit) for 12 h. (F) Flow cytometry of PMA-pretreated HMS-1 cells in the presence of cystatin, top; PMA-pretreated HMS-1 cells in the presence of SCCA-1, middle; PMA-pretreated HMS-1 cells in the presence of SCCA-2, bottom. Only SCCA-2 inhibited the CD44 shedding. Peak 1, FITC-IgG; Peak 2, FITC-CD44 mAb.

chymotrypsin-like sheddase as the mechanism of PMA-induced CD44 shedding in myoepithelial cells.

## DISCUSSION

Cancer cells come under the influence of important paracrine regulation from the host microenvironment (11). Both positive (fibroblast, myofibroblast and endothelial cell) and negative (tumor infiltrating lymphocytes and cytotoxic macrophages) cellular regulators exist that profoundly affect tumor cell behavior *in vivo* (12–14). One host cell, the myoepithelial cell, appears to belong to the negative cellular regulator group (1). Our previous studies have shown that myoepithelial

**TABLE I**

Representative Inhibitory Spectrum of the Putative Myoepithelial CD44 Sheddase

| Proteinase | Inhibitory class | Activity |
|---|---|---|
| EDTA | M | −−−−− |
| TIMP-1 | M | −−−−− |
| 1,10-Phenanthroline | M | −−−−− |
| BB2516 | M | −−−−− |
| TAPI | M | −−−−− |
| PAI-1 | S | −−−−− |
| 3,4 DCI | S | −−−−− |
| TLCK | S | −−−−− |
| TPCK | S | +++ |
| SCCA-2 | S | +++ |
| $\alpha$-1-Antichymotrypsin | S | +++ |
| Steffin A | C | −−−−− |
| SCCA-1 | C | −−−−− |
| Chymostatin | S, C | ++++ |
| CA-074 | C | −−−−− |
| E-64 | C | −−−−− |
| Cystatin | C | −−−−− |

*Note.* M, metalloproteinase inhibitor; S, serine proteinase inhibitor; C, cysteine proteinase inhibitor.

cells and derived cell lines exert multiple suppressive effects on carcinoma cells through secretion of a number of different anti-invasive, antiproliferative, and antiangiogenic molecules (1, 7). Another candidate paracrine suppressor molecules is shed CD44. The present study has examined the mechanism of myoepithelial CD44 shedding.

CD44 is a cell surface receptor for several extracellular matrix components predominant of which is hyaluronan but which also include collagen, laminin, fibronectin and chondroitin sulfate proteoglycan (15). The binding of hyaluronan to CD44 is thought to mediate a number of different biological processes including lymphocytic homing, endothelial chemotaxis (angiogenesis) and tumor cell haptotaxis, invasion and metastasis (16–18). A number of different regulatory mechanisms exist which can influence the efficacy of CD44–hyaluronan binding and the biological processes which are dependent on this interaction. Variant isoforms of CD44 resulting from alternative splicing can influence the affinity of cellular binding (19–24); phosphorylation of the CD44's cytoplasmic domain can influence the binding properties of its ectodomains (20–24); and increased shedding or secretion of the CD44 molecule through various mechanisms can either reduce or enhance binding to hyaluronan depending on the specifics and the dynamics of the situation (21–29).

Our present studies demonstrate that myoepithelial cells shed CD44. The shedding is myoepithelial cell CD44 specific. Increased shedding of ectodomains of molecules such as CD44 can be produced by either extrinsic or intrinsic mechanisms (21, 22, 25–28). Hyaluronidase, for example, can digest both hyaluronan and the variant isoforms of cell-associated CD44, altering the growth, motility and metastasizing properties of tumor cells (25, 26). Alternately CD44 can be cleaved by intrinsic membrane secretases or sheddases (21, 22).

Our studies addressing the mechanism of the PMA-induced CD44 shedding in myoepithelial cells indicate that a putative chymotrypsin-like sheddase is involved. There has been a recent interest in membrane sheddases or membrane convertases as they are sometimes designated (30–41). Membrane sheddases have been implicated in the shedding of a number of different membrane and cell surface molecules which include a diverse range of membrane proteins of Type I or Type II topologies (31). Examples of molecules shed by sheddase mechanisms include Alzheimer's amyloid precursor protein, angiotensin converting enzyme, TGF-$\alpha$, the tumor necrosis ligand and receptor superfamilies (33) and cell adhesion molecules such as L-selectin (38, 39) and CD44 (21, 22, 36, 37). Most of the sheddases identified to date have been metalloproteinases but not necessarily matrix metalloproteinases (33, 38, 39). The identification of these sheddases has rested mainly on indirect evidence as ours has, which is based on the specificities of a broad spectrum of proteinase inhibitors. The vast majority of putative sheddases have not been purified or cloned. The one exception has been tumor necrosis factor-$\alpha$ converting enzyme (TACE), a metalloproteinase-disintegrin sheddase, demonstrated to have catalytic function, and thought responsible for the shedding of diverse cell surface proteins including, in addition to TNF-$\alpha$, TGF$\alpha$ and L-selectin but interestingly not CD44 (35). CD44 has been reported to be cleaved and shed, on the other hand, by stimulated human granulocytes and certain malignant cell lines including glioblastoma lines. Both cellular sheddings were induced by PMA and inhibited by metalloproteinase inhibitors including TIMP-1 and 1,10-phenanthroline (21, 22, 36, 37). In our studies with myoepithelial cells, diverse metalloproteinase inhibitors including TIMP-1, 1,10-phenanthroline, EDTA, and hydroxamate-based inhibitors, TAPI and BB2516 were not effective in inhibiting PMA-induced CD44 shedding; rather diverse chymotrypsin-like serine proteinase inhibitors but not trypsin-like serine or papain-like cysteine proteinase inhibitors were effective. When we first began investigating the inhibitory spectrum of our putative sheddase, we began with general inhibitors of each proteinase class. We initially noted that chymostatin exhibited the greatest inhibitory activity. Because this proteinase inhibitor had general inhibitory activity against both chymotrypsin-like serine proteinases as well as cysteine proteases, we further defined the inhibitory spectrum of our putative sheddase by investigating two additional proteinase inhibitors: cystatin and $\alpha_1$-antichymotrypsin.

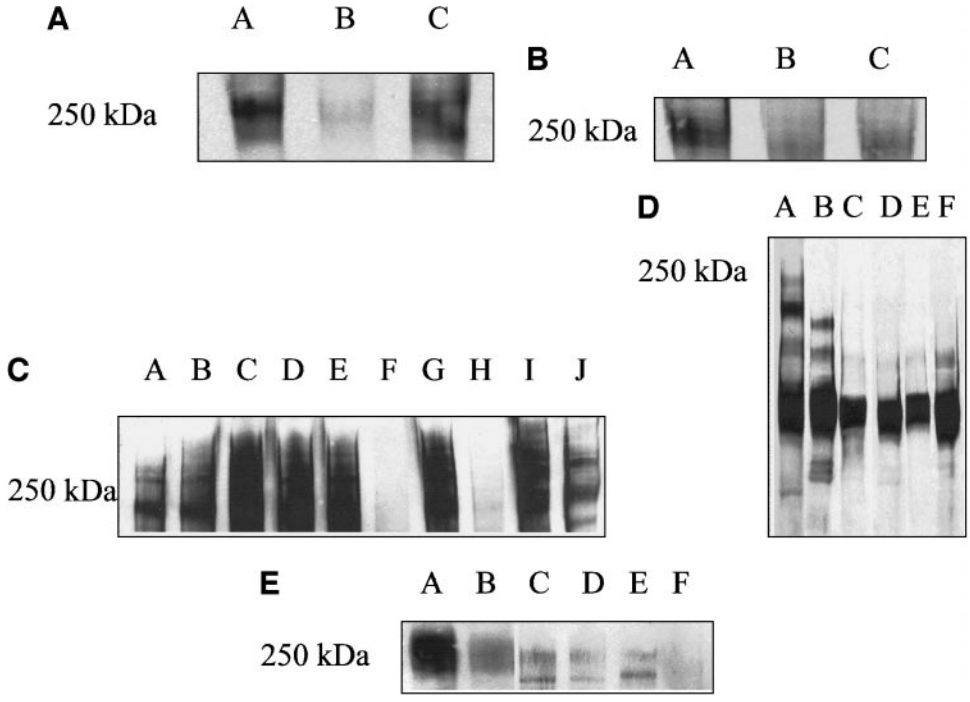


**FIG. 2.** Western blots of HMS-1 fractions: (A) HMS-1 CD44 sheds following PMA pretreatment but this activity cannot be transferred in CM: Lane A, untreated HMS-1 cell; lane B, PMA-pretreated HMS-1 cells; lane C, CM from HMS-1 cells pretreated with PMA and transferred to untreated HMS-1 cells followed by the usual cellular harvest. (B) HMS-1 CD44 sheds following PMA pretreatment but this activity can not be abolished by hourly removal of CM over the ensuing 24 h from HMS-1 cells following PMA-pretreatment. Lane A, untreated HMS-1 cells; lane B, PMA-pretreated HMS-1 cells; lane C, HMS-1 cells pretreated with PMA whose CM was removed hourly for 24 h followed by the usual cellular harvest. (C) Susceptibility of myoepithelial CD44 to different proteases: Cathepsin G (lane A); cathepsin L (lane B); cathepsin D (lane C); cathepsin B (lane D); trypsin (lane E); chymotrypsin (lane F); hyaluronidase (lane G); elastase (lane H); MMP-1 (lane I); pronase (lane J). No enzyme other than chymotrypsin and elastase cleaved myoepithelial CD44. (D) Exogenous chymotrypsin CD44 cleavage is specific for myoepithelial cells: Lane A, untreated Hs578T cells; lane B, chymotrypsin-treated Hs578T cells; lane C, untreated A253 cells; lane D, chymotrypsin-treated A253 cells; lane E, untreated A431 cells; lane F, chymotrypsin-treated A431 cells. Although these nonmyoepithelial cells express alternate forms of CD44, their CD44 does not exhibit chymotrypsin cleavage. (E) CD44 shedding in membrane preparations of PMA-pretreated HMS-1 cells. A progressive decrease in CD44 is observed over time: Lane A, no pretreatment; lane B, PMA pretreatment followed by 1 h incubation; lane C, PMA pretreatment followed by 2 h incubation; lane D, PMA pretreatment followed by 3 h incubation; lane E, PMA pretreatment followed by 4 h incubation; lane F, PMA pretreatment followed by 8 h incubation.

The former had inhibitory activity against papain-like cysteine proteases where the latter had inhibitory activity against serine proteinase inhibitors. We observed inhibitory activity only with $\alpha_1$-antichymotrypsin. Then we compared SCCA-2 with SCCA-1. These latter inhibitors are proteinase inhibitors that map to a serpin cluster at 18q21.3 but differ in inhibitory spectrum (42, 43). SCCA-1 inhibits only papain-like cysteine proteases, whereas SCC2-A inhibits chymotrypsin-like serine proteinases but interestingly not purified chymotrypsin (42, 43). Yet SCCA-2 was active against our putative myoepithelial sheddase. Finally we compared TPCK, an inhibitor of chymotrypsin-like serine proteinases with TLCK, an inhibitor of trypsin-like serine proteinases and found that only TPCK was active against our putative myoepithelial sheddase. These four separate lines of evidence, although indirect, all point to a putative chymotrypsin-like sheddase.

From our studies, three requirements were necessary for CD44 shedding to occur (1): The substrate, CD44, had to be susceptible to cleavage in the first place. The susceptibility of a particular isoform of CD44 to cleavage may not necessarily be a function of its primary amino acid sequence determined by its specific splice variation but rather a function of its tertiary structure and/or degree of glycosylation. CD44 molecules on nonmyoepithelial cells were not susceptible to cleavage by exogenous purified chymotrypsin suggesting that their CD44 might not be susceptible to shedding from an endogenous chymotrypsin-like sheddase even if one existed in those cells (2). There must be activation of the sheddase. In our past studies, we have shown that PMA activates and causes membrane association of protein kinase C (44). Presumably this association activates the sheddase responsible for CD44 shedding. The mechanism of activation remains unknown but could be due to direct activation of the

sheddase by phosphorylation, increased access to the substrate as a result of phosphorylation, or conformational modification of the substrate itself making it susceptible to cleavage. From our studies we can not yet determine whether nonmyoepithelial cell lines lack the sheddase or lack the activating response to PMA or both since their CD44 does not appear to be susceptible to chymotrypsin cleavage in the first place (3). Even if the CD44 is of the type which is susceptible to cleavage and even if the membrane sheddase exists and can be activated, both sheddase and CD44 substrate must be in *cis* orientation. Actually this requirement is confirming evidence that our putative chymotrypsin-like activity is indeed a sheddase. Since the sheddase is not secreted, transfer of activity or abolishment of activity by removal of CM could not be achieved. Transfer of sheddase activity likewise could not be achieved by cell–cell contact or membrane extracts because both produce a *trans* orientation.

Our observations that myoepithelial cell CD44 is equally susceptible to both exogenous chymotrypsin cleavage as well as endogenous PMA-induced shedding is again indirect evidence that we are dealing with a chymotrypsin-like sheddase. Certainly we do not know whether both exogenous and endogenous molecules cleave the same sites of CD44, only that CD44 is susceptible to cleavage by both molecules. Obviously the exogenous chymotrypsin is not restricted by the *cis* orientation requirement.

The significance of CD44 shedding from myoepithelial cells would be anticipated to have paracrine tumor suppressive effects on both carcinoma cells themselves as well as on endothelial cells (angiogenesis) from recent studies (45, 46). These studies have demonstrated that soluble CD44 can have autocrine suppressive effects on tumor cells: soluble CD44 originating either extrinsically (soluble wild-type CD44–Ig fusion protein) or intrinsically (transfection of cDNAs encoding soluble isoforms of CD44) can compete with tumor cell membrane CD44 for hyaluronan binding sites and exert antitumoral effects including decreased tumorigenicity and increased apoptosis (18, 45, 46). Since both carcinoma cell invasion and angiogenesis are dependent upon membrane CD44-hyaluronan interactions (27–29, 45, 46), myoepithelial cell specific shedding of CD44 could reduce the carcinoma and endothelial cell CD44-hyaluronan interactions critical to invasion and angiogenesis *in vivo*. CD44 shedding from myoepithelial cells would therefore be anticipated to have paracrine suppressive effects on tumors.

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