Stimulated Shedding of Vascular Cell Adhesion Molecule 1 (VCAM-1) Is Mediated by Tumor Necrosis Factor-α-converting Enzyme (ADAM 17)*

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A variety of cell surface adhesion molecules can exist as both transmembrane proteins and soluble circulating forms. Increases in the levels of soluble adhesion molecules have been correlated with a variety of inflammatory diseases, suggesting a pathological role. Although soluble forms are thought to result from proteolytic cleavage from the cell surface, relatively little is known about the proteases responsible for their release. In this report we demonstrate that under normal culture conditions, cells expressing vascular cell adhesion molecule 1 (VCAM-1) release a soluble form of the extracellular domain that is generated by metalloproteinase-mediated cleavage. VCAM-1 release can be rapidly simulated by phorbol 12-myristate 13-acetate (PMA), and this induced VCAM-1 shedding is mediated by metalloproteinase cleavage of VCAM-1 near the transmembrane domain. PMA-induced VCAM-1 shedding occurs as the result of activation of a specific pathway, as the generation of soluble forms of three other adhesion molecules, E-selectin, platelet-endothelial cell adhesion molecule 1, and intercellular adhesion molecule 1, are not altered by PMA stimulation. Using cells derived from genetically deficient mice, we identify tumor necrosis factor-α-converting enzyme (TACE or ADAM 17) as the protease responsible for PMA-induced VCAM-1 release, including shedding of endogenously expressed VCAM-1 by murine endothelial cells. Therefore, TACE-mediated shedding of VCAM-1 may be important for the regulation of VCAM-1 function at the cell surface.

The proteolytic cleavage and release of transmembrane cell surface proteins, termed ectodomain shedding, has emerged as an important post-translational mechanism for regulating the function of cell surface proteins (1). A wide variety of structurally diverse proteins including cytokines, growth factors, and adhesion molecules can be shed from the cell surface. In many cases, these shed ectodomains are biologically active. Ectodomain shedding can be mediated by both membrane-bound as well as soluble proteases. To date, members of the Zn2+-dependent protease superfamily, including the matrix metalloproteinases (MMPs), membrane-tethered MMPs (MT-MMPs), and the disintegrin metalloproteinases (ADAMs), have been shown to be responsible for the cleavage of the majority of shed proteins identified. In addition, soluble neutrophil-derived proteases including neutrophil elastase, cathepsin G, and proteinase-3 have also been implicated in the shedding of cell surface proteins (2). Of the disintegrin and metalloproteinase (ADAM) family of proteases, tumor necrosis factor-α-converting enzyme (TACE; ADAM 17) has emerged as a central mammalian ectodomain sheddase (3). TACE-deficient mice are not viable and show multiple developmental defects (4). Furthermore, cells isolated from TACE-deficient mice lack shedding of several unrelated cell surface proteins including tumor necrosis factor-α, tumor necrosis factor-α receptor, several epidermal growth factor receptor ligands, Notch-1, amyloid precursor protein, L-selectin, and fractalkine (4–10).

A role for ectodomain shedding in the regulation of adhesion molecule function was first suggested by the observation that L-selectin is rapidly shed from the surface of leukocytes upon activation (11). Subsequent studies have shown that the generation of soluble L-selectin is metalloproteinase-dependent, and cells derived from TACE-deficient mice lack L-selectin shedding (4). It has also been shown that cleavage of the adhesion molecule L1 is mediated by ADAM 10 and is required for L1-mediated cell migration (12). Similarly, cleavage of the adhesion receptor CD44 by MT1-MMP plays an important role in CD44-mediated migration in vitro (13). We have recently shown that cleavage and shedding of the endothelial adhesion molecule, fractalkine, is mediated by TACE (ADAM 17) (10). Together, these findings suggest that metalloproteinase-dependent processing of cell surface adhesion molecules may represent an important novel regulatory mechanism.

A variety of cell adhesion molecules have been shown to exist as cell-associated as well as soluble proteins that are thought to

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** The abbreviations used are: MMP, matrix metalloproteinase; MT-MMP, membrane-tethered MMP; ADAM, a disintegrin and metalloproteinase; TACE, tumor necrosis factor-α-converting enzyme; EGF, enhanced green fluorescent protein; HA, hemagglutinin epitope tag; IFN-γ, interferon-γ; ICAM-1, intercellular adhesion molecule 1; IRES, internal ribosomal entry site; PE, phycoerythrin; PECAM-1, platelet-endothelial cell adhesion molecule 1; PMA, phorbol 12-myristate 13-acetate; VCAM-1, vascular cell adhesion molecule 1; ELISA, enzyme-linked immunosorbent assay; FACS, fluorescence-activated cell sorter; WT, wild type.
by cleavage from the cell surface (14). Examples include the selectins E-selectin and P-selectin and members of the Ig superfamily including intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and platelet-endothelial cell adhesion molecule 1 (PECAM-1). Levels of these soluble adhesion molecules are elevated in vivo in a variety of inflammatory diseases (14). In most cases, relatively little is known about the protease(s) responsible for adhesion molecule shedding. However, the finding that TACE (ADAM 17) mediates cleavage of the selectin family of molecules has suggested that ADAM-family proteases may play an important role in the generation of these soluble adhesion molecules.

In this report we have used in vitro cell-based systems to characterize factors that regulate the generation of soluble VCAM-1. We find that VCAM-1 can undergo metalloproteinase-mediated cleavage and shedding from the cell surface and that this can be rapidly stimulated by phorbol esters. We additionally demonstrate that TACE (ADAM 17) function is required for VCAM-1 shedding. The possible implications of TACE-mediated cleavage and shedding for VCAM-1 function are also discussed.

MATERIALS AND METHODS

Reagents and Antibodies—The following antibodies were used: polyclonal rabbit anti-hemagglutinin epitope tag (HA) (Zymed Laboratories Inc.); anti-human VCAM-1 (BIOSOURCE) and anti-mouse VCAM-1 (R&D Systems); anti-endoglin monoclonal antibody MJ7/1 (15) using supernatant from hybridomas (University of Iowa Hybridoma Bank); phycoerythrin (PE)-labeled anti-VCAM-1 (Pharmingen); PE-labeled anti-rat IgG (Biomedia); PE- and peroxidase-conjugated anti-goat and anti-rabbit IgG (Jackson Immuno Research). Phorbol-12-myristate 13-acetate (PMA) and all other chemicals not specified were from Sigma.

GM6001 was purchased from Elastin Products Co. Genotyping for ADAM 9 was by Southern blotting (16) and by PCR for the NEO sequence. F1 mice that were positive for the presence of the gaaagcgaaggag-3' mutation in ADAM 17 (TACE) were by Southern blotting (19). The heart and aorta were aseptically removed and from crosses with ADAM-deficient mice generated as described previously (10). Lysates were separated by SDS-PAGE under reducing conditions.

Protein Analysis—Protein extracts from the 80% confluent cells were confirmed by cytokeratin staining (monoclonal antibody LE61 to keratin 18, Dr. E. B. Hay, University of Dundee, UK) after cold acetone fixation for 15 min. The presence of a characteristic basket weave staining pattern in the cytoplasm was considered evidence of the presence of the keratin fibers indicative of epithelial cells.

Dermal fibroblasts were prepared from mouse skin by digestion in 0.2% trypsin overnight at 4°C to allow separation of the epidermis from the dermis. Fibroblasts were isolated from the dermis by incubation on a sterile pad prequillified with Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum and 0.2% type III collagenase (In-vitrogen) at 37°C for 2 h (newborn) or at 4°C overnight (adult). After digestion, debris was removed, and the cells were plated in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum.

Endothelial cells were isolated using a modified version of a published protocol (19). The heart and aorta were aseptically removed and minced in digestion buffer (serum-free M199 media, 0.024 M Hepes buffer, pH 7.4, and 0.2% type IA collagenase) before digestion for 30 min at 37°C with gentle agitation. After filtering through a 70-μm cell strainer, cells from a single mouse were plated in M199 containing 20% fetal calf serum, ECM2 supplement (Clonetics), and 5 units/ml IFN-γ in a well of a 6-well tray (gelatin coated). After a 16-h incubation at 32–32°C, cells were washed to remove non-adherent cells, and cultures were subsequently maintained at 32–33°C. Two cycles of cell sorting were utilized to isolate cells greater than 90% positive for endoglin (12).

Staining Protocol—All cells cultured in medium contained streptomycin. NIH3T3 cells were maintained in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum. Stomach epithelial and dermal fibroblast cell lines from ADAM 9- and ADAM 17-deficient cells were maintained at 32–33°C in Dulbecco’s modified Eagle’s medium plus 10% fetal calf serum plus 5 units/ml IFN-γ, whereas endothelial cells were kept in M199 containing 20% fetal calf serum, ECM2 supplement (Clonetics), and 5 units/ml IFN-γ in gelatin-coated tissue culture plasticware. For experiments, cells were plated in the absence of IFN and grown at 37°C for 36 h to arrest immortalization by the SV40 large T antigen.

Generation of Expression Constructs and Retroviral Infection—HA epitope-tagged adhesion molecules were generated by reverse transcription-PCR using primers containing 5' and 3' Sp6/IIT sites for subcloning into the pBM-IARES-PURO retroviral vector. All PCR-generated constructs were verified by DNA sequencing. Retroviral expression plasmids were constructed using the pBM-IARES-EGFP vector (G. Nolan, Stanford University) or the pBM-IARES-PURO vector (20). High titer retrovirus was prepared as previously described (21). For infection, 4 × 10^5 NIH3T3 or ADAM-deficient cells were plated into 25-cm² tissue culture flasks 24 h before infection by incubation with 5 ml of virus stock for 12 h in the presence of 4 μg/ml Polybrene. After infection, retroviral supernatant was replaced with fresh medium, and cells were cultured for at least 48 h before use in subsequent experiments. For p126 large T antigen, the NIH3T3 cells were subsequently cultured in the presence of 1–2 μg/ml puromycin for 48 h.

Adhesion Molecule Shedding Assays—Cells were plated at a density of 6 × 10^5 cells/60-mm dish in complete growth medium 36 h before stimulation. Cells were pretreated for 15 min by the addition of GM6001 (50 μM final) or dimethyl sulfoxide (MeSO) vehicle control directly to the culture medium. Cells were washed with serum-free medium and stimulated with 2.5 ml of serum-free medium with or without PMA (100 ng/ml), GM6001 (50 μM), or MeSO control followed by incubation at 37°C for 45 min or the indicated time. After stimulation, supernatants were removed, and cells were washed once with cold phosphate-buffered saline and subsequently lysed with 1 ml of RIPA buffer, 1% Nonidet P-40, 0.2% sodium deoxycholate, 0.1% SDS, 2 μg/ml aprotinin, 2 μg/ml leupeptin, 1 μg/ml pepstatin, 100 μg/ml phenylmethylsulfonyl fluoride, and 0.5 mM iodoacetamide). Resulting cell supernatants and lysates were cleared by centrifugation at 15,000 × g and stored at −80°C until analysis. Adhesion molecule concentrations were determined by ELISA using commercially available ELISA kits (Cytoskeleton Inc., Denver, R&D Systems) or matched ELISA antibody pairs (ICAM-1 and human VCAM-1, BIOSOURCE; anti-mouse VCAM-1, R&D Systems). The levels of soluble adhesion molecules were determined for triplicate dishes and reported as the mean ± S.D. All experiments shown are representative of duplicate experiments.

Analysis—Cells expressing HA epitope-tagged human adhesion molecules were plated, stimulated, and lysed as described previously (10). Lysates were separated by SDS-PAGE under reducing conditions, transferred to Immobilon polyvinylidene difluoride membranes (Millipore), and subsequently immunoblotted with specific antibodies.

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prior to visualization by enhanced chemiluminescence (ECL, Amer sham Biosciences).

**Flow Cytometry and Cell Sorting**—Cell surface levels of adhesion molecules were confirmed after retroviral infections by flow cytometric analyses (FACScan; BD Biosciences) of cells stained with specific PE-conjugated antibodies (Phar mingen). For reconstitution experiments, TACE-deficient cells were infected with an IRES-EGFP retrovirus encoding TACE, a catalytically inactive TACE (Glu → Ala) mutant, or an empty vector control. Enhanced green fluorescent protein (EGFP)-positive cells were isolated by fluorescence-activated cell sorting (FACS) using a FACS Vantage cell sorter (BD Biosciences). Sorted cells were subsequently superinfected with pBM-VCAM1HA-IRES-PURO retrovirus and selected with puromycin as above. To isolate purified endothelial cells from mixed cultures, cells were stained with anti-endoglin monoclonal antibody MJ7/18 (15) followed by PE-labeled anti-rat IgG (Biomedia). Two cycles of cell sorting using a MoFlo cytometer (Cytomation, Inc.) were utilized to isolate populations with greater than 98% cells positive for endoglin.

**RESULTS**

**PMA Induces Rapid Cleavage and Shedding of VCAM-1**—The release of a variety of proteins from the cell surface can be stimulated by the phorbol ester PMA through activation of a metalloproteinase-dependent ectodomain sheddase machinery, a pathway that appears to be conserved across multiple cell types. We have previously shown that NIH3T3 cells can be used to study PMA-induced shedding (10) and, therefore, decided to characterize factors that regulate the shedding of VCAM-1 and other adhesion molecules in this cell line.

A cDNA encoding full-length VCAM-1 with a cytoplasmic tail HA epitope tag (Fig. 1A) was cloned into the pBM-IRES-PURO retroviral vector (20). After infection and selection with puromycin, flow cytometry was used to confirm that VCAM-1 is efficiently expressed and appropriately targeted to the cell surface (Fig. 1B). 3T3-VCAM-1 cells were subsequently cultured in the presence or absence of PMA for 45 min, and levels of soluble VCAM-1 released into the medium were determined by ELISA. Stimulation of 3T3-VCAM-1 cells with PMA leads to a 16-fold increase in the amount of soluble VCAM-1 (Fig. 1C).

To confirm that PMA stimulation did not simply induce the release of proteins from the surface of NIH3T3 cells, we also determined the effect of PMA on shedding of three additional adhesion molecules: ICAM-1, E-selectin, and PECAM-1. Despite efficient cell surface expression as determined by flow cytometry (Fig. 1B), PMA stimulation has no effect on the shedding of ICAM-1, E-selectin, or PECAM-1 from retrovirally infected NIH3T3 cells (Fig. 1C). This finding suggests that among these proteins, VCAM-1 is part of a specific shedding pathway that can be rapidly activated by PMA stimulation.

We next determined whether the release of soluble VCAM-1 in response to PMA occurs by cleavage of the transmembrane form of VCAM-1. Western blot analysis using polyclonal antibodies against the VCAM-1 extracellular domain or the cytoplasmic tail HA epitope tag shows that cell-associated VCAM-1 exists predominantly as a 110-kDa species (Fig. 1D). As expected, cellular VCAM-1 is recognized by antibodies against both the extracellular domain and the intracellular HA epitope-tag (Fig. 1D). In contrast, after release from the cell surface in the presence or absence of PMA, soluble VCAM-1 exists as a 100-kDa species that is recognized by the antibody against the extracellular domain but not by an antibody against the cytoplasmic tail HA epitope tag (Fig. 1D). This finding confirms that soluble VCAM-1 is generated by cleavage of full-length VCAM-1 near the transmembrane domain, leading to removal of the cytoplasmic tail and release of the majority of the ectodomain.

**Constitutive and PMA-inducible VCAM-1 Shedding Are Metalloproteinase-dependent**—To determine whether constitutive and/or PMA-inducible shedding of VCAM-1 from NIH3T3
cells were infected with an IRES-EGFP retrovirus encoding either WT-TACE, a catalytically inactive TACE (Glu → Ala) mutant, or an empty vector control. Expression of WT-TACE specifically rescues the loss of PMA-inducible VCAM-1 shedding in TACE-deficient cells (Fig. 3B). In addition, overexpression of WT-TACE increases basal VCAM-1 shedding, suggesting that TACE is not efficiently regulated when overexpressed in this cell type, similar to what we have observed with fractalkine shedding (10).

To determine whether constitutive VCAM-1 shedding in TACE-deficient cells is also mediated by a metallocproteinase, we evaluated the effect of GM6001 on the release of soluble VCAM-1. TACE-deficient stomach cells showed constitutive release of VCAM-1 that was partially, but not completely, blocked by GM6001 (Fig. 3C). Thus, TACE function is absolutely required for PMA-induced VCAM-1 shedding but not for basal VCAM-1 shedding. However, constitutive shedding is partially mediated by a metalloproteinase that does not appear to be ADAM 9 since basal VCAM-1 shedding is maintained in cells genetically deficient for ADAM 9 (Fig. 3 and data not shown).

To confirm that TACE is required for the shedding of endogenous VCAM-1, we isolated endothelial cells from WT and TACE-deficient mice intercrossed with the Immortomouse. Polyclonal populations of primary endothelial cells were isolated by FACs sorting based on endoglin expression (19). Similar to previous studies with murine endothelial cells, both WT and TACE-deficient cells expressed significant and comparable levels of VCAM-1 on their cell surface (data not shown). Endothelial cells specifically lacking TACE function failed to shed endogenous VCAM-1 in response to PMA stimulation (Fig. 3D and data not shown). Furthermore, retroviral expression of wild type TACE, but not the catalytically inactive TACE (Glu → Ala) mutant, efficiently restored PMA-induced VCAM-1 shedding that is completely blocked by GM6001 (Fig. 3D). Therefore, the shedding of VCAM-1 by TACE is not an artifact due to increased substrate availability after retroviral expression in NIH3T3 cells, and shedding of endogenously expressed VCAM-1 is also mediated by TACE in murine endothelial cells.

**DISCUSSION**

Although the existence of a soluble form of VCAM-1 has been known for more than a decade, relatively little is known about the mechanism(s) by which VCAM-1 can be shed from the cell surface. In this study, we show for the first time that VCAM-1 shedding can be mediated by two distinct metalloproteinase activities, a constitutive VCAM-1 sheddase that is active under normal cell culture conditions and a PMA-inducible protease that we identify as TACE (ADAM 17). Similar to other TACE substrates, we show that cleavage of VCAM-1 occurs near the transmembrane region, leading to release of an intact VCAM-1 ectodomain. In addition, we show that the proteolytic release of soluble VCAM-1 from the cell surface by TACE is not simply due to ubiquitous and nonspecific protein shedding as PMA fails to alter the levels of soluble ICAM-1, E-selectin, and PECAM-1.

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predicted to affect its function at several levels. First, cleavage of VCAM-1 by TACE may play a role in regulating the adhesive function of VCAM-1 by rapidly decreasing its levels at the cell surface. In support of this model, it has been shown that L-selectin, another TACE substrate, is rapidly shed from the surface of leukocytes during emigration into sites of inflammation (22), and MMP inhibitors have been shown to decrease the rolling velocity of leukocytes on microvessels in vivo by 40%, although the exact mechanism is not yet clear (23, 24). These and other studies suggest novel roles for proteases in the dynamic regulation of adhesion molecule function. A second potential implication of VCAM-1 shedding is that the soluble ectodomain generated by TACE cleavage may remain functionally active. For example, soluble ICAM-1 retains its ability to bind to leukocyte integrins and reduces leukocyte recruitment in diverse in vivo models of inflammation (14, 25). Recent studies have additionally shown that the cell-associated transmembrane cleavage fragments generated by ectodomain cleavage may have unique functions. For example, MMP-mediated cleavage of the CD44 ectodomain is followed by further cleavage of the membrane-anchored fragment by a γ-secretase-like protease, leading to the release of an intracellular cytoplasmic tail fragment (26). The resulting CD44 tail fragment subsequently translocates to the nucleus, where it can function as a transcriptional activator (26). It will be interesting to determine whether any of these secondary functions are also seen after TACE-mediated cleavage and shedding of VCAM-1. Furthermore, our understanding of the functional significance of TACE-mediated VCAM-1 shedding will be further clarified by identifying more physiologically relevant stimuli of TACE activity.

Although the in vivo significance of VCAM-1 cleavage and shedding is not yet known, the observation of high levels of soluble VCAM-1 in human serum suggests a likely physiological role (27). Soluble VCAM-1 present in both human and mouse serum (data not shown) exists as a 100-kDa protein similar to that released by NIH3T3 cells in vitro and suggests that it is likely generated by juxtamembrane cleavage. Recent studies report that VCAM-1 can also be cleaved from the cell surface by the soluble protease neutrophil elastase (27), and it has been proposed from in vitro data that neutrophil elastase cleavage of VCAM-1 from the surface of bone marrow stromal cells plays a role in the mobilization of hematopoietic progenitor cells (27). We also find that neutrophil elastase cleaves VCAM-1 from NIH3T3 cells, generating a 65-kDa form of soluble VCAM-1 distinct from the 100 kDa form generated by TACE-mediated shedding (data not shown). However, the predominant form of VCAM-1 seen in serum is 100 kDa (data not shown), inconsistent with cleavage by neutrophil elastase but compatible with its generation by a juxtamembrane sheddase.

Through the data presented in this paper we clearly show that TACE can be added to the list of proteases that can mediate the cleavage and shedding of VCAM-1. However, more definitive in vivo experiments are needed to determine when neutrophil elastase, TACE, or other proteases are responsible for the generation of soluble VCAM-1 in vivo. It will also be important to understand whether the proteases lead to the generation of different forms of soluble VCAM-1 with distinct biological activities and functions.

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