Cleavage of human 7-domain VCAM-1 (CD106) by thrombin

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Summary

Vascular cell adhesion molecule 1 (VCAM-1, CD106) is expressed as a type I transmembrane integrin counter-receptor on activated endothelium and mediates white blood cell attachment. The alternatively spliced 7-domain (7d) form of VCAM-1 contains a potential thrombin cleavage site. Thrombin proteolysis of 7d-VCAM-1 may help regulate adhesive activity of VCAM-1. We determined whether 7d-VCAM-1 is proteolyzed and rendered inactive by thrombin. Recombinant extracellular domain of 7d-VCAM-1 was cleaved by thrombin to generate 33- and 44-kDa products. Cleavage was in the sequence PGPR/IAAQIG near the N-terminal border of the alternatively spliced fourth immunoglobulin (Ig)-like module. There was no cleavage of 6d-VCAM-1 lacking the fourth module. Expression of full-length 7d-VCAM-1 presented on Chinese hamster ovary (CHO) monolayers, as detected by flow cytometry with an antibody directed to Ig-like modules 1–3, was reduced by thrombin treatment whereas there was no reduction in the expression of full-length 6d-VCAM-1. Adhesion of blood eosinophils to full-length 7d-VCAM-1 was reduced after treatment of CHO cells with thrombin, whereas adhesion to full-length 6d-VCAM-1 was not affected. We conclude that cleavage of 7d-VCAM-1 by thrombin is a potential mechanism for differential regulation of VCAM-1 splice forms in white blood cell adhesion and trafficking.

Keywords

Adhesion molecules, adhesion receptors/integrins, leukocyte function/activation, leukocyte trafficking/recruitment, thrombin

Introduction

Vascular cell adhesion molecule 1 (VCAM-1) is a type I transmembrane integrin counter-receptor on activated endothelium and bone marrow stromal cells (1–3). Cell-surface VCAM-1 mediates rolling, firm arrest, and migration of white blood cells to sites of tissue injury or inflammation (4, 5) and egress of hematopoietic progenitor cells from bone marrow into the circulation (6, 7). Integrins that engage VCAM-1 include α4β1 (CD49d/29) (8–10), αβ7 (CD49d/β7) (9–12), αδβ2 (αD/CD18) (8), and αMβ2 (CD11b/18; our unpublished results). In addition to the transmembrane form, soluble or circulating forms of VCAM-1 have been identified in the culture supernatant of cytokine-treated endothelial cells, human serum, and synovial fluid of patients with rheumatoid arthritis (13–16). The origins of soluble VCAM-1 in various situations are not clear, although the most common mechanism is likely proteolytic cleavage of membrane-tethered VCAM-1 from cell surfaces (15).

Two major splice variants of VCAM-1 are present in humans (17). 7d-VCAM-1 contains putative integrin ligation sites in immunoglobulin (Ig)-like modules 1 and 4, rendering 7d-VCAM-1 potentially bivalent (17). The alternatively spliced 6-domain form, 6d-VCAM-1, is missing module 4 (17). Rotary shadowing electron microscopy demonstrates a characteristic bend near the middle of recombinant soluble 7d-VCAM-1, suggesting that the region around module 4 is flexible and particularly susceptible to proteolysis (18). Indeed, endoprotease Glu-C cleaves 7d-VCAM-1 within the sequence FTV/ISP at the N-terminus of module 4 (19). Furthermore, chymotrypsin or pepsin generate 50-kDa and 35-kDa products from 7d-VCAM-1, compatible with cleavages in or near module 4 (19).

Thrombin is a highly specific serine protease that cleaves a limited number of natural substrates preferentially on the carboxyl side of arginine residues (20, 21) at sites determined in part by amino acids preceding or surrounding the scissile bond (20). In particular, the arginine at the site of thrombin cleavage is often preceded by proline (20). In the course of purifying 6d-VCAM-1 and 7d-VCAM-1 with a C-terminal His-tag linked by a thrombin-sensitive sequence, we noted that 7d-VCAM-1, but not 6d-VCAM-1, was cleaved internally. Inspection of the sequence...
of 7d-VCAM-1 revealed the potential thrombin cleavage sequence PGR/IAAQIG located near the N-terminal border of module 4 (17). The goals of the present study were to characterize the thrombin cleavage and determine whether the cleavage regulates white blood cell adhesion.

Materials and methods

Materials

Several of the monoclonal antibodies (mAbs) have been described previously (22). MAb IC3.21 was generated against recombinantly expressed 7d-VCAM-1 by Dr. Mary Ann Accavitti at the Hybridoma Core Facility, University of Alabama-Birmingham. This mAb recognizes VCAM-1 modules 5–6 based on reactivity with VCAM-1 constructs in Western blot and ELISA assays (not shown); anti-VCAM-1 mAb clone 106C04 (Ab-4) from Neomarkers (Fremont, CA, USA) and 1.G11B1 from Chemicon (Temecula, CA, USA) recognize VCAM-1 modules 1–3 based on reactivity with VCAM-1 constructs in Western blots and/or ELISA (not shown). Others include: FITC-conjugated goat anti-mouse, (Chemicon); alkaline phosphatase-conjugated donkey anti-mouse IgG (H+L), Jackson ImmunoResearch Laboratories (West Grove, PA, USA); and rabbit thrombomodulin, Haematologic Technologies, Inc. (Essex Junction, VT, USA).

Expression of VCAM-1 constructs

Recombinant soluble 7d-VCAM-1 was produced in High 5 insect cells transfected with recombinant baculovirus (22). For generation of recombinant soluble 6d-VCAM-1, total RNA was purified from stably transfected Chinese hamster ovary (CHO) cells expressing full-length human 6d-VCAM-1, which were a kind donation, along with CHO cells expressing 7d-VCAM-1, from Biogen (Cambridge, MA). All subsequent steps for the production of 6d-VCAM-1 were identical to that of 7d-VCAM-1. Sequences in the expressed and processed protein that were introduced from pCOCO and are not present in VCAM-1 were: ADPG at the N-terminus and the LELVPR/GSAAGHHHHHH thrombin cleavage and His-tag sequence at the C-terminus. Secreted proteins were purified from medium with Ni-NTA beads (Qiagen, Valencia, CA, USA). The 6d-VCAM-1 and 7d-VCAM-1 proteins (Fig. 1A) were partly multimerized on non-reduced SDS-PAGE gels. To enrich for monomers, small amounts of Ni-NTA beads were added to protein solutions in order to preferentially bind and remove multimers, leaving monomers behind in solution. Yields of pure monomeric 6d-VCAM-1 and 7d-VCAM-1 were identical to that of 7d-VCAM-1. Sequences in the expressed and processed protein that were introduced from pCOCO and are not present in VCAM-1 were: ADPG at the N-terminus and the LELVPR/GSAAGHHHHHH thrombin cleavage and His-tag sequence at the C-terminus. Secreted proteins were purified from medium with Ni-NTA beads (Qiagen, Valencia, CA, USA). The 6d-VCAM-1 and 7d-VCAM-1 proteins (Fig. 1A) were partly multimerized on non-reduced SDS-PAGE gels. To enrich for monomers, small amounts of Ni-NTA beads were added to protein solutions in order to preferentially bind and remove multimers, leaving monomers behind in solution. Yields of pure monomeric 6d-VCAM-1 and 7d-VCAM-1 were between 5–20 mg/l of conditioned medium. The 6d-VCAM-1 and 7d-VCAM-1 constructs migrated with expected molecular mass sizes of approximately 66 kDa and 76 kDa, respectively, on non-reduced SDS-PAGE gels (Fig. 1B). Smaller VCAM-1 constructs were made using a similar strategy in order to localize antigenic epitopes of anti-VCAM-1 mAbs.

Thrombin cleavage of recombinant soluble 6d-VCAM-1 and 7d-VCAM-1

Recombinant soluble 6d-VCAM-1 and 7d-VCAM-1 were dialyzed into thrombin cleavage buffer (150 mM NaCl, 2.5 mM CaCl₂, 20 mM Tris-HCl, pH 8.4), and 1.5 units of biotinylated human thrombin (Novagen, Madison, WI, USA) was incubated with 1 mg of either 6d-VCAM-1 or 7d-VCAM-1 in a total volume of 700 µl at 22°C for 24 h. Alternatively, VCAM-1 was incubated with non-biotinylated human thrombin (generously provided by John Fenton II, New York State Department of Health).
for 1–2 hrs at 22°C or 37°C. To remove the biotinylated human thrombin, 15 µl of streptavidin agarose beads (Novagen) were incubated with the cleavage reaction for 30 min at 22°C and gently mixed every 5 min and pelleted by centrifugation at 1500 x g for 5 min. The supernatant was aspirated, transferred to a clean tube, and 15 µl of streptavidin agarose beads were again added, incubated as before, and pelleted, resulting in the supernatant containing 6d-VCAM-1 or 7d-VCAM-1 and/or cleavage products free of thrombin.

Purification of thrombin cleavage products by size exclusion chromatography

The thrombin cleavage products in 700 µl volume (1 mg total) were passed over twin 2.5 x 85 cm columns connected in tandem and filled with Sephacryl S-100. The columns were equilibrated and eluted with 20 mM Tris, pH 8.0 at a flow rate of 115 µl/min at 4°C. Undigested VCAM-1 and thrombin cleavage products eluted as distinct peaks (not shown). The purity of products was verified by SDS-PAGE (Fig. 1B). Fractions containing the products were lyophilized, reconstituted with water, and dialyzed against 10 mM Tris, 150 mM NaCl, pH 7.4.

Characterization of thrombin cleavage products

Products of thrombin cleavage were electrophoresed on a 10% reducing SDS-PAGE gel, stained with GelCode Blue Staining Reagent (Pierce Biotechnology, Milwaukee, WI, USA), and bands were excised, reduced, alkylated, trypsinized, extracted, and analyzed on a Bruker BIFLEX III Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) mass spectrometer (Bruker Daltonics, Billerica, MA, USA). These analyses were performed at the University of Wisconsin-Madison Biotechnology Center. Mass to charge values for the various cleavage products were entered into the Mascot Peptide Mass Fingerprint website (http://www.matrixscience.com) and used to search VCAM-1 sequences. The excised 44-kDa cleavage product was also subjected to N-terminal sequence analysis at Utah State University (care of Melissa Hassebrock) by Edman degradation.

Characterization of CHO monolayers expressing full-length 6d-VCAM-1 or 7d-VCAM-1

Transfected CHO cells stably expressing full-length 6d-VCAM-1 or 7d-VCAM-1 (23) were grown in α-MEM (Gibco, Carlsbad, CA, USA), 10% FBS, with 800 nM methotrexate (MTX) and passaged by 1:6 dilution twice a week. The two populations were subjected to fluorescence activated cell sorting (FACS) on the Triple-laser FACS Vantage SE sorter (BD Biosciences, Franklin Lakes, NJ, USA; available through the Flow Cytometry Facility, Comprehensive Cancer Center, University of Wisconsin, Madison), and the top 5% VCAM-1 expressers based on fluorescence intensity with mAb 1.G11B1 recognizing modules 1–3 were collected and cultured. Three consecutive sortings were performed in order to obtain pure populations expressing equal densities of 6d-VCAM-1 and 7d-VCAM-1.

Expression of cell surface VCAM-1 by CHO cells in monolayer culture was assayed by ELISA. Polystyrene 96-well tissue culture treated plates with black sides and clear bottoms (Corning, Acton, MA, USA) were seeded overnight at 37°C in 200 µl of α-MEM/10% FBS with 6.6 x 10^4 transfected or untransfected CHO cells expressing or not expressing full-length 6d-VCAM-1 or 7d-VCAM-1. CHO monolayers were confluent the following day. Growth medium was aspirated, and CHO monolayers were fixed by addition of 100 µl of 4% paraformaldehyde at 22°C for 20 min. Monolayers were blocked for 15 min at 37°C with 1% BSA, washed with PBS, and then incubated with 100 µl of mAb 1.G11B1 at 0.05 µg/ml in PBS, pH 7.4 for 1 h. Wells were washed, incubated for 1 h with 100 µl of horse radish peroxidase-conjugated goat anti-mouse diluted 1:3,000 in PBS, pH 7.4, washed twice, and then incubated in 100 µl ortho-Phenylenediamine Dihydrochloride (OPD) substrate solution (Sigma-Aldrich, St. Louis, MO, USA) for 15 min. The reaction was stopped by addition of 50 µl of 4 M H₂SO₄, and absorbances were read at 490 nm on an EL microplate reader (Bio-Tek Instruments, Winooski, VT, USA).

Thrombin treatment of full-length 6d-VCAM-1 and 7d-VCAM-1 on CHO monolayers

CHO monolayers sorted by FACS and expressing equal densities of full-length 6d-VCAM-1 or 7d-VCAM-1 were grown to confluence, washed twice with PBS, incubated with EDTA, and 5x10⁶ CHO cells were resuspended in 100 µl of α-MEM without FBS in an eppendorf tube with or without non-biotinylated human thrombin (2 units) or 100 µl of 0.1% trypsin and incubated overnight at 37°C. The following day, CHO cells were pelleted, the supernatant was aspirated, and cells were resuspended on ice in 0.25 ml FACS buffer (PBS containing 2% BSA and 0.2% NaN₃) along with 5 µg/ml final concentration of mAb 1.G11B1 for 30 min at 4°C. Cells were pelleted, washed with 0.25 ml FACS buffer, resuspended in 0.25 ml FACS buffer, incubated with FITC-labeled secondary mAb for 30 min at 4°C in the dark, pelleted, fixed by resuspension in 250 µl FACS fix (1% paraformaldehyde, 67.5 mM sodium cacodylate, 113 mM NaCl, pH 7.2), and then, within one week, washed with PBS, resuspended in FACS buffer, and analyzed. Measurements were collected on a FACS Calibur (BD Biosciences; available through the Flow Cytometry Facility, Comprehensive Cancer Center, University of Wisconsin, Madison). Data were collected from 10,000 cells per condition and analyzed using the FlowJo software (Tree Star, Inc., Ashland, OR, USA). The geometric mean channel fluorescence (gMCF) is reported.

Eosinophil (EOS) isolation

Human EOS were isolated from peripheral blood by anti-CD16 magnetic bead selection, Miltenyi Biotec (Auburn, CA, USA) (24, 25). Samples were from normal, allergic rhinitic, or allergic asthmatic volunteers. The purity of the eosinophils was at least 99% as determined by Diff-Quik staining. Viability was at least 99% as assessed by staining with propidium iodide and annexin V-FITC (BD Biosciences). The University of Wisconsin Human Subjects Committee approved the study, and informed consent was obtained before participation of volunteers.

Adhesion of EOS to purified thrombin cleavage products and thrombin-treated CHO cells

EOS adhesion to polystyrene 96-well non-tissue culture treated plates (BD Biosciences) coated with 500 nM of solutions of int-
act 7d-VCAM-1 or VCAM-1 thrombin cleavage products was assayed as described (26). To assay adhesion to protease-treated CHO monolayers, polystyrene 96-well tissue culture treated plates with black sides and clear bottoms (Corning) were seeded in 200 µl of α-MEM/10% FBS with 6.6 x 10^4 CHO cells either not expressing or expressing full-length 7d-VCAM-1 or 6d-VCAM-1. Monolayers became confluent overnight and were washed twice with PBS, pH 7.4, placed in 100 µl of α-MEM without FBS, and incubated with or without 2 units of non-biotinylated human thrombin overnight or 100 µl of 0.1% trypsin for 15 min at 37°C. EOS were prepared for adhesion by fluorescent labeling with calcein AM (Molecular Probes, Eugene, OR, USA). Labeling was in RPMI/0.2% BSA at 22°C for 30 min with a calcein AM concentration of 5 µg/ml. EOS were then pelleted, washed once in 5 ml RPMI/0.2% BSA to remove excess dye, and then resuspended in RPMI/0.2% BSA. EOS were added to CHO monolayers in 100 µl of 3 x 10^5 cells per well for 50 min at 37°C. CHO monolayers were washed three times followed by addition of 100 µl RPMI 1640. Fluorescence (485 nm excitation; 535 nm emission) was measured on a Genios Pro plate reader (Tecan, Research Triangle Park, NC, USA) at 0.1 s intervals.

**Statistics**

Results were analyzed by one-way ANOVA with Dunnnett’s or Tukey-Kramer multiple comparison post test on GraphPad Prism software version 3.02 (GraphPad Software, Inc., San Diego, CA, USA).
Results

7d-VCAM-1 is cleaved by thrombin

7d-VCAM-1 contains a potential thrombin cleavage site near the N-terminal border of module 4 near the site of a bend in 7d-VCAM-1 that has been noted by rotary shadowing electron microscopy (Fig. 1A). The alternatively spliced 6d-VCAM-1 variant (Fig. 1A), which lacks module 4, is missing this sequence. When recombinant soluble 7d-VCAM-1, 1.4 mg/ml, was incubated with 2 units/ml biotinylated or non-biotinylated human thrombin at 22°C, two products of approximate molecular masses of 33-kDa and 44-kDa were generated (Fig. 1B). When the temperature was increased to 37°C or the concentration of thrombin was increased to 80 units/ml, the level of cleavage of 7d-VCAM-1 shown in Figure 1B was achieved by only a 1- to 2-hour incubation (not shown). In contrast, recombinant soluble 6d-VCAM-1 was not cleaved by thrombin (Fig. 1B).

Trypsin reduced the expression of both full-length 6d-VCAM-1 and 7d-VCAM-1 splice forms based on activity and recombinant soluble VCAM-1 constructs (not shown). The thrombine cleavage product yielded peptides from modules 1–3, whereas the 44-kDa cleavage product yielded peptides from modules 4–7 (Table 1). Thrombomodulin, which enhances thrombin cleavage of protein C and inhibits thrombin cleavage of fibrinogen (27, 28), inhibited cleavage of 7d-VCAM-1 by thrombin (not shown). These results demonstrate a cleavage site recognized by thrombin near the N-terminal border of module 4 of 7d-VCAM-1.

To learn whether thrombin cleaves cell surface VCAM-1, we tested effects of thrombin on transfected CHO cells stably expressing the transmembrane form of 6d-VCAM-1 or 7d-VCAM-1. The cells were sorted by fluorescence activated cell sorting (FACS) to yield populations with matching 6d-VCAM-1 and 7d-VCAM-1 protein expression levels in flow cytometric assays with mAb 1.G11B1 (Fig. 2A,B). This antibody recognizes an epitope in modules 1–3 common to both the 6d-VCAM-1 and 7d-VCAM-1 splice forms based on reactivity in Western blot and ELISA assays of thrombin cleavage products and recombinant soluble VCAM-1 constructs (not shown). ELISA indicated that the expression of 7d-VCAM-1 or 6d-VCAM-1 on CHO monolayers was equivalent based on the signal intensity generated by detection with mAb 1.G11B1 (not shown). There was an approximately two-fold reduction in the geometric mean channel fluorescence (gMCF) of CHO cells expressing 7d-VCAM-1 following incubation with thrombin (Fig. 2B). In contrast, there was no decrease in the expression of full-length 6d-VCAM-1 in response to thrombin treatment (Fig. 2B). Trypsin reduced the expression of both full-length 6d-VCAM-1 and 7d-VCAM-1 on CHO surfaces (Fig. 2B). These results indicate that full-length 7d-VCAM-1 expressed on CHO surfaces is cleaved by thrombin, whereas thrombin does not cleave the transmembrane form of 6d-VCAM-1.

Figure 3: Adhesion of white blood cells to thrombin cleavage products. Products from thrombin cleavage of recombinant soluble 7d-VCAM-1 were coated at 500 nM onto 96-well polystyrene microtitre plates in triplicate and assayed in cell adhesion of EOS. *, p<0.05 and **, p<0.01 comparing adhesion to 7d-VCAM-1. Results are the mean and standard deviation of adhesion from three separate donors (9 wells), one-way ANOVA with Dunnett’s post test.

The thrombin cleavage products support adhesion of white blood cells

7d-VCAM-1 contains two putative integrin ligation sites in modules 1 and 4, each containing an IDSPL recognition sequence (29, 30). Thrombin cleaves 7d-VCAM-1 such that the first site in module 1 is present in the 33-kDa product whereas the second site in module 4 is present in the 44-kDa cleavage product (Fig. 1A). The two thrombin cleavage products were purified by size exclusion chromatography (Fig. 1B). The 33-kDa product supported specific adhesion of EOS better than the 44-kDa protein when immobilized onto polystyrene wells (Fig. 3). Although the 33-kDa protein better reproduced the adhesion of intact 7d-VCAM-1, the fact that both thrombin cleavage products mediated adhesion of EOS indicates that the two integrin recognition sites in 7d-VCAM-1 are individually active in the cleavage products.

We then determined how proteolysis by thrombin regulates white blood cell adhesion to the transmembrane forms of 7d-VCAM-1 or 6d-VCAM-1 presented on CHO monolayers. EOS (8–10) and other white blood cells (31) interact with VCAM-1 via a4β1 integrin. EOS adhered in equal percentages to CHO monolayers expressing 7d-VCAM-1 or 6d-VCAM-1, reaching maximal adhesion at 10-15 minutes (Fig. 4A,B). Pre-incubation of CHO monolayers expressing 7d-VCAM-1 with thrombin caused a reduction in the adhesion of EOS nearly to the level of adhesion found on untransfected CHO cells (Fig. 4C). There was no decrease in the adhesion to the transmembrane form of 6d-VCAM-1 following incubation with thrombin (Fig. 4D). Incubation with trypsin reduced adhesion of EOS to monolayers expressing either splice form (Figs. 4C,D). To demon-
strate that the density of intact VCAM-1 determines adhesion, we also tested adhesion to unsorted CHO cells that expressed 5-fold less cell surface VCAM-1 in comparison to sorted CHO cells based on reactivity with mAb 1.G11B1 (Fig. 2A). EOS adhered in lower numbers to monolayers of unsorted cells in comparison to monolayer cells that had been sorted and expressed higher levels of VCAM-1 splice forms (Figs. 4C,D).

Discussion
In this study, we show that thrombin cleaves the soluble form of 7d-VCAM-1 in the sequence PGPR/IAAQIG near the N-terminal border of module 4. This result is consistent with the finding that module 4 is susceptible to cleavage by other proteases (19) and with electron microscopy images that demonstrate a flexible bend in this region (18). Both fragments generated from thrombin cleavage mediated adhesion of EOS, compatible with the known integrin ligation sites in module 1 and 4, although adhesion was stronger to the fragment containing module 1. Thrombin treatment of CHO monolayers expressing full-length 7d-VCAM-1 caused a reduction in the expression of the epitope in modules 1–3 recognized by mAb 1.G11B1 and attenuated adhesion of EOS. In contrast, the soluble or transmembrane forms of 6d-VCAM-1, which are missing module 4, were not cleaved by thrombin, and there was no attenuation of white blood cell adhesion to CHO cells expressing the 6d-VCAM-1 splice variant after thrombin treatment. Thrombin proteolysis, therefore, is a potential regulator of the adhesive activity of 7d-VCAM-1.

Studies of bronchoalveolar lavage (BAL) fluid after segmental antigen challenge of patients with asthma have demonstrated increased thrombin activity (32) and levels of soluble VCAM-1 (33). These findings raise the possibility that thrombin may be physiologically important in the generation of soluble forms of VCAM-1 in BAL fluid. Other serine proteases in addition to thrombin, including neutrophil elastase and cathepsin G, also cleave VCAM-1 in vitro (6), indicating that several proteases are perhaps important in VCAM-1 proteolysis. Cleavage of

Figure 4: Effect of thrombin on adhesion of white blood cells to CHO monolayers expressing full-length 7d-VCAM-1 or 6d-VCAM-1. A) Time-dependent specific adhesion of EOS labeled with the fluorescent dye, calcine AM, to CHO monolayers expressing full-length 7d-VCAM-1, 6d-VCAM-1, or to monolayers expressing undetectable levels of VCAM-1. Results are representative of adhesions performed in triplicate wells on three separate occasions with the standard deviation indicated. B) Fluorescence microscopy photomicrographs of calcine AM-labeled EOS adherent to CHO monolayers; Scale bar = 50 μm. C and D) CHO monolayers expressing full-length 7d-VCAM-1 (C) or 6d-VCAM-1 (D) were incubated with buffer, thrombin, or trypsin and assayed in adhesion of calcine AM-labeled EOS. CHO monolayers expressing a five-fold lower gMCF level of 7d-VCAM-1 or 6d-VCAM-1 or untransfected CHO monolayers expressing undetectable levels of VCAM-1 were used to study the density-dependence of VCAM-1 on cell adhesion. Results are the mean and standard deviation of adhesion experiments performed in triplicate on three separate occasions (9 wells). *, p<0.05, indicates inhibition comparing adhesion to buffer-treated sorted CHO cells; †, p<0.05, adhesion to thrombin treated CHOS compared to untransfected CHO, one-way ANOVA with Dunnett’s post test.
7d-VCAM-1 by thrombin was inhibited by thrombomodulin. Thus, the cleavage is a function of the "procoagulant" form of thrombin (27, 28).

Thrombin cleavage of full-length 7d-VCAM-1 presumably results in release of a fragment comprising modules 1–3 but retention of the remaining 4 modules on the cell surface (Fig. 1A). Thus, functional consequences of the cleavage may include binding to cells of the soluble fragment via its integrin binding site in module 1 and interaction of cells to the foreshortened membrane-tethered VCAM-1 via its integrin binding site in module 4.

Soluble forms of VCAM-1 have been demonstrated to bind CD16+ natural killer cells, Jurkat cells, and T-lymphocytes following activation with MnCl₂ or PMA (15); to purified EOS in suspension (our unpublished data); and T lymphocytes in synovial fluid of patients with rheumatoid arthritis (34). Soluble VCAM-1 is chemotactic for T cells (35) and induces chemotaxis and tyrosine phosphorylation of monocytes via G protein and protein kinase C pathways (36). Finally, the enhanced viability demonstrated for EOS purified from BAL fluid following segmental bronchoprovocation (37) can be replicated in vitro by incubation of purified blood EOS with soluble VCAM-1, whereas a second integrin ligand in soluble form, plasma fibronectin, has no effect on EOS survival (25).

Integrin recognition of module 4 of the remnant left on the cell surface likely differs from recognition of module 1 in 6d- and 7d-VCAM-1 in two regards. First, module 4 is closer to the cell surface. Rotary shadowing of 7d-VCAM-1 demonstrated a length of 25.9 nm, or 3.7 nm per Ig-like module (18). Thus, the module 4–7 remnant is calculated to extend approximately 15 nm from the cell surface, as compared to 22 nm and 26 nm for 6d-VCAM-1 and 7d-VCAM-1, respectively. A similarly sized truncated version of VCAM-1 comprising modules 1–3 is generated in mice by alternative splicing and tethered to the membrane by a glycosphatidylinositol (GPI) anchor (38, 39). Interestingly, the residue homologous to arginine (a potential cleavage site) is glutamine in mouse and rat 7d-VCAM-1. Therefore, one would predict that mouse and rat 7d-VCAM-1 cannot be cleaved by thrombin to yield the shorter remnant. Second, integrin recognition of module 4 has been shown to differ from recognition of module 1 (40–42). We recently found that α4β2 on EOS cooperates with α4β1 to mediate adhesion to module 4, whereas α4β1 on EOS is primarily responsible for adhesion to module 1 (our unpublished results). Such differences in recognition of modules 1 and 4 likely explain why unsorted CHO cells expressing 5-fold less intact 7d-VCAM-1 bound a similar number of EOS as CHO cells with a 2-fold reduction of intact 7d-VCAM-1 due to thrombin cleavage.

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References


