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In Vitro Evaluation of Functional Interaction of Integrin αvβ3 and Matrix Metalloprotease-2

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Abstract: Integrin αvβ3 and matrix metalloprotease-2 (MMP-2) are two established molecular targets of angiogenesis. Basic understanding of various forms of functional interaction of integrin αvβ3 and active MMP-2 may be used to develop therapeutic approaches. Based upon the idea that integrins are present on the surface of invasive cells and MMP-2 may be localized to this and other cell-surface receptors, we investigated the hypothesis that integrin binding will alter cleavage of MMP-2 substrates. To investigate this hypothesis, integrin-binding and MMP-2 cleavable motifs were combined in a single peptide, MMP-RGD, designed with fluorescent probes for monitoring peptide cleavage. MMP-RGD was bound to integrin αvβ3 with equal affinity compared to the integrin-binding motif and was cleaved with equal specificity by active MMP-2. MMP-RGD bound to human umbilical vein endothelial cells (HUVECs). MMP-2 from HUVECs cleaved MMP-RGD, but the cleavage was not altered due to integrin binding. Our results indicate that integrin αvβ3 and active MMP-2 may not be as functionally collaborative for substrate cleavage as expected based on the current knowledge of their cell surface colocalization.

Keywords: Integrin; αvβ3; matrix metalloprotease; MMP-2; molecular targets; angiogenesis; HUVEC; RGD peptide

Introduction

Therapeutic approaches can benefit from exploiting functionality of two or more co-overexpressed molecular targets in, on, or around the cell.[1,2] However, fundamental understanding of functional interactions of the combination of targets is very important for rational design of therapeutic strategies. Therapeutic strategies can be small molecule drugs, macromolecular drugs, prodrugs, or drug delivery systems, and the knowledge of how the co-overexpressed targets interact will allow the design of specific approaches that utilize the combined effect of the two targets.

One such potentially useful pair of targets is integrin αvβ3 cell surface receptor and matrix metalloprotease-2 (MMP-2) extracellular protease in angiogenesis. Angiogenesis, the development of new blood vessels, is the common underlying pathology of many otherwise unrelated diseases, including cancer, macular degeneration, rheumatoid arthritis and atherosclerosis. Therefore, molecular targets of angiogenic blood vessels, such as integrin αvβ3 and MMP-2, are therapeutically important for all angiogenesis-dependent diseases.[3]

Integrin αvβ3 is a member of the integrin family of heterodimeric cell surface receptors. Many extracellular

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matrix (ECM) proteins interact with cells via integrins. A consensus tripeptide sequence, arginine-glycine-aspartic acid (RGD), is the cell attachment site of a large number of ECM proteins, and many integrins recognize this sequence.[4] Specifically, integrin αvβ3 binds ECM ligands including vitronectin, fibrinogen, von Willebrand factor, and thrombospondin through amino acid sequences that contain the RGD sequence.[5] Integrin αvβ3 is overexpressed in the ligand-binding activated state on the surface of angiogenic endothelial cells in response to angiogenic growth factors. Integrin αvβ3 mediates the cell adhesive events required for migration of endothelial cells to the newly forming blood vessels.[6,7]

Integrin αvβ3 has been explored as a molecular target in many different ways. Integrin antagonists, such as a humanized version of integrin αvβ3 monoclonal antibody, LM609,[8] and a cyclic RGD peptide, cilengitide (EMD 121974),[9] are currently in phase II clinical trials as antiangiogenic agents for cancer therapy. Various radiolabeled[10] and fluorescently labeled[11] RGD peptides have been developed as tracers for imaging tumors. Different RGD sequence-containing peptides binding integrin αvβ3 have also been successfully incorporated into drug delivery systems for targeting therapeutics to tumor neovasculature[12] based upon this specific integrin up regulation and overactivation in angiogenesis. But binding to the extracellular matrix is only one component of endothelial cell-invasive and migratory behavior that could be exploited in therapeutic approaches.

Invasive endothelial cells also secrete and activate MMP-2, a proteolytic enzyme that is part of the family of matrix metalloproteases (MMPs). MMPs are zinc-dependent human endopeptidases that are together capable of degrading all components of the extracellular matrix (ECM) and many other proteins.[13] MMP-2 aids endothelial cell migration by causing degradation of basal lamina and extracellular matrix (ECM)[14,15] and releasing pro-invasive components from the ECM.[16] After MMPs, primarily MMP-2 and -9, were linked to tumor invasion and metastasis, peptidomimetic and zinc-binding MMP-inhibitors were tested in clinical trials as anticancer drugs.[17] However, all MMP-inhibitors failed to reach the end point of increased survival in phase III trials. Several factors, such as lack of selectivity, mechanism of MMP-inhibitor activity, trial design, and side effects, may have caused the failure of MMP-inhibitors.[18,19] In fact, MMPs such as MMP-3 and MMP-8 are now known to possess antitumorigenic actions and hence are characterized as MMP antitargets. Even the pro-invasion MMPs have been implicated in antiangiogenic agent activation. Therefore, discovery of new chemical leads that selectively inhibit target MMPs and spare the antitarget MMPs is required to revive the future development of MMP-inhibitors.[20] Despite the disappointment of MMP-inhibitors as therapeutic molecules, other approaches that utilize MMPs, specifically MMP-2, to activate drugs have been investigated in recent years. MMP-2 has been utilized for designing prodrugs and delivery systems that require cleavage of MMP-2 substrate peptides for activating drug molecules attached to the peptides.[21,22]

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MMP-2 is secreted as inactive proMMP-2, which is then activated—at least in part—on the cell surface in a trimolecular complex of membrane type-1 MMP (MT1-MMP), tissue inhibitor of metalloproteases-2 (TIMP-2) and proMMP-2.[23] Integrin $\alpha v\beta 3$ is also implicated as being involved in the later stages of activation of MMP-2 by $\alpha v\beta 3$ expressing cancer and endothelial cells.[24,25] Although an earlier paradigm considered MMP-2 to be a soluble enzyme secreted into interstitial fluid, more recent evidence suggests that the active form of MMP-2 is held on the surface of migrating cells, primarily at the cell invadopodia, thus providing a mechanism for restricted and localized ECM degradation.[26−28] Integrin $\alpha v\beta 3$ and MMP-2 colocalize on angiogenic blood vessels and specifically on endothelial cells of human glioma-associated vasculature.[29] Integrin $\alpha v\beta 3$ and MMP-2 were also observed to be localized in the same microdomains of endothelial cell membrane *in vitro*.[30] It has been further proposed that the active form of MMP-2 is held on the surface of endothelial cells by binding to integrin $\alpha v\beta 3$.[31,32] A fragment of MMP-2, PEX, and a small molecule, TSRI265, have been shown to disrupt angiogenesis by inhibiting MMP-2 binding to integrin $\alpha v\beta 3$.[33,34] However, such direct physical interaction of MMP-2 and integrin $\alpha v\beta 3$ has been questioned, and the cell surface receptor of MMP-2 has not been verified.[35]

Based upon the observation that integrin $\alpha v\beta 3$ and MMP-2 are colocalized and co-overactive on the surface of endothelial cells, the hypothesis that integrin binding may assist to enhance the cleavage of MMP-2 substrate peptide was generated (Figure 1). Based on this hypothesis, at least six possibilities exist for MMP cleavage of a substrate. The MMP can be bound to integrin $\alpha v\beta 3$ (1 and 5), some other surface receptor (3 and 4), or not bound to any receptor (2 and 6). Also, the peptide can be either bound to integrin $\alpha v\beta 3$ (1, 2, and 3) or not bound to a receptor (4, 5, and 6). We hypothesized that integrin-binding of the peptide can facilitate the cleavage of MMP-2 substrate peptide, and hence the cleavage of the integrin-bound form of the peptide (1, 2, and 3) will dictate the overall cleavage of the peptide. Such functional interaction of integrin $\alpha v\beta 3$ and MMP-2 of angiogenic endothelial cells has implications for the design of novel therapeutic strategies, including MMP-2 cleavable prodrugs.

To our knowledge, there is only one published report that has investigated integrin-binding, MMP-2 cleavable doxorubicin conjugates.[36] The primary purpose of this work was to clarify the structural requirements that a doxorubicin−RGD conjugate should have for effective vascular targeting. Although the MMP-2 cleavable doxorubicin conjugate showed higher activity compared to the noncleavable conjugate *in vitro*, minimal, if any, effect was observed on antitumor efficacy *in vivo*. While phenomenological end points, specifically cell proliferation and cell sprouting, were evaluated, no mechanistic evaluation of interactions of the

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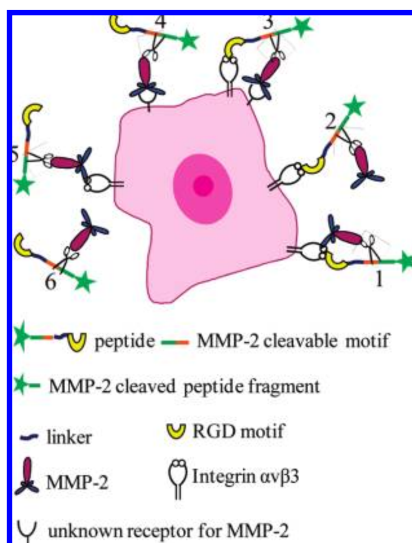

Figure 1. Schematic depicting proposed cleavage of integrin αvβ3 binding, MMP-2-cleavable peptide in pericellular space. An MMP-2 substrate can bind to integrin αvβ3 overexpressed on the surface of endothelial cells via RGD motif (1, 2, and 3). Then, the MMP-2 cleavable motif can be cleaved by integrin αvβ3-bound MMP-2 (1), soluble MMP-2 in the pericellular space (2), or MMP-2 bound to any other cell surface receptor (3). Alternatively, the peptide can be cleaved in the unbound state by cell-bound MMP-2 through any receptor (4) or integrin αvβ3 (5) or soluble MMP-2 (6). Our hypothesis that integrin αvβ3 binding can enhance the cleavage of MMP-2 substrate peptide was generated to test if the mechanisms 1, 2, and 3 dictate the cleavage.

conjugates with integrin αvβ3 and MMP-2 was carried out.[36] Independently, we have been studying the concept of complementary functionalities of integrin αvβ3 and MMP-2. Our focus in the current work was to systematically investigate the functional interactions of integrin αvβ3 and MMP-2 in purified form and in endothelial cell model using an integrin binding, MMP-2 substrate. We have particularly probed into the effect of integrin binding on the cleavage of MMP-2 substrate by the endothelial cells which does not appear to be altered by integrin binding.

Experimental Section

Peptides. An integrin binding, MMP-2 cleavable peptide (MMP-RGD) was used, FAM-GPLG∼VRGK(TAMRA)-aeea-GRGDS-amide, denoted using single letter nomenclature of amino acids where FAM, TAMRA and aeea denote 5-carboxyfluorescein, 5-carboxytetramethylrhodamine, and amino-ethyloxy-ethyloxyacetic acid, respectively. GPLG∼VRGK, a MMP-2 substrate, was cleaved by MMP-2 between G and V and previously examined for *in vivo* imaging of MMP-2 expression and inhibition.[37,38] GRGDS was the integrin binding motif.[5] As a linker between MMP-cleavable motif and RGD motif, aeea was included.[39,40] FAM and TAMRA were included as fluorescent probes that comprise a fluorescence resonance energy transfer (FRET) pair.[41] In the intact peptide, fluorescence of FAM was partially quenched by TAMRA and the cleavage of peptide between G and V by MMP-2 could be followed by increase in fluorescence of FAM upon separation from TAMRA. MMP-2 substrate sequence, FAM-GPLG∼VRGK(TAMRA), without RGD-motif (MMP-control), and GRGDS without MMP-substrate motif (RGD-control) were used as the controls. MMP-RGD and MMP-control peptides were synthesized by New England Peptide LLC (Gardner, MA). GRGDS-amide was purchased from AnaSpec (San Jose, CA).

Binding to Purified Integrin αvβ3. Binding of MMP-RGD with purified integrin αvβ3 was assessed using competitive displacement with vitronectin,[42] a natural ECM ligand of integrin αvβ3, and compared with binding of RGD-control. Purified vitronectin (Millipore, Billerica, MA) was biotinylated using EZ-link micro sulfo-NHS-LC biotinylation kit (Pierce, Rockford, IL) as per manufacturer's instructions. Purified integrin αvβ3 (1 μg/mL, Millipore, Billerica, MA) was adsorbed to 96-well Immulux HB microtiter plates (Dynex Technologies, Chantilly, VA) overnight at 4 °C in tris buffered saline, pH 7.6, containing 1 mM Ca^{2+}, Mg^{2+}, and Mn^{2+} (TBS^{3+}). Plates were then blocked with 1% BSA in TBS^{3+}. After removing blocking buffer and rinsing the wells, mixtures of 5 nM biotinylated vitronectin (bVn) and varying concentrations of peptides in TBS^{3+} were added to the integrin coated wells and incubated for 2 h at room temperature. Unbound bVn and peptides were washed away, and bound bVn was detected using peroxidase conjugated antibiotin goat polyclonal antibody (Calbiochem, EMD, San Diego, CA). Assays were performed in triplicate, the mean

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percent bVn bound was fitted to a sigmoidal curve using GraphPad Prism v.3.0, and the IC_{50} was derived for each peptide.

Cleavage by Purified, Active MMP-2. Cleavage of peptides between G and V by purified active MMP-2 was confirmed by high pressure liquid chromatography−mass spectroscopy (HPLC−MS). Cleavage of the peptides was assessed by measuring decreased FRET quenching between FAM and TAMRA. Reverse-phase HPLC analysis of peptides was performed after incubation with 9 nM human recombinant active MMP-2 (Calbiochem, EMD, San Diego, CA) in tris buffer saline (pH 7.6) containing 10 mM Ca^{2+}, 0.05 mM Zn^{2+} and 0.05% Brij-35 (TBS/Zn) for 2 h at 37 °C. Peptides incubated in the buffer without MMP-2 and in the presence of 10 μM GM6001, MMP-inhibitor (Millipore, Billerica, MA), were used as the controls. Fluorescence of FAM in the samples was also continuously measured at excitation and emission wavelengths of 490 and 535 nm using fluorescence plate reader (Spectramax, Gemini XS, Molecular Devices, Sunnyvale, CA). HPLC was performed using Zorbax Extend-C18 column (4.6 × 150 mm, 3.5 μm, Agilent technologies, Santa Clara, CA) with a Waters 600 controller and 474 scanning fluorescence detector (Waters, Milford, MA). The mobile phase for elution was a linear gradient of initially 20% acetonitrile and 80% water both with 0.1% trifluoroacetic acid changing over 60 min to 80% acetonitrile and 20% water both with 0.1% trifluoraceitic acid. Peptides and their fragments eluting from the column were detected at excitation and emission wavelengths of 490 and 535 nm (FAM), 490 and 570 nm (TAMRA FRET transfer) or 540 and 570 nm (TAMRA) and collected for MALDI-TOF mass spectroscopy.

Kinetics of cleavage of MMP-RGD peptide was followed by measuring the increase in fluorescence of FAM and compared with that of the MMP-control peptide. Stock solutions of peptides (1 mM) were prepared in DMSO and further diluted in TBS/Zn buffer to prepare substrate solutions of varying concentrations. Substrate solutions were then transferred to a black 96-well plate, and active MMP-2 at a final concentration of 9 nM was added for each substrate concentration. Immediately after addition of MMP-2, the plate was transferred to a fluorescence plate reader and the fluorescence of FAM was measured every 5 min over 2 h while incubating the mixtures at 37 °C. Initial velocity of enzymatic cleavage of the peptides was defined as rate for the cleavage over first 20 min and determined as change in fluorescence per minute (ΔRFU/min). Enzyme cleavage kinetics of peptides was then modeled by the Michaelis−Menten equation, and kinetic parameters V_{max} (maximum velocity) and K_M (Michaelis−Menten constant) were determined by nonlinear regression analysis on a plot of initial velocity (v) versus substrate concentration (S) using GraphPad Prism v.3.0.

Effect of Integrin αvβ3 Binding on MMP-2 Cleavage. Peptide cleavage by purified active MMP-2 was studied in the presence and absence of immobilized integrin αvβ3 to evaluate the effect of integrin binding on the cleavage. Purified integrin αvβ3 (1 μg/mL) was adsorbed to 96-well Fluorolux HB black microtiter plates (Dynex Technologies, Chantilly, VA) overnight at 4 °C in TBS^{3+}. TBS^{3+} was added for uncoated control wells. Plates were then blocked with 1% BSA in TBS^{3+}. After removing blocking buffer and rinsing the wells, MMP-RGD solutions (0.01, 0.3, and 10 μM) prepared in tris buffer saline containing 10 mM Ca^{2+}, 1 mM Mg^{2+}, 1 mM Mn^{2+}, 0.05 mM Zn^{2+} and 0.05% Brij-35 (TBS^{4+}) were added and allowed to bind for 2 h at room temperature. Active MMP-2 at a final concentration of 9 nM was then added to the peptide solutions, and the cleavage was followed as described. Peptide solutions treated with MMP-2 in the uncoated wells were used as the unbound controls.

Cell Culture. Human umbilical vein endothelial cells (HUVECs) were used as a model of angiogenic endothelial cells. HUVECs (HUVEC-2, BD Biosciences, San Jose, CA) were grown in Clonetics EGM-2 endothelial cell growth medium (Lonza, Walkersville, MD) in 75 cm^2 tissue culture treated flasks (BD Falcon, BD Biosciences, San Jose, CA). Before reaching confluence, cells were trypsinized and split at the ratio of 1:4 or 1:8. Cells between passages 1 and 3 were used for the experiments. HUVECs used for the experiments were treated with 50 ng/mL phorbol 12-myristate 13-acetate (PMA, Sigma, St. Louis, MO) for 30 min or more in order to induce activation of proMMP-2 in the cells.[43]

Expression of Active MMP-2 in HUVEC Culture. Gelatin zymography was used to confirm the presence of the active form of MMP-2 on the surface and in the supernatant culture medium of HUVECs. HUVECs were trypsinized and resuspended in EGM-2, and 4 × 10^5 cells per well were plated in 6-well tissue culture-treated plates (BD Falcon, BD Biosciences, San Jose, CA). After overnight incubation, cells were washed with Dulbecco's phosphate buffered saline with calcium and magnesium (DPBS) followed by incubation with serum-free EGM-2(SF EGM-2) containing PMA for 2, 8, and 24 h or SF EGM-2 for 24 h. At each time point, culture medium was collected and centrifuged at 10,000 rpm for 20 min at 4 °C, and the supernatant was collected as conditioned medium. Cells were washed with DPBS and lysed by incubating with lysis buffer (50 mM tris buffer, pH 8.0 containing 300 mM sodium chloride, 1% Triton X-100 and 10% v/v protease inhibitor cocktail; Sigma, St. Louis, MO) for 20 min at 4 °C followed by ultrasonic homogenization. Cell homogenates were centrifuged at 10,000 rpm for 20 min at 4 °C to remove cell debris, and the supernatant was collected as cell lysate sample. Cell lysates were analyzed for their total protein content using bicinchoninic acid assay (BCA Protein Assay Kit, Thermo Fisher Scientific, Rockford, IL). Samples were mixed 1:1 with zymogram sample buffer and electrophoresed on precast 10% zymogram gels (Bio-Rad Laboratories,

(43) Tatin, F.; Varon, C.; Genot, E.; Moreau, V. A Signalling Cascade Involving Pkc, Src and Cdc42 Regulates Podosome Assembly in Cultured Endothelial Cells in Response to Phorbol Ester. *J. Cell Sci.* **2006**, *119* (4), 769–81.

Hercules, CA). Gels were washed for 30 min in 2.5% Triton X-100 and developed overnight at 37 °C in 50 mM Tris buffer, pH 7.5 containing 200 mM sodium chloride, 5 mM calcium chloride and 0.02% Brij-35. Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie blue followed by destaining. Human recombinant pro and active MMP-2 (Calbiochem, EMD, San Diego, CA) were used as standards. The integrated density of each band was determined by analyzing digital images of the gels using Quantity One software (Bio-Rad) applying correction for the background. The integrated density was reported in volume units of pixel intensity times mm^2 by the software. The final quantification of MMP-2 bands from conditioned media and cell lysates was expressed as the integrated density per μg of protein per unit sample volume (pixel intensity × $mm^2/\mu g/\mu L$), referred to as relative MMP concentration (Relative [MMP]), so that MMP-2 concentrations in the conditioned media and cell lysates could be compared.

Expression of Integrin αvβ3 on HUVECs. Immunofluorescence microscopy was used to demonstrate expression of integrin αvβ3 on the surface of HUVECs. HUVECs were trypsinized and resuspended in EGM-2, and 5 × 10^4 cells per well were plated in 48-well tissue culture-treated plates (BD Falcon, BD Biosciences, San Jose, CA). After overnight incubation, cells were washed with DPBS and incubated with PMA/SF EGM-2 at 37 °C for 24 h. Cells were then fixed for 20 min with 4% paraformaldehyde in PBS and subsequently permeabilized for 3 min with 0.1% Triton-X-100 in PBS. After fixation and permeabilization, the cells were blocked (1% BSA in PBS) followed by incubation with 10 μg/mL R-phycoerythrin-conjugated mouse antihuman integrin αvβ3 monoclonal antibody (PE-LM609, Millipore, Billerica, MA) for 1 h at room temperature. For negative control, R-phycoerythrin-conjugated mouse isotype control IgG1 (PE-IgG1, Millipore, Billerica, MA) was used at the same concentration. Cells were then washed three times using PBS and rinsed once with deionized water. Staining was observed using Olympus IX70 epifluorescent imaging microscope, and images were recorded using a Retiga 1300C cooled CCD camera (Q-imaging, Surrey, BC) and processed with IP Lab software (Scanalytics, Fairfax, VA).

Peptide Binding to HUVECs. Inhibition of HUVEC adhesion to vitronectin by RGD-containing peptides was used to assess the binding of peptides to integrin αvβ3 on the surface of HUVECs. The assay was performed using 96-well Cytomatrix human vitronectin cell adhesion strips (Millipore, Billerica, MA). Trypsinized cells were resuspended in SF EGM-2, pretreated with PMA for 30 min and then incubated with varying concentrations of peptides for 15 min at room temperature. Peptide-treated cells were then incubated in vitronectin-coated wells at 37 °C for 1.5 h. Untreated cells plated in vitronectin- and BSA-coated wells served as controls. Unattached cells were removed by rinsing the wells with DPBS. Attached cells were fixed with 70% ethanol and stained with 0.2 mg/mL crystal violet. After removal of excess stain, cell-bound stain was solubilized in a 75/25 mixture of 0.06 M NaH_2PO_4 at pH of 4.5 and ethanol. The absorbance of the solution was determined at 560 nm using UV−vis microplate reader (Thermo Fisher Labsystems Multiskan Plus). Cell adhesion was calculated for each peptide concentration considering cell adhesion in the absence of any peptide as 100%.

Peptide Cleavage in HUVEC Culture. Peptide cleavage by MMP-2 from HUVECs was analyzed using HPLC−MS and confirmed using a MMP-inhibitor, GM6001. HUVECs were trypsinized and resuspended in EGM-2, and 5 × 10^4 cells per well were plated in 48-well tissue culture-treated plates (BD Falcon, BD Biosciences, San Jose, CA). After overnight incubation, the cells were washed with DPBS and incubated with PMA/SF EGM-2 at 37 °C for 30 min. For GM6001 treated controls, the cells were further pretreated with 25 μM GM6001 at 37 °C for an additional 30 min. Cells were then incubated with 10 μM peptide at 37 °C for 24 h. At 24 h, supernatant medium was collected, filtered through 0.45 μm syringe filter (Millex- LH, Millipore, Billerica, MA) and analyzed by HPLC−MS using the method described. Cleavage was also followed by measuring fluorescence of FAM in the collected media samples using fluorescence plate reader.

Effect of Integrin Binding on Peptide Cleavage in HUVEC Culture. Cleavage of MMP-RGD in HUVECs was studied in the presence and absence of excess RGD-control peptide to evaluate the effect of integrin binding on the cleavage by MMP-2 from HUVECs. HUVECs were trypsinized and resuspended in EGM-2, and 5 × 10^4 cells per well were plated in 48-well tissue culture-treated plates. After overnight incubation, the cells were washed with DPBS and incubated with PMA/SF EGM-2 at 37 °C for 30 min. The cells were further pretreated with DPBS or 500 μM RGD-control at 37 °C for additional 30 min followed by addition of MMP-RGD at a final concentration of 50 μM. The cells were incubated with MMP-RGD at 37 °C for 2, 8, and 24 h. At each time point, supernatant medium was collected and filtered through 0.45 μm filter. MMP-RGD solutions prepared and treated similarly in the absence of cells were used as the uncleaved controls. The cleavage of MMP-RGD was detected by measuring the fluorescence of FAM at excitation and emission wavelengths of 490 and 535 nm using a fluorescence plate reader.

Effect of Peptides on HUVEC Viability. The effect of RGD-containing peptides on the viability of HUVECs under conditions similar to those used to test the effect of integrin binding on the peptide cleavage was examined using the CellTiter 96 AQ_{ueous} One Solution Cell Proliferation Assay (Promega, Madison, WI). HUVECs were trypsinized and resuspended in EGM-2, and 5 × 10^4 cells per well were plated in 48-well tissue culture-treated plates. After overnight incubation, the cells were washed with DPBS and incubated with PMA/SF EGM-2 at 37 °C for 30 min. Cells were further pretreated with DPBS or 500 μM RGD-control at 37 °C for an additional 30 min followed by addition of DPBS or MMP-RGD at a final concentration of 50 μM. The cells were incubated with the peptides at 37 °C for 24 h. After removal of the peptides, cells were incubated with a 1:5 mixture of

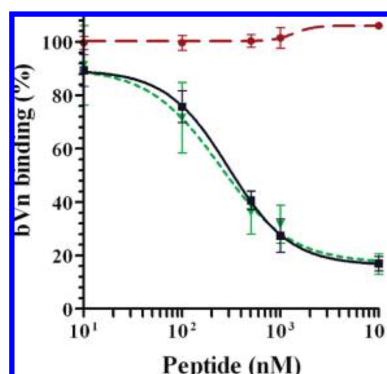

Figure 2. Binding of MMP-RGD (blue squares), RGD-control (green upside-down triangles) and MMP-control (red circles) to integrin αvβ3 as measured by competitive displacement of biotinylated vitronectin (bVn). MMP-RGD bound to integrin αvβ3 in a similar concentration dependent manner as RGD-control whereas MMP-control did not bind to integrin. (n = 3, average ± SD.)

CellTiter 96 AQ_{ueous} One Solution reagent (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium, inner salt; MTS) and SF EGM-2 at 37 °C for 3 h. The absorbance of the MTS formazan product formed due to chemical reduction of MTS by the live cells was measured at 492 nm using a UV−vis microplate reader (Thermo Fisher Labsystems, Multiskan Plus).

Results

Peptide Interaction with Purified Integrin αvβ3 and MMP-2. *Integrin αvβ3 Binding.* Both MMP-RGD and RGD-control inhibited binding of bVn to integrin αvβ3 in a concentration-dependent manner. No such inhibition was observed with MMP-control peptide that did not have the RGD sequence even at high concentration (Figure 2). There was no significant difference between IC_{50} values of MMP-RGD (0.272 ± 0.064 μM) and RGD-control (0.294 ± 0.114 μM) with a p-value of 0.848. These results confirmed that MMP-RGD peptide bound to integrin αvβ3 via the GRGDS motif and the attachment of MMP-2 substrate motif did not affect the binding of GRGDS motif with integrin αvβ3.

MMP-2 Cleavage. Intact MMP-RGD and the peptide fragments formed upon cleavage eluted with retention times of 16.4, 15.4, and 6.9 min, respectively. The fragments, FAM-GPLG (MW = 701) and VRGK(TAMRA)-Aeea-GRGDS (MW = 1485), were confirmed by mass spectroscopy of the eluted HPLC fractions (Figure 3). Similarly, MMP-control peptide and the peptide fragments, FAM-GPLG (MW = 701) and VRGK(TAMRA) (MW = 870), formed upon cleavage by MMP-2 eluted with retention times of 18.9, 15.4, and 9.0 min, respectively.

Cleavage of MMP-RGD and MMP-control peptides could be followed using FRET between FAM and TAMRA. Fluorescence of FAM increased when it was

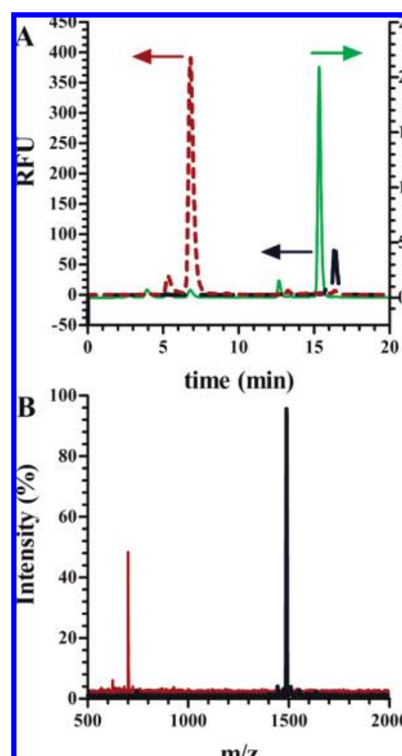

Figure 3. Chromatograms (A) and mass spectra (B) of the fragments formed upon cleavage of MMP-RGD by purified active MMP-2. Elution of uncleaved MMP-RGD (blue — —, 16.4 min) was monitored using λ_{Ex} 490 nm/λ_{Em} 570 nm. Elution of VRGK(TAMRA)-Aeea-GRGDS (red — —, 6.9 min) and FAM-GPLG (green —, 15.4 min) fragments was monitored using λ_{Ex} 540 nm/λ_{Em} 570 nm and λ_{Ex} 490 nm/λ_{Em} 535 nm, respectively. Fragments were confirmed to be FAM-GPLG (red —; MW = 701) and VRGK(TAMRA)-Aeea-GRGDS (black —; MW = 1485) by mass spectroscopy. (Data are representative of three independent experiments.)

separated from TAMRA upon cleavage of the peptides by MMP-2, and the observed cleavage was inhibited by MMP-inhibitor, GM6001 (Figure 4).

The cleavage kinetics of MMP-RGD peptide (V_{max}/K_M = 23.80 ± 1.42 ΔRFU/min/μM) was similar to that of the control MMP-2 substrate motif (V_{max}/K_M = 29.31 ± 3.35 ΔRFU/min/μM) with a p-value of 0.173 (Figure 5). Thus, the cleavage of MMP-2 substrate motif was not affected by the presence of linker and GRGDS motif in MMP-RGD.

Effect of Integrin Binding on MMP-2 Cleavage. Three concentrations of MMP-RGD (0.01, 0.3, and 10 μM) that had shown 10, 50 and 80% inhibition, respectively, of bVn binding to integrin αvβ3 were chosen. Inhibition of bVn binding to integrin αvβ3 by MMP-RGD was considered to be an indication of the extent of binding of MMP-RGD to integrin αvβ3; thus, greater inhibition indicated higher occupancy of RGD-binding integrin αvβ3 sites by MMP-RGD. The rate and extent of cleavage of MMP-RGD by MMP-2 in the integrin-coated wells was similar to that observed in the uncoated wells irrespective of the extent of

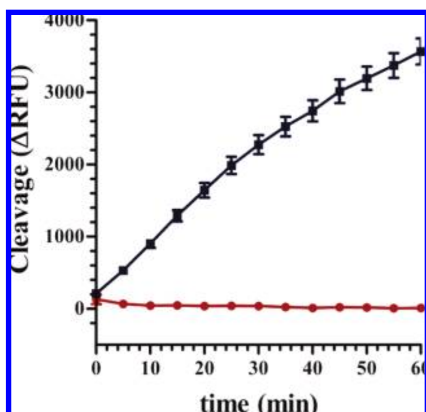

Figure 4. Fluorescence resonance energy transfer (FRET) property of MMP-RGD. Increase in fluorescence (ΔRFU) of FAM (λ_{Ex} 490 nm/λ_{Em} 535 nm) upon cleavage of MMP-RGD by MMP-2 (blue squares) and inhibition of the cleavage by MMP-inhibitor, GM6001 (red circles). Similar results were obtained for MMP-control (not shown). (n = 3, average ± SD.)

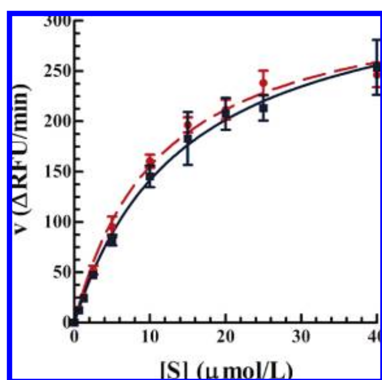

Figure 5. Michaelis–Menten plot modeling kinetics of cleavage of MMP-RGD (blue squares) and MMP-control (red circles) peptides by purified active MMP-2. Initial velocity of enzymatic cleavage of the peptides (v) was measured as increase in fluorescence (ΔRFU) of FAM (λ_{Ex} 490 nm/λ_{Em} 535 nm) per minute and plotted against substrate concentration [S]. Cleavage of MMP-RGD by active MMP-2 followed similar kinetics as that of MMP-control without RGD-motif. (n = 3, average ± SD.)

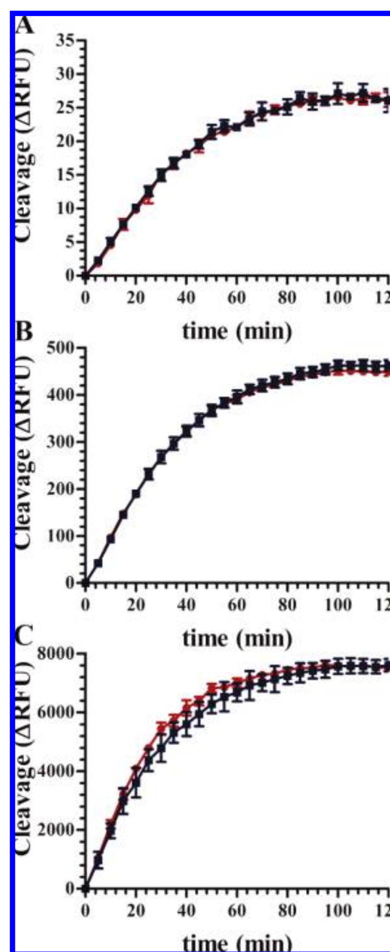

Figure 6. Effect of binding of MMP-RGD to immobilized integrin αvβ3 on the cleavage of MMP-RGD by purified active MMP-2. MMP-RGD (A, 0.01 μM; B, 0.3 μM; C, 10 μM) was added to uncoated (red circles) or integrin αvβ3-coated (blue squares) wells, followed by addition of active MMP-2, and the cleavage of MMP-RGD was followed by measuring increase in fluorescence (ΔRFU) of FAM (λ_{Ex} 490 nm/λ_{Em} 535 nm) over 2 h. (n = 3, average ± SD.)

peptide binding (Figure 6). Thus, there was no detectable effect of integrin αvβ3 binding on the cleavage of MMP-RGD by soluble MMP-2.

Interaction of Peptide with Integrin αvβ3 and MMP-2 in HUVEC Culture. *HUVECs as a Model of Angiogenic Endothelial Cells.* HUVECs cultured on tissue culture treated plastic do not endogenously activate proMMP-2 (Figure 7, PMA −); hence, HUVECs treated with phorbol ester (PMA), an inducer of MMP-2 activation, were used as the cell culture model of angiogenic endothelial cells. Active MMP-2 was present in the conditioned media and cell lysates obtained from HUVECs treated with PMA at 24 h (Figure 7). Active MMP-2 in the cell lysates confirmed the presence of cell-associated active MMP-2 on the surface of PMA-treated HUVECs at 24 h. Since proMMP-2 is activated on the external cell surface, a significant fraction of the cell-associated active MMP-2 was expected, and is observed, to be present on the cell surface. When quantified, the active MMP-2 from the cell lysate of 24 h PMA-treated HUVECs was significantly higher than that from the respective conditioned medium sample (p-value = 0.042, Figure 8), indicating a higher concentration of active MMP-2 at the cell surface than in the culture medium at 24 h post-PMA treatment of HUVECs. Thus, even though active MMP-2 was detected in the conditioned medium after treating HUVECs with PMA for 24 h, MMP-2 activity was concentrated at the cell surface. Integrin αvβ3 is present on the surface of angiogenic endothelial cells,[7] and integrin αvβ3 was confirmed to be present on PMA-treated HUVECs (Figure 9).

Peptide Binding to HUVECs. MMP-RGD showed concentration-dependent inhibition of adhesion of HUVECs to vitronectin

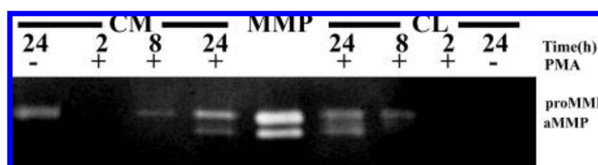

Figure 7. Analysis of pro and active MMP-2 in the culture of PMA-treated HUVECs. Gelatin zymography was used to analyze pro and active forms of MMP-2 in the conditioned media (CM) and cell lysates (CL) obtained from HUVECs cultured in serum-free media for 24 h (PMA −) and HUVECs treated with PMA for 2, 8, and 24 h (PMA +). Equal volumes and equal amounts of protein were loaded for CM and CL, respectively. Human recombinant proMMP-2 (upper band) and active MMP-2 (lower band) were used as standards (MMP/center lane). Zymogram is representative of three independent experiments.

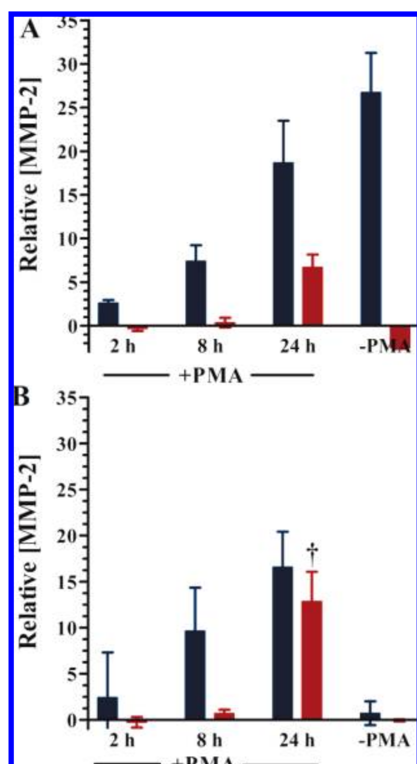

Figure 8. Quantification of pro and active MMP-2. The gelatin zymogram bands of proMMP-2 (dark blue) and active MMP-2 (red) from the conditioned media (CM, A) and cell lysates (CL, B) obtained from HUVECs cultured in serum free media for 24 h (−PMA) and HUVECs treated with PMA for 2, 8, and 24 h (+PMA) were quantified as the integrated band density per unit sample volume after normalizing for the total protein content of the cell lysates. (†, $p < 0.05$ compared to the CM +PMA 24 h active MMP-2 group; $n = 3$, average ± SD.)

above 10 μM (Figure 10). Effect of MMP-RGD and RGD-control on adhesion of HUVECs to vitronectin was similar at all concentrations tested, confirming that MMP-2 substrate motif did not affect the binding of MMP-RGD to HUVECs.

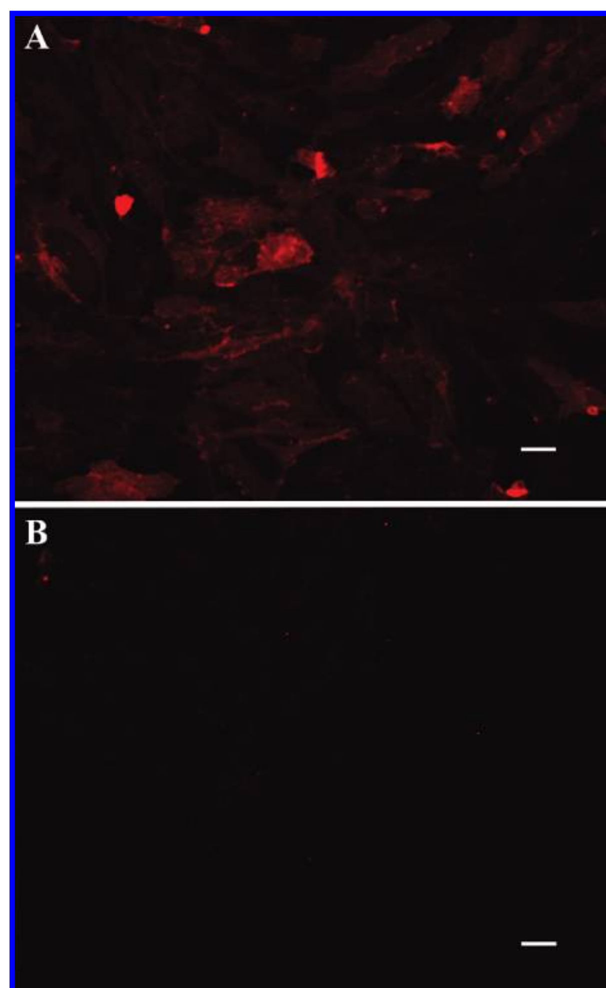

Figure 9. Expression of integrin $\alpha v\beta 3$ on the surface of PMA treated HUVECs. Immunofluorescence microscopy was used to confirm expression of integrin $\alpha v\beta 3$ on the surface of (A) PMA-treated HUVECs using a phycoerythrin-labeled mouse antihuman integrin $\alpha v\beta 3$ monoclonal antibody, PE-LM609. (B) PMA-treated HUVECs stained with phycoerythrin-labeled mouse isotype control IgG1 were used as negative control. Images are representative fields from one of three experiments. Scale bar = 50 μm.

Peptide Cleavage by HUVECs. For HUVECs treated with PMA and exposed to the MMP-RGD peptide for 24 h, the supernatant media showed the presence of the cleaved fragment FAM-GPLG (MW = 701). Cleavage of MMP-RGD by HUVECs could also be followed by measuring increase in fluorescence of FAM in the supernatant media (Figure 11). Cleavage of MMP-RGD by PMA-treated HUVECs observed at 24 h was inhibited in the presence of GM6001 (p-value equal to 0.041). Fluorescence of GM6001 treated culture medium containing MMP-RGD was similar to that measured with the uncleaved control (p-value equal to 0.44). The expected fragment formed by MMP-2 cleavage of MMP-RGD was observed by MS, which suggested

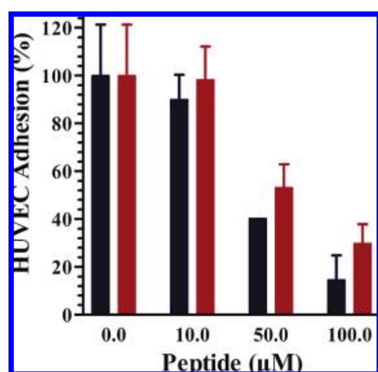

Figure 10. Binding of MMP-RGD to integrin αvβ3 on the surface of HUVECs assessed by cell adhesion assay. Inhibition of adhesion of the PMA-treated HUVECs to vitronectin by increasing concentrations of MMP-RGD (dark blue) and RGD-control (red) was measured. No statistically significant difference was observed between percent cell adhesion values obtained with MMP-RGD and RGD-control, *p*-value of greater than 0.05 at all concentrations. ($n = 3$, average ± SD.)

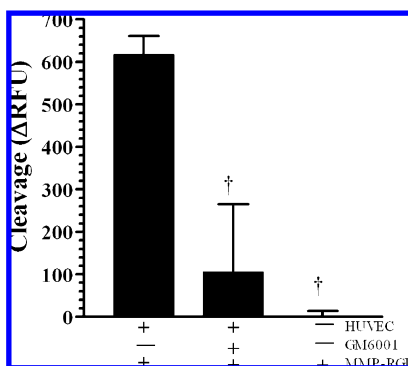

Figure 11. Cleavage of MMP-RGD by MMP-2 from PMA-treated HUVECs. PMA-treated HUVECs were incubated with 10 μM MMP-RGD in the presence or absence of MMP-inhibitor, GM6001, where † indicates *p* less than 0.05 compared to the first group. Cleavage of MMP-RGD by cells was monitored by measuring increase in fluorescence (ΔRFU) of FAM (λ_{Ex} 490 nm/ λ_{Em} 535 nm) in the supernatant media. ($n = 3$, average ± SD.)

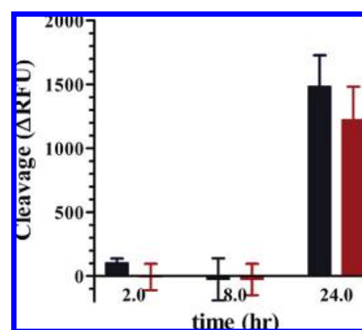

Figure 12. Effect of integrin binding on the cleavage of MMP-RGD by PMA-treated HUVECs. Cleavage of 50 μM MMP-RGD by active MMP-2 from PMA-treated HUVECs was examined in the absence (dark blue) and presence (red) of 500 μM RGD-control. Cleavage of MMP-RGD by HUVECs was not altered by blocking integrin binding with excess RGD-control. ($n = 3$, average ± SD.)

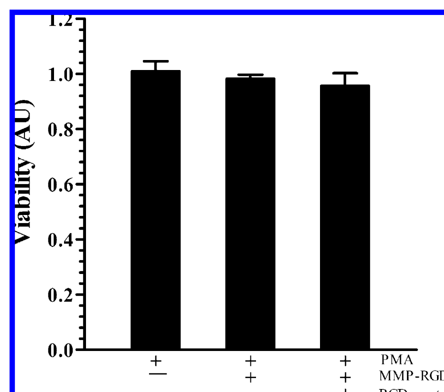

Figure 13. Effect of RGD-containing peptides on the viability of PMA-treated HUVECs. Viability of PMA-treated HUVECs was examined after 24 h treatment with 50 μM MMP-RGD in the presence or absence of 500 μM RGD-control. The RGD-containing peptides did not have any significant effect on the viability of HUVECs at the concentrations that were used to assess the effect of integrin binding on MMP-RGD cleavage. ($n = 3$, average ± SD.)

cleavage by MMP-2. In addition, inhibition of the cleavage by a MMP-inhibitor further confirmed that MMP-RGD was cleaved by MMP-2 from HUVECs.

Effect of Integrin Binding on the Cleavage by HUVECs. Blocking integrin binding by 500 μM RGD-control peptide did not affect the cleavage of MMP-RGD by PMA-treated HUVECs at 2, 8, or 24 h as measured by the increase of FAM fluorescence in the culture medium (*p*-value equal to 0.11, 1.00, and 0.19 at 2, 8, and 24 h, respectively, Figure 12). These results indicated that integrin binding did not enhance the cleavage of MMP-RGD by MMP-2 from HUVECs as expected. Further, it should be noted that significant cleavage was not observed until 24 h as would be expected from the activation of MMP (Figures 7 and 8).

Effect of Peptides on HUVEC Viability. Incubation of PMA-treated HUVECs with 50 μM MMP-RGD in the presence or absence of 500 μM RGD-control peptide for 24 h did not influence cell viability (*p*-value = 0.17 and 0.11 for MMP-RGD alone and MMP-RGD with RGD-control, respectively, compared to no peptide treatment, Figure 13). Thus, the viability of PMA-treated HUVECs was not decreased by the test MMP-RGD peptide or excess RGD-control peptide used to block the integrin binding of MMP-RGD. These results ruled out the possibility of differential cell viability being a reason behind the observation that blocking integrin binding by 500 μM RGD-control peptide did not affect the cleavage of MMP-RGD by PMA-treated HUVECs as described above.

Discussion

Co-overactivity and proximity of integrin $\alpha v\beta 3$ and MMP-2 on the endothelial cell surface suggests that there should be linkage of their functions. As one possible link, integrin binding of MMP-2 substrate was hypothesized to increase the cleavage efficiency by MMP-2. With this hypothesis in mind, we used an integrin binding MMP-2 substrate peptide to assess functional association of integrin $\alpha v\beta 3$ and MMP-2 for MMP-2 substrate cleavage.

Combining MMP-2 substrate and GRGDS motifs in MMP-RGD did not affect their interaction with purified MMP-2 and integrin $\alpha v\beta 3$, respectively. Maintenance of substrate and ligand properties was important for assessing MMP-RGD as a tool to analyze functional association of MMP-2 and integrin $\alpha v\beta 3$. Binding of MMP-RGD with immobilized $\alpha v\beta 3$ did not have any detectable effect on MMP-RGD cleavage by soluble active MMP-2. Integrin-binding did not cause a major change in presentation of the cleavage site of the peptide to soluble active MMP-2. Since active MMP-2 is bound to the surface of invasive integrin $\alpha v\beta 3$-expressing cells,[26,30] whether surface-bound MMP-2 can alter cleavage of substrates remains to be investigated. The cell membrane receptor for active MMP-2 has not been unambiguously confirmed, and several receptors are known;[35,44,45] therefore, it was not possible to study all receptor-bound active MMP-2 at this time. MMP-2 has been proposed to interact with integrin $\alpha v\beta 3$ on the surface of invasive cells.[32] The binding of MMP-2 to integrin may limit the cleavage of substrate that is bound to the same receptor. Alternatively, MMP-2 bound to other receptors may not gain access to the integrin-bound substrate. Continued efforts to verify the membrane receptor that holds active MMP-2 at the cell surface are required so that substrate cleavage by receptor bound conformation of active MMP-2 can be examined. At this point, cultured cells induced to activate MMP-2 were deemed the best available *in vitro* model for examining proteolytic cleavage by the cell surface receptor-bound active MMP-2.

Phorbol ester (PMA) treated HUVECs have been shown to present the migratory phenotype of angiogenic endothelial cells. Treatment of HUVECs with PMA caused activation of integrin $\alpha v\beta 3$ receptors and proMMP-2 on the cell surface.[43,46,47] Confirmation of the presence of integrin $\alpha v\beta 3$ and active MMP-2 on the cell surface in our laboratory validated PMA-treated HUVECs as cell culture model. MMP-RGD bound to integrin $\alpha v\beta 3$ on the surface of HUVECs with affinity similar to that of RGD-control peptide. MMP-RGD was also cleaved by active MMP-2 from PMA-treated HUVECs.

We proposed that binding of MMP-RGD to the surface of HUVECs would increase local concentration of the MMP-2 substrate motif thereby increasing the probability of interaction between the substrate and active MMP-2 on the cell surface. Integrin $\alpha v\beta 3$ binding was also proposed to make the MMP-2 substrate more accessible to the colocalized MMP-2. Hence, we hypothesized that integrin-binding can enhance efficiency of MMP-RGD cleavage by active MMP-2 from HUVECs. Cleavage of MMP-RGD by MMP-2 from PMA-treated HUVECs was detected at 24 h but not at 2 and 8 h after incubation of the peptide with the cells. Similar results were obtained when the integrin-binding was blocked with excess RGD-control peptide. It was also confirmed that treatment of PMA-treated HUVECs with these RGD-containing peptides did not affect the cell viability thereby allowing direct comparison of the MMP-RGD cleavage with or without blocking the integrin-binding. Active MMP-2 was detected in cell lysate as well as conditioned medium at 24 h post-PMA treatment of HUVECs. Also, concentration of active MMP-2 in the cell lysate was significantly higher than in the conditioned medium at this time. Presence of active MMP-2 in the cell lysate indicated active MMP-2 on the cell surface. Thus, in spite of the concentration of MMP-2 activity on the surface of PMA-treated HUVECs, cleavage of MMP-RGD observed at 24 h was not affected by blocking integrin-binding with excess RGD-control peptide; integrin binding did not enhance the cleavage of MMP-RGD by active MMP-2 in the cell model.

Localization of MMP-2 cleavable prodrugs on the cell surface has been previously shown to aid in the activation of prodrugs caused by MMP-2 cleavage.[48,49] In one example, targeting of MMP-2 cleavable tumor necrosis factor (TNF) prodrug to the surface of human fibrosarcoma (HT-1080) cells using single-chain antibody variable fragment binding fibroblast activation protein on the surface of HT-1080 cells was required for activation of TNF.[48] Various reasons, as outlined below, can explain the lack of similar enhancement

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of the cleavage of MMP-2 substrate by HUVECs due to integrin binding.

We have shown that the binding of MMP-RGD to purified integrin αvβ3 does not affect cleavage of the peptide by soluble purified active MMP-2. However, it is possible that integrin-binding affects presentation of the peptide cleavage site to active MMP-2 bound to cell surface integrin αvβ3 or another currently unknown receptor. In such a scenario, the cell surface localized active MMP-2 (mechanisms 1 and 3 in Figure 1) may not contribute significantly to the cleavage of integrin-bound peptide and hence will not result in enhancement of the cleavage. Altering the length and type of linker between integrin-binding and MMP-2 substrate motifs would allow one to probe into optimum presentation of the peptide for cleavage by cell-bound active MMP-2. A shorter linker to position the substrate closer to the MMP-2 on the cell surface or longer linker to offer the flexibility required for accessing MMP-2 may be required. Also, the unbound form of the peptide in the supernatant culture medium may be more easily accessible and cleavable by either the cell-bound or soluble MMP-2. In such a case, cleavage of the unbound peptide (mechanisms 4, 5, and 6 in Figure 1) may dominate over the cleavage of cell-bound peptide (mechanisms 1, 2, and 3 in Figure 1).

Another plausible explanation is that the binding of linear GRGDS motif to the surface of HUVECs may not be strong enough to tightly hold MMP-RGD molecules on the surface. However, MMP-2 cleavable and noncleavable doxorubicin-conjugates that contained divalent cyclic RGD peptides help to rule out this possibility.[36] Divalent cyclic RGD peptides have higher affinity for integrin αvβ3 compared to linear RGD.[50,51] No increase in antiproliferative activity of MMP-2 cleavable doxorubicin conjugate was observed due to the presence of integrin binding divalent cyclic RGD peptide. Lack of MMP-based increased activity suggests that the MMP-2 activity was not altered when the integrin binding affinity was increased.

Although integrin αvβ3 binding did not enhance the cleavage of MMP-2 substrate under our experimental conditions, it must be noted that the two molecular targets may collaborate by other mechanisms as well. Integrin binding can provide a mechanism for homing MMP-2 cleavable peptide prodrug to angiogenic vasculature *in vivo* thereby preventing nonlocal activation. Also, multiple therapeutics with different mechanisms of action can be attached to the two motifs of the peptide, for example, an antiangiogenic agent to integrin binding motif and an anticancer drug to MMP-2 cleavable motif.

In summary, integrin-binding and MMP-2 substrate motifs when combined in a single peptide retained their individual interactions with integrin αvβ3 and active MMP-2. However, our results described herein did not show alteration of MMP-2 substrate cleavage due to integrin αvβ3 binding despite the previously described colocalization of two target proteins on the surface of endothelial cells. The results implicate that integrin αvβ3 and active MMP-2 may not be functionally collaborative molecular targets in a way that is speculated based simply on their co-overexpression on the surface of angiogenic endothelial cells. MMP-2 substrate cleavage is not enhanced by increasing local concentration of the MMP-2 substrate available to active MMP-2 on the cell surface due to integrin αvβ3 binding of the substrate. It is possible that a specific spatial orientation is required between integrin αvβ3-targeting component and MMP-2 substrate component to obtain the MMP-2 substrate cleavage enhancement due to its integrin αvβ3 binding on the cell surface. Further assessment of functional collaboration of integrin αvβ3 and MMP-2 is suggested, but greater understanding of the molecular positioning of MMP-2 in relation to integrin αvβ3 is needed to design the therapeutic systems that can exploit the proposed collaborating targets.

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