Biochemical and Biophysical Research Communications 460 (2015) 596–602

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Fluid shear stress promotes proprotein convertase-dependent activation of MT1-MMP

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ARTICLE INFO

Article history:
Received 6 March 2015
Available online 20 March 2015

Keywords:
Angiogenesis
Mechanotransduction
Sprouting
Furin

ABSTRACT

During angiogenesis, endothelial cells (ECs[1]) initiate new blood vessel growth and invade into the extracellular matrix (ECM). Membrane type-1 matrix metalloproteinase (MT1-MMP) facilitates this process and translocates to the plasma membrane following activation to promote ECM cleavage. The N-terminal pro-domain within MT1-MMP must be processed for complete activity of the proteinase. This study investigated whether MT1-MMP activation was altered by sphingosine 1-phosphate (S1P) and wall shear stress (WSS), which combine to stimulate EC invasion in three dimensional (3D) collagen matrices. MT1-MMP was activated rapidly and completely by WSS but not S1P. Proprotein convertases (PCs) promoted MT1-MMP processing, prompting us to test whether WSS or S1P treatments increased PC activity. Like MT1-MMP, PC activity increased with WSS, while S1P had no effect. A pharmacological PC inhibitor completely blocked S1P- and WSS-induced EC invasion and MT1-MMP translocation to the plasma membrane. Further, a recombinant PC inhibitor reduced MT1-MMP activation and decreased lumen formation in invading ECs, a process known to be controlled by MT1-MMP. Thus, we conclude that PC and MT1-MMP activation are mechanosensitive events that are required for EC invasion into 3D collagen matrices.

1. Introduction

Angiogenesis is the formation of new capillaries from pre-existing vessels and is important during physiological and pathological events such as wound healing, embryonic development, the female reproductive cycle, and tumor vascularization [1]. During angiogenesis, endothelial cells (ECs) respond to biochemical factors such as vascular endothelial growth factor (VEGF) [2], basic fibroblast growth factor (bFGF) [3], placental growth factor [4], and sphingosine 1-phosphate (S1P) [5], as well as mechanical shear forces created by blood flow [6]. Wall shear stress (WSS) rates have been estimated at 1–8 dyn/cm^2 for the microcirculation, the site of angiogenic initiation [7]. Although multiple signaling pathways are activated by growth factors, lipids, and mechanical forces [4,6,8,9], the precise underlying signals and intracellular events that control EC sprouting in response to shear forces remain incompletely understood.

Membrane type-1 matrix metalloproteinase (MT1-MMP) is a member of the matrix metalloproteinase family of enzymes and is vital during angiogenesis. Mice deficient in MT1-MMP demonstrate defective vascular infiltration of cartilage [10] and corneal angiogenesis [10]. Also, MT1-MMP exclusively promoted endothelial-dependent vessel formation *in vitro* and *in vivo* [11], indicating a clear requirement for MT1-MMP in initiating new blood vessel growth. In addition, MT1-MMP is required for EC tubulogenesis and lumen formation [12–15]. Together, these studies establish a critical role for MT1-MMP in angiogenic responses.

To be functional, the propeptide sequence of MT1-MMP must be removed by one of several intracellular proprotein convertases (PCs), which include furin, PC1/3, PC2, PACE4, PC4, PC5/6, and PC7 [16,17]. Of these, furin, PC5/6, PC7, and PACE4 cleave the first 111 amino acids of MT1-MMP at a defined site (Arg-Arg-Lys-Arg^{111}) to unmask the catalytic domain and promote surface localization of MT1-MMP [18,19]. Furin, PC1, PC6, and PC7 mRNA have been

Abbreviations: ECs, endothelial cells; MT1-MMP, membrane type-1 matrix metalloproteinase; ECM, extracellular matrix; WSS, wall shear stress; S1P, sphingosine 1-phosphate; PC, proprotein convertase; 3D, three-dimensional; HUVEC, human umbilical vein endothelial cell; GFP, green fluorescent protein; GAPDH, glyceraldehyde phosphate dehydrogenase; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor.

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http://dx.doi.org/10.1016/j.bbrc.2015.03.075
0006-291X/

detected in ECs [20], and furin colocalized with MT1-MMP in the trans-Golgi network [21]. Although it is well-recognized that MT1-MMP promotes vessel outgrowth and lumen formation [11,13] and proprotein convertases activate MT1-MMP [18,19], it is not known whether WSS affects MT1-MMP activation. We observed here that WSS activated proprotein convertases, and proprotein convertases activated MT1-MMP to facilitate EC invasion of 3D collagen matrices.

2. Materials and methods

2.1. Endothelial cell invasion stimulated by shear stress

Human umbilical vein endothelial cells (HUVECs) (Lonza Bio-Products) were used at passage 4–6. Collagen type I was isolated as previously described [22]. Experiments applying 1 μM S1P and 5.3 dyn/cm^2 WSS were conducted as previously described [23,24].

2.2. Quantification of endothelial sprouting responses stimulated by shear stress

Invading cell cultures were fixed in 3% glutaraldehyde in PBS overnight and stained (15 min) with 0.1% toluidine blue in 30% methanol. Invasion density was quantified as the average number of structures invading beneath the monolayer per standardized 1 mm^2 field ($n > 3$ fields). The percentage of cells forming lumens and the lumen diameter were quantified from images taken of a side view of invasion. For each treatment group, $n > 100$ cells were measured.

2.3. Proprotein convertase inhibition

ECs were pre-incubated with 25 μM of proprotein convertase inhibitor, decanoyl-RVKR-chloromethylketone (ALX-260-022, Enzo) or vehicle control (DMSO) during attachment to collagen matrices (1 h) and for the duration of WSS application (24 h).

2.4. Proprotein convertase activity

Cells were seeded on polymerized collagen matrices containing S1P, exposed to 5.3 dyn/cm^2 WSS, and allowed to invade for 3 h before homogenization in lysis buffer [100 mM HEPES (pH 7.5), 0.5% TX-100, 1 mM $CaCl_2$, 1 mM 2-mercaptoethanol, Complete Protease Inhibitor Cocktail (Roche), and Halt Phosphatase Inhibitor Cocktail (Pierce)] at 4 °C for 10 min. Samples were vortexed every 5 min for 20 min and centrifuged at 13,000 × g at 4 °C for 10 min. Supernatants were collected and stored at −80 °C until use. Assay buffer, fluorogenic substrate peptides (Boc-RVRR-AMC, ALX-260-040, Enzo), and reactants were prepared according to manufacturer's instructions and measured for fluorescence intensity at excitation/emission wavelengths of 380(±20 nm)/520(±20 nm) using a Victor X3 plate reader (PerkinElmer Life Sciences) in triplicate wells.

2.5. Immunoblotting

Immunoblotting was conducted as previously described [23,24]. Band intensities were measured using ImageJ. Antisera used in this study were raised against GAPDH (ab8245, Abcam), MT1-MMP (SC30074, Santa Cruz; MAB3328, Millipore), Zyxin (cs3553, Cell Signaling), and antitrypsin (ab9400, Abcam). In all figures, densitometric analyses of band intensities were compiled from 3 independent experiments. Pro and active MT1-MMP levels were normalized to GAPDH. Data presented are mean values ±S.D.

2.6. Cell transfection and immunofluorescence analyses

Transient transfections of MT1-MMP-GFP expression plasmids were performed and quantified as previously described [24,25]. Transfection efficiency was approximately 20%. ECs were serum-starved for 1 h and then untreated or treated with the proprotein convertase inhibitor in the presence of 1 μM S1P and WSS for 2 h. Cells were fixed in 4% paraformaldehyde before quantifying MT1-MMP-GFP localization to the cell periphery as previously described [24,25]. Data shown were averaged from 3 experiments ($n = 39$ cells total/group).

2.7. Proprotein convertase inhibitor cloning and lentiviral transduction

Full-length α1-antitrypsin (α_1-AT), α1-antitrypsin variant Pittsburgh (α_1-PIT), and α_1-antitrypsin variant Portland (α_1-PDX) constructs were kind gifts from Gary Thomas (Oregon Health Sciences University) [26]. The inserts were subcloned into the pIEx-5 vector (Novagen) using the *Acc65I* and *HindIII* sites, generating a C-terminal S-tag. Positive clones confirmed by sequence analysis were subcloned into the pENTR4 vector (Invitrogen) using the *Acc65I* and *XhoI* sites and recombined into the pLenti6/V5 DEST vector (Invitrogen) using the GATEWAY system. Lentiviruses were generated as previously described [24,25]. ECs were transduced for 3d and selected with blasticidin (1 μg/ml) for 8d. Blasticidin was removed for 24 h and invasion assays were conducted.

2.8. Statistical analyses

All data are presented as the mean ± standard deviation (S.D.) or standard error of the mean (SEM) for each group. Individual statistical analyses were performed using SAS software. Lowercase letters denote groupings from one-way ANOVA followed by post hoc pairwise comparison testing using Tukey's method ($p < 0.05$).

3. Results

3.1. MT1-MMP activation increases with time during EC invasion in 3D collagen matrices

We have shown that surface translocation of MT1-MMP facilitates EC invasion in multiple assay systems [24,25]. However, whether WSS directly affects MT1-MMP activation has not been investigated. We used an established model [23,24], where 5.3 dyn/cm^2 WSS combined with 1 μM S1P promoted EC sprouting. Robust sprouting required S1P and WSS to promote EC invasion of 3D collagen matrices (Fig. 1A). Extracts were collected from invading cultures at 0, 1, 6, 12, and 18 h following stimulation with S1P and WSS. MT1-MMP was converted from the pro (63 kDa) to an active, lower molecular weight form (60 kDa) almost completely by 12 h (Fig. 1B). Quantification of band intensities from multiple experiments revealed a significant decrease in pro MT1-MMP with time in S1P- and WSS-treated ECs (Fig. 1C), which corresponded with an increase in active MT1-MMP over time (Fig. 1D). To test the effects of S1P and WSS separately, ECs were treated with or without WSS in the presence or absence of S1P. We observed increased active MT1-MMP in all groups exposed to WSS, while S1P appeared to have no effect on MT1-MMP activation (Fig. 2A). Quantification of both pro and active forms of MT1-MMP revealed that pro MT1-MMP levels were decreased significantly at 2 h compared to control treatment (Fig. 2B). At 6 h WSS treatment, pro MT1-MMP was nearly undetectable (Fig. 2B). In accordance with decreased levels of pro MT1-MMP, active MT1-MMP levels increased significantly with 2 h and 6 h WSS treatment compared to controls (Fig. 2C). No