

Endothelial semaphorin 7A promotes seawater aspiration-induced acute lung injury through plexin C1 and $\beta 1$ integrin

MINLONG ZHANG^{1*}, XUE YAN^{2*}, WEI LIU¹, RUILIN SUN¹, YONGHONG XIE¹ and FAGUANG JIN¹

¹Department of Respiration, Tangdu Hospital, Fourth Military Medical University, Xi'an, Shaanxi 710038;

²School of Medicine, Xianyang Vocational and Technical College, Xianyang, Shaanxi 712000, P.R. China

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Abstract. Inflammation and edema are two main characteristics in seawater aspiration-induced acute lung injury (ALI). In a previous study of the authors, it was demonstrated that endothelial semaphorin 7A (SEMA7A) serves an important role in the development of seawater-induced inflammation and edema. However, the mechanism of endothelial SEMA7A-mediated ALI remains unclear. Therefore, the authors explored the effect of SEMA7A in rat pulmonary microvascular endothelial cells (RPMVECs) and the interaction between endothelial SEMA7A and alveolar macrophages during seawater aspiration-induced ALI. The role of SEMA7A in endothelial permeability was detected using plexin C1 blocking antibody or SEMA7A small interfering (si)RNA. In addition, RPMVECs were co-cultured with rat alveolar macrophage cell line-NR8383 cells and pro-inflammatory cytokine production was detected. Interaction between the $\beta 1$ integrin and SEMA7A was detected using the $\beta 1$ integrin blocking antibody or SEMA7A siRNA. Seawater stimulation induced endothelial cytoskeleton remodeling, endothelial permeability, phosphorylation of cofilin, and increased the vascular endothelial growth factor (VEGF) expression in RPMVECs. Moreover, seawater stimulation led to expression of proinflammatory cytokines and activated the nuclear factor- κ B pathway in co-cultured cells. However, blockage with the plexin C1 antibody inhibited endothelial cytoskeleton remodeling, endothelial permeability, phosphorylation of cofilin, and treatment with SEMA7A siRNA inhibited expression of VEGF in RPMVECs. In addition, blockage with $\beta 1$ integrin antibody reduced expression of proinflammatory cytokines and inhibited activation of NF- κ B in co-culture

cells. These results suggest that SEMA7A promotes seawater induced lung edema via plexin C1 and stimulates seawater induced lung inflammation via $\beta 1$ integrin.

Introduction

Seawater drowning is a common accident and the injuries following drowning have caused serious social and public medical problems. Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common complications (1). Inflammation and lung epithelial-endothelial barrier injury are two main characteristics in seawater aspiration induced ALI (2). In seawater aspiration-induced ALI animal models, pulmonary inflammation is induced by infiltrated inflammatory cells. It has been reported that hypoxia-enhanced expression of the nuclear factor (NF)- κ B is important in the release of pro-inflammatory cytokines (3,4). In addition, several studies also indicated that the increased pulmonary endothelial permeability triggered the infiltration of inflammatory cells and edema fluid (2,5).

Semaphorins consist of a large class of membrane-anchored or secreted proteins that are divided into 8 subclasses on account of structural features and sequence similarities (6-8). Semaphorin 7A (SEMA7A) is a membrane-associated GPI-linked protein and it can also be present in the form of secretion. Originally, SEMA7A was identified as neuronal guidance proteins (7). Recent studies demonstrated that SEMA7A has the effect of promoting the production of cytokines in inflammatory cells (9,10). In addition, SEMA7A also has the effect of reorganizing the cytoskeleton in several cell types and this effect is necessary for cell spreading and migration (11-13). SEMA7A can interact with two different receptors, the $\beta 1$ integrin and plexin C1. Several studies have shown that $\beta 1$ integrin was associated with immunomodulatory and inflammation function (14,15). However, plexin C1 was associated with cell adhesion, dendricity and cytoskeletal reorganization (16,17).

Earlier work from this laboratory (Respiratory Medicine Laboratory, Tangdu Hospital, Fourth Military Medical University, Shaanxi, China) determining that seawater administration increased the expression of SEMA7A both in lung tissue and RPMVECs and suppression of SEMA7A inhibited inflammation process and pulmonary edema by ameliorating endothelial barrier permeability (18). Moreover, it was also

Correspondence to: Dr Faguang Jin or Dr Yonghong Xie, Department of Respiration, Tangdu Hospital, Fourth Military Medical University, 1 Xinsi Road, Xi'an, Shaanxi 710038, P.R. China
E-mail: jinfag@fmmu.edu.cn
E-mail: xieyh65@fmmu.edu.cn

*Contributed equally

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identified that seawater stimulation induced HIF-1 α led to the expression of SEMA7A. Although SEMA7A was shown to be critical in the development of lung inflammation and pulmonary edema in seawater aspiration-induced ALI, the specific mechanism of molecular interaction is not clear.

Therefore, the authors studied the effect of interaction between endothelial SEMA7A and plexin C1 in RPMVECs permeability and explored the interaction between endothelial SEMA7A and β 1 integrin in NR8383 cells. In addition, the changes of inflammation and cell transmigration related signal pathways were also investigated. The result of the present study suggested that, in seawater aspiration-induced ALI, endothelial SEMA7A promoted the increasing of endothelial barrier permeability and induced the expression of endothelial permeability related protein vascular endothelial growth factor (VEGF) via interaction with plexin C1. Furthermore, the co-culture experiments indicated that endothelial SEMA7A stimulated release of pro-inflammatory cytokines by alveolar macrophages and activated NF- κ B pathway via interaction with β 1 integrin.

Materials and methods

Animals preparation. Sprague-Dawley (SD) rats (male, 5-7 weeks old, 200 \pm 20 g; n=32) were obtained from the Animal Center of Fourth Military Medical University (Xi'an, China). The feeding environment of rats includes temperature-controlled house with 12 h light-dark cycles, free access to standard laboratory diet and water *ad libitum*. All the animal experiments were approved by the Animal Care and Use Committee of the Fourth Military Medical University and in accordance with the Declaration of the National Institutes of Health Guide for Care and Use of Laboratory Animals (Bethesda, MD, USA; publication No. 85-23, revised 1985).

Drug and reagents. Seawater (osmolality 1,300 mmol/l, pH 8.2, relative density 1.05, NaCl 6.518 g/l, MgSO₄ 3.305 g/l, MgCl₂ 2.447 g/l, CaCl₂ 1.141 g/l, KCl 0.725 g/l, NaHCO₃ 0.202 g/l, NaBr 0.083 g/l, diluted by deionized water) was obtained according to the major composition of the East China Sea provided by Chinese Ocean Bureau (Beijing, China). ELISA kits for tumor necrosis factor (TNF)- α (cat. no. PRTA00) and interleukin (IL)-1 β (cat. no. PRLB00) were obtained from R&D Systems, Inc. (Minneapolis, MN, USA). Recombinant rat SEMA7A (rSEMA7A) was obtained from R&D Systems, Inc. Antibody for plexin C1 (cat. no. AF5375) and β 1 integrin (cat. no. AF2405) were obtained from R&D Systems, Inc. Anti-NF- κ B p65 (cat. no. 3033), anti-pNF- κ B p65 (cat. no. 3036), anti-p-cofilin (cat. no. 5175), anti-cofilin (cat. no. 3313) antibodies were obtained from Cell Signaling Technology, Inc. (Cell Signaling Technology, Inc., Danvers, MA, USA). The anti- β -actin (cat. no. sc-47778) antibody were obtained from Santa Cruz Biotechnology, Inc. (Dallas, TX, USA). Endothelial cell growth supplement was obtained from Upstate Biotechnology, Inc. (Lake Placid, NY, USA).

RPMVEC culture and siRNA transfection. Primary RPMVEC isolation and culture were performed according to previous methods with some modification (19). Firstly, the pleura and outer edges of washed (with PBS) fresh rat

lung lobe were cut off. Then, the 1.5 mm³ specimens of tissue cut from lung surface were carefully plated into cell culture dishes (containing DMEM supplemented with 20% FBS, 25 μ g/ml endothelial cell growth supplement and 100 U/ml penicillin-streptomycin). These tissues were cultured at 37°C in a humidified atmosphere with 5% CO₂ and 95% air. The residue specimens were removed following 60 h. The cells were passed (with 0.25% trypsin) when monolayer cells were achieved and all experimental cells were between passage 2 and 3. Primary RPMVECs were identified according to their characteristic morphology and staining with anti-CD31 antibody (18). In some of these experiments, RPMVECs were pre-treated with or without anti-plexin C1 antibody (5 μ g/ml) or isotype antibody (5 μ g/ml) for 1 h at 37°C before stimulation. The plexin C1 antibody based blocking was performed as previously described (14). SEMA7A small interfering (si)RNA (the sequence is presented in Table I) and scramble control siRNA were obtained from GeneChem Co., Ltd. (Shanghai, China). The siRNA was transiently transfected into RPMVECs with Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to manufacturer's instructions and suppression efficiency was detected by reverse transcription-quantitative polymerase chain reaction. Total RNA was extracted with TRIzol reagent (cat no. 9108; Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. RNA concentration was tested by spectrometric analysis. The PrimeScript RT reagent kit (cat no. RR037A; Takara Biotechnology Co., Ltd.) and the One Step SYBR PrimeScript RT-PCR kit (cat no. RR066A; Takara Biotechnology Co., Ltd.) were used. SEMA7A and β -actin were examined using a Real Time PCR instrument following the manufacturer's protocol (Takara Biotechnology Co., Ltd.). Amplification and detection were carried out using a Bio-Rad My iQ detection system (Edinburgh Biological Science and Technology Development co. Berkeley, CA, USA). The PCR cycling conditions were as follows: Pre-denaturation for 1 min at 94°C, denaturation for 30 sec at 94°C, annealing for 30 sec at 55°C, extension for 1 min at 72°C, for a total of 30 cycles. The sequences of the rat SEMA7A primers were: 5'-CGAGTGGCCCAGTTATGCAG-3' (forward) and 5'-AAACCGCATGGCTTTCAGGA-3' (reverse). The sequences of the rat β -actin primers were 5'-ACGGTCAGGTCATCACTATCGG-3' (forward) and 5'-GCACTGTGTTGGCATAGAGGTC-3' (reverse). The 2^{- $\Delta\Delta$ C_t} method was used for quantification (20). The knockdown efficiency of the siRNA was verified in our previous study (18). After 48 h transfection, RPMVECs were stimulated with 25% seawater or co-culture, and 25% deionized water stimulation was used as a control.

NR8383 cells co-culture with RPMVECs. RPMVECs (5 \times 10⁵ cells/well in a six-well plate) were stimulated with or without 25% seawater for 24 h and fixed with 2% paraformaldehyde. Following washing, fixed RPMVECs were co-cultured with NR8383 cells (American Type Culture Collection, Manassas, VA, USA; 5 \times 10⁵ cells/ml) for 24 h. In some experiments, NR8383 cells were pre-treated with or without anti- β 1 integrin antibody or isotype antibody (5 μ g/ml) for 1 h before co-culture. The β 1 integrin antibody based blocking was performed as previously described (14). In addition,

Table I. SEMA7A small interfering RNA sequence.

ID	5'-sequence	Stem sequence	Loop sequence	Stem sequence	3'-sequence
Sema7a-RNAi-a	GATCCC	caGGACCAAGCCTATGATGAT	CTCGAG	ATCATCATAGGCTTGGTCCTG	TTTTTGGAT
Sema7a-RNAi-b	AGCTATCCAAAA	caGGACCAAGCCTATGATGAT	CTCGAG	ATCATCATAGGCTTGGTCCTG	GG
SEMA7A, semaphorin 7A.					

RPMVECs or NR8383 cells were preconditioned by culturing with rSEMA7A (5 µg/ml) and incubated for 24 h before stimulation in other experiments.

Detection of proinflammatory cytokines. TNF-α and IL-1β obtained from culture medium were detected with ELISA kits according to the manufacturer's protocol. To detect the proinflammatory cytokine concentration in cells, the culture medium (100 µl) from NR8383 cells was collected. The concentration of proinflammatory cytokines was detected in each sample by microplate reader at 450 nm.

Western blot analysis. Cells proteins (1x10⁶ cells/ml) were extracted according to instructions of protein extraction kit (cat no. P0013; Beyotime Institute of Biotechnology, Haimen, China) and protein concentration was determined with a bicinchoninic acid protein assay kit (cat no. PA115; TianGen Biotech Co., Ltd., Beijing, China). Proteins (35 µg per sample) were separated in 10% SDS-PAGE and electrotransferred to nitrocellulose membranes. The membrane was incubated at 4°C overnight with rabbit monoclonal antibodies for β-actin (1:5,000 dilution), SEMA7A (1:1,000 dilution), VEGF (1:1,000 dilution), cofilin (1:1,000 dilution), p-cofilin (1:1,000 dilution), NF-κB (1:1,000 dilution) or pNF-κB (1:1,000 dilution). The secondary antibody (horse radish peroxidase-goat anti-rabbit IgG; 1:5,000 in TBST; cat no. A0216; Beyotime Institute of Biotechnology) was incubated at 37°C for 1 h. Detection of target protein uses enhanced chemiluminescence detection system (GE Healthcare Life Sciences, Chalfont, UK).

Immunofluorescence staining. RPMVECs (2x10⁵ cells/well in a six-well plate) were seeded in growth medium onto sterile glass slides and incubated for 24 h. Following administration, RPMVECs were fixed with 4% paraformaldehyde (pH 7.4) for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and incubated with 2% BSA for 30 min. Then cells were stained with Alexa488-Phalloidin (Molecular Probes; Thermo Fisher Scientific, Inc.) for 60 min. Nuclei were stained with DAPI. Slides were mounted with ProLong Gold anti-fade reagent and read with confocal microscope.

RPMVEC monolayer permeability measurement. The monolayer permeability was measured according to previous method (21). RPMVECs (2x10⁵ cells/well) were cultured on collagen coated Transwell insert which were placed into 24-well plates containing 500 µl medium. 100 µl fluorescein isothiocyanate (FITC)-dextran (2,000-kDa, Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was added into the insert following monolayer formation. At 1 h, the insert was removed and 100 µl medium was obtained from the bottom chamber. The fluorescent density was measured at a wavelength of 520 nm using a fluorospectrophotometer (LS-50B; PerkinElmer, Waltham, MA, USA) and GraphPad Prism version 5.0 software (GraphPad software, Inc. La Jolla, CA, USA).

Statistical analysis. All data are expressed as mean ± standard deviation and statistical analysis were performed with one-way analysis of variance followed by Dunnett's test. P<0.05 was considered to indicate a statistically significant difference.

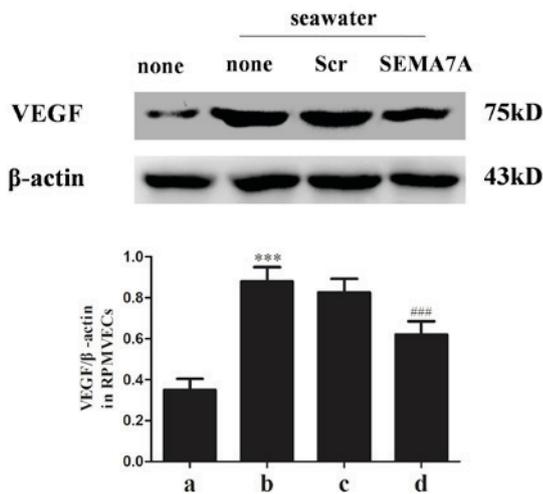


Figure 1. Endothelial SEMA7A promotes the expression of VEGF in seawater-stimulated RPMVECs. All data were obtained at 6 h following seawater administration. (A) Normal group, (B) seawater group, (C) seawater + negative scrambled control (Scr siRNA) group, (D) seawater + SEMA7A siRNA group (n=8). ^{***}P<0.001 vs. group A; ^{###}P<0.001 vs. group C. VEGF, vascular endothelial growth factor; SEMA7A, semaphorin 7A; Scr, scramble; RPMVECs, rat pulmonary microvascular endothelial cells; siRNA, small interfering RNA.

Results

Endothelial SEMA7A promotes VEGF expression in RPMVECs stimulated by seawater. VEGF is vascular permeability- and cell transmigration-related protein. To assess the role of SEMA7A on endothelial permeability, the authors explored the effect of SEMA7A in seawater-stimulated VEGF expression in RPMVECs using SEMA7A siRNA. The expression of VEGF increased significantly in seawater group. However, in SEMA7A group, the expression of VEGF was significantly inhibited in seawater stimulated RPMVECs (Fig. 1).

Endothelial SEMA7A promotes cytoskeletal remodeling monolayer permeability and phosphorylation of cofilin in RPMVECs stimulated by seawater via interaction with plexin C1. To investigate the role of receptor plexin C1 on cytoskeletal remodeling in RPMVECs, the authors stained F-actin on cells (Fig. 2A). Seawater or rSEMA7A exposure both induced evident motile phenotype characterized by contraction of stress fibers and enlargement of gap between cells. However, cells were pretreated with the plexin C1 antibody led to a significant reduction in cytoskeletal remodeling.

To assess the role of plexin C1 on monolayer RPMVECs permeability, the FITC-dextran flux across the monolayer was detected (Fig. 2B). Seawater stimulation and rSEMA7A both resulted in FITC-dextran flux increase (P<0.001). Blockage of plexin C1 inhibited the increase of FITC-dextran flux (P<0.05).

In addition, the phosphorylation of cofilin was assessed (Fig. 2C). Seawater stimulation or rSEMA7A both led to the phosphorylation of cofilin (P<0.001). Blockage of plexin C1 inhibited the increase of p-cofilin (P<0.001).

Endothelial SEMA7A promotes proinflammatory cytokine expression in NR8383 cells stimulated by seawater. On the basis of increasing expression of endothelial SEMA7A

stimulated by seawater, and given that seawater stimulation resulted in robust inflammatory responses, the authors investigated the role of endothelial SEMA7A on the expression of proinflammatory cytokines. In inflammation, the vast majority of proinflammatory cytokines are released by inflammatory cells. Therefore, RPMVECs were co-cultured with rat alveolar macrophage cell line-NR8383 cells. In this co-culture system, the RPMVECs were fixed with paraformaldehyde to inhibit cytokine production and preserve cell surface molecules. As presented in the Fig. 3, expression of TNF- α and IL-1 β were significantly increased in seawater group (P<0.001). Administration of SEMA7A siRNA significantly reduced TNF- α and IL-1 β produced by NR8383 cells (P<0.001).

Endothelial SEMA7A promotes proinflammatory cytokine production in seawater stimulated NR8383 cells via interaction with β 1 integrin and activation of NF- κ B pathway. To investigate the role of NF- κ B pathway and β 1 integrin on the proinflammatory cytokines production by NR8383 cells, the β 1 integrin antibody was used in this co-culture cell system. NF- κ B activity in NR8383 cells was measured by western blotting. In the co-culture system, seawater induced phosphorylation of NF- κ B (Fig. 4A) and the expression of TNF- α and IL-1 β (Fig. 4B) in NR8383 cells. Blockage of β 1 integrin attenuated the increase of pNF- κ B and the expression of TNF- α and IL-1 β following seawater stimulation. In addition, as a control, the rSEMA7A was directly administrated to the cultured NR8383 cells. As presented in Fig. 4, the rSEMA7A also significantly led to the phosphorylation of NF- κ B and the expression of TNF- α and IL-1 β in NR8383 cells. Blockage of β 1 integrin attenuated the increase of pNF- κ B and the expression of TNF- α and IL-1 β following rSEMA7A administration.

Discussion

In the current study, the authors investigated the role of SEMA7A in pulmonary microvascular endothelial cells and the interaction between endothelial SEMA7A and alveolar macrophages during seawater aspiration-induced ALI. These results demonstrated that seawater stimulation induced endothelial cytoskeleton remodeling, endothelial permeability and increased expression of VEGF in RPMVECs. Seawater stimulation also led to expression of proinflammatory cytokines and activated the NF- κ B pathway in NR8383 cells. Meanwhile, blockage with the plexin C1 antibody inhibited endothelial cytoskeleton remodeling, endothelial permeability, phosphorylation of cofilin and treatment with SEMA7A siRNA inhibited expression of VEGF in RPMVECs following seawater stimulation. Moreover, blockage with the β 1 integrin antibody reduced expression of proinflammatory cytokines and inhibited activation of NF- κ B in co-cultured NR8383 cells.

SEMA7A was originally identified to regulate neurite growth and axon track formation during embryonic development (22). During the recent past years, several studies have also suggested that SEMA7A is involved in immune responses. These studies have demonstrated that SEMA7A regulates inflammatory responses through T cell function modulation, macrophage recruitment, stimulation of pro-inflammatory cytokine production, chemokine expression regulation and

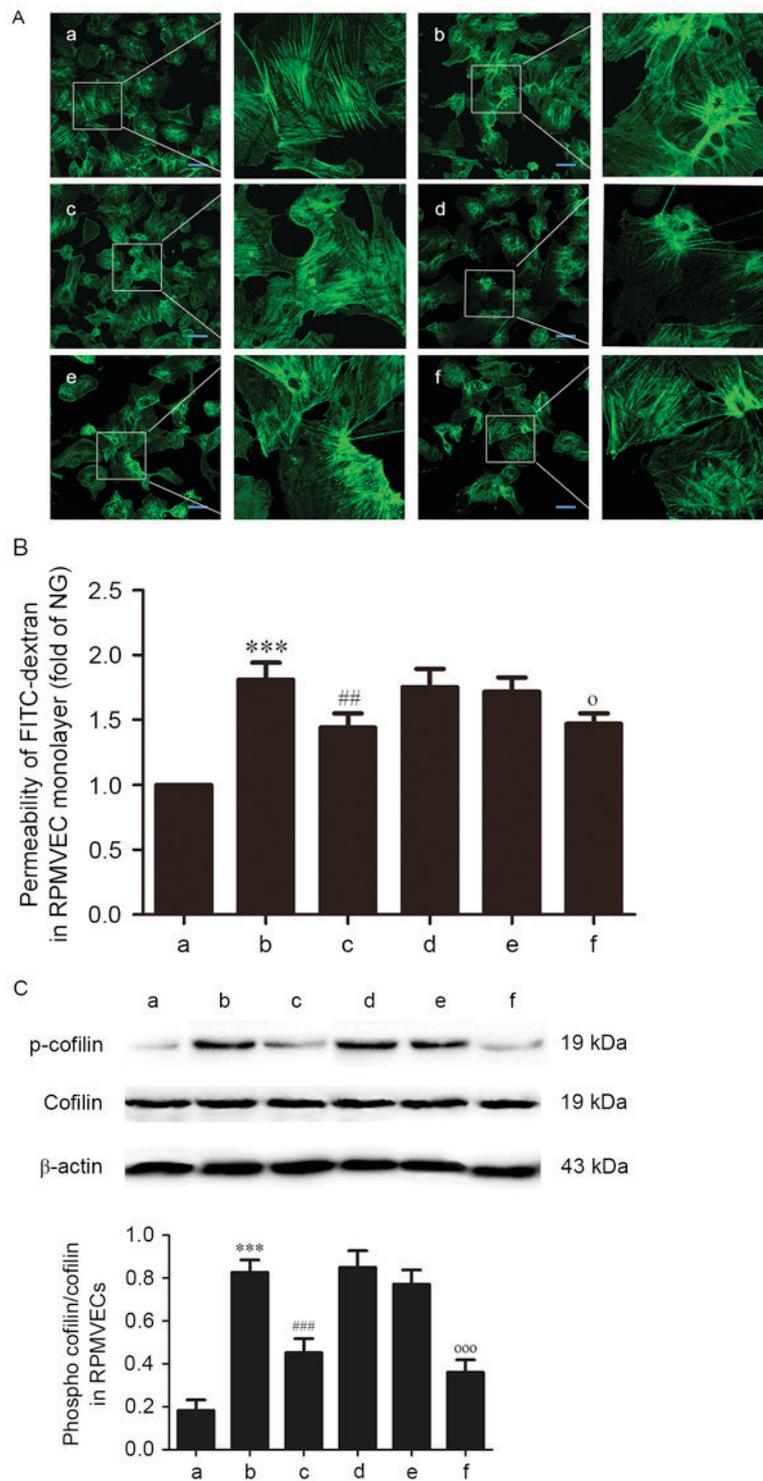


Figure 2. Endothelial SEMA7A promotes cytoskeletal remodeling, monolayer permeability and phosphorylation of cofilin in RPMVECs stimulated by seawater via interaction with plexin C1. All data were obtained at 6 h following seawater aspiration. (A) Phenotype of cultured RPMVECs was examined by confocal microscopy following staining for F-actin (scale bar=20 μ m). The right images represent the enlarged image in the white box. (B) Following RPMVEC monolayer formation, cells were treated with seawater. The permeability was shown as fold of the NG. (C) Endothelial SEMA7A activated phosphorylation of cofilin via interaction with plexin C1 in seawater stimulated RPMVECs. (a) Normal group, (b) seawater group, (c) seawater + plexin C1 antibody group, (d) seawater + control antibody group, (e) rSEMA7A+ control antibody group and (f) rSEMA7A + plexin C1 antibody group (n=8). ***P<0.001 vs. group a. ##P<0.01, ###P<0.001 vs. group b; °P<0.05, °°°P<0.001 vs. group e. SEMA7A, semaphorin 7A; RPMVECs, rat pulmonary microvascular endothelial cells; FITC, fluorescein isothiocyanate; NG, normal group.

dendritic cell migration (23,24). In hypoxia-induced lung inflammation, hypoxia increased expression of SEMA7A on endothelial cells and SEMA7A enhanced the activity of inflammatory cells (25).

Inflammation and lung epithelial-endothelial barrier injury are two primary characteristics in seawater aspiration induced ALI. The NF- κ B pathway was activated in the inflammation process. In addition, seawater stimulation also

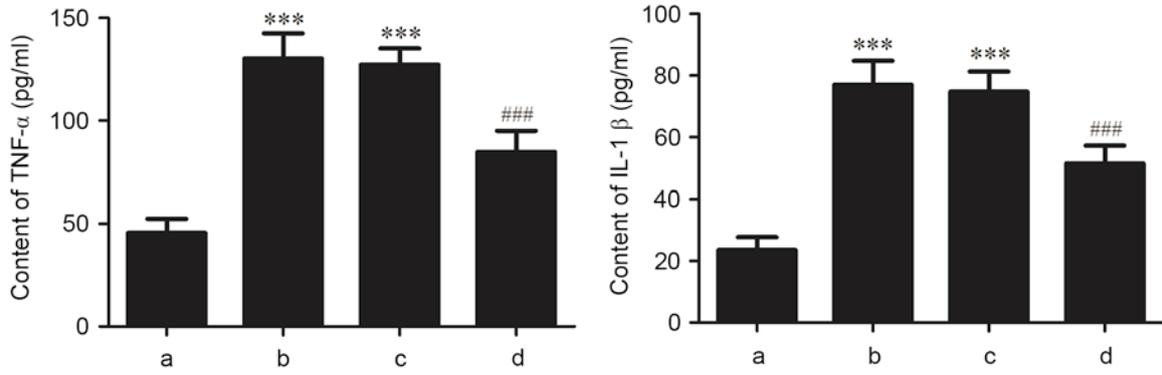


Figure 3. Endothelial SEMA7A promotes proinflammatory cytokines production in seawater stimulated co-culture NR8383 cells. All data were obtained at 6 h following seawater administration. (a) Normal group, (b) seawater group, (c) seawater + negative scrambled control (Scr siRNA) group, (d) seawater + SEMA7A siRNA group (n=8). ***P<0.001 vs. group a; ###P<0.001 vs. group b. SEMA7A, semaphorin 7A; siRNA, small interfering RNA; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β .

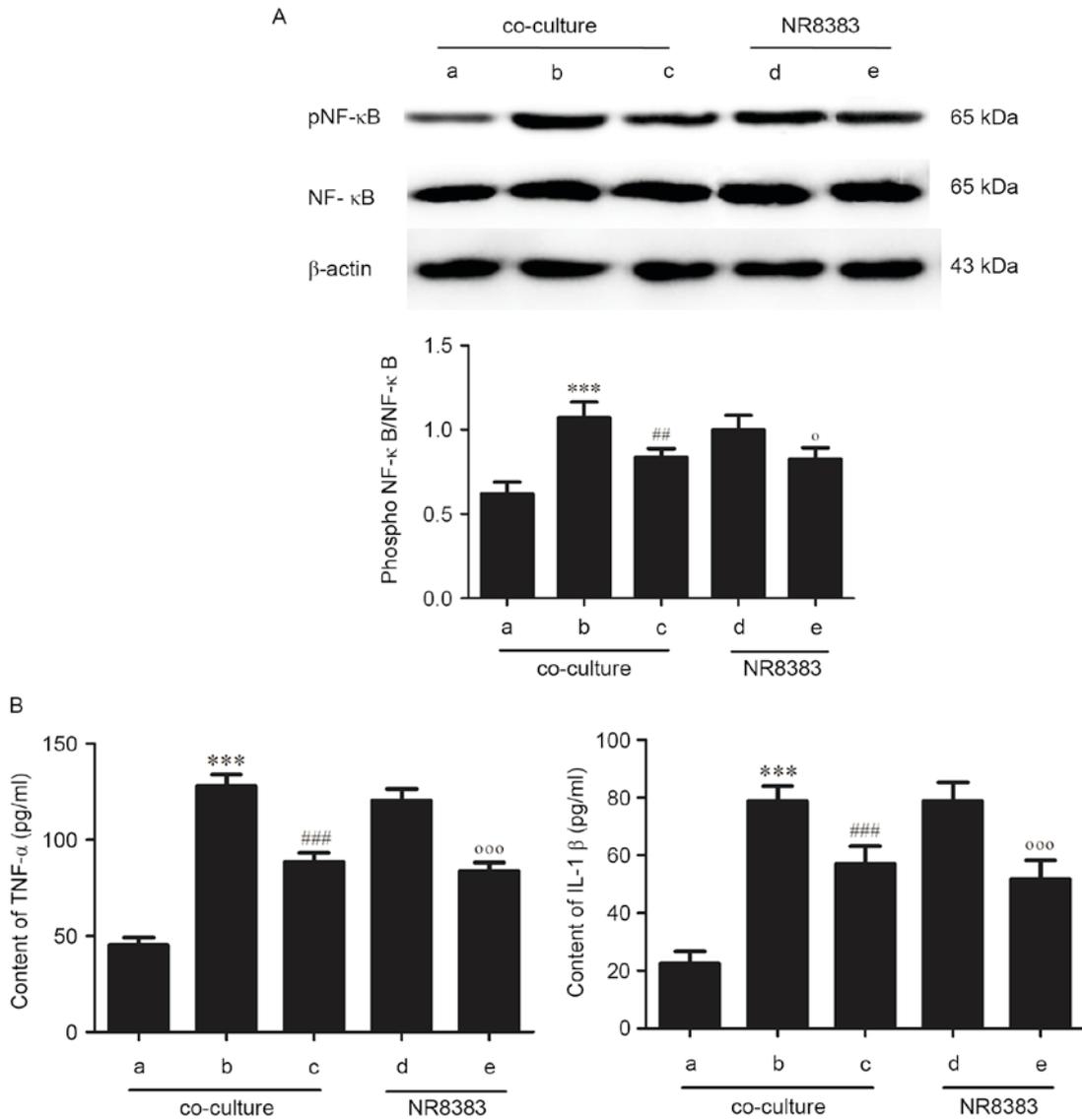


Figure 4. Endothelial SEMA7A promotes proinflammatory cytokines production in seawater stimulated NR8383 cells via interaction with β 1 integrin and activation of the NF- κ B pathway. All data were obtained at 6 h following seawater administration. (A) Endothelial SEMA7A activated the NF- κ B pathway via interaction with β 1 integrin in seawater stimulated NR8383 cells. (B) Endothelial SEMA7A promotes proinflammatory cytokine production in seawater stimulated NR8383 cells via interaction with β 1 integrin. (a-c: co-culture cells system, d-e: NR8383 cells only). (a) Normal group, (b) seawater + control antibody group, (c) seawater + β 1 integrin antibody group, (d) rSEMA7A + control antibody group, (e) rSEMA7A + β 1 integrin antibody group (n=8). ***P<0.001 vs. group a; ##P<0.01, ###P<0.001 vs. group b, °P<0.05, °°°P<0.001 vs. group d. SEMA7A, semaphorin 7A; NF- κ B, nuclear factor- κ B; TNF- α , tumor necrosis factor- α ; IL-1 β , interleukin-1 β .

led to the pulmonary epithelial-endothelial barrier injury and the increase of lung tissue permeability. In this process, HIF-1 α -VEGF expression was significantly induced (26). In a previous study of the authors, it was shown that seawater stimulation induced the expression of SEMA7A and administration of SEMA7A siRNA protected the endothelial barrier and inhibited the inflammation following seawater stimulation (18). In the present study, the authors further explored the mechanism of endothelial SEMA7A-mediated ALI induced by seawater. SEMA7A can interact with two different receptors, plexin C1 and β 1 integrin. As regards to the SEMA7A receptors involved in the inflammation process, most of the published studies associate the β 1 integrin receptor with immunomodulatory functions (14,27). However, plexin C1 receptor has been associated with adhesion, transmigration and changes in the cytoskeleton (17). Seawater stimulation also led to inflammation responses. In our present study, blockage with β 1 integrin antibody reduced expression of proinflammatory cytokines and inhibited activation of NF- κ B in co-culture NR8383 cells. Meanwhile, administration of SEMA7A siRNA inhibited the proinflammatory cytokine production following seawater stimulation. Furthermore, blockage with β 1 integrin antibody also inhibited activation of the NF- κ B pathway in co-cultured NR8383 cells. Endothelial barrier injury and endothelial permeability increase serve important roles in the process of inflammation infiltration. The present study demonstrated that blockage with the plexin C1 antibody inhibited endothelial cytoskeleton remodeling, endothelial permeability and treatment with SEMA7A siRNA inhibited expression of VEGF in RPMVECs following seawater stimulation.

Our experiments demonstrated the roles of SEMA7A and its two receptors in seawater aspiration-induced ALI. These results showed that SEMA7A triggers the inflammatory responses by increasing the microvascular permeability via interaction with the plexin C1 receptor and enhancing inflammatory cell activity via interaction with β 1 integrin receptor during seawater aspiration-induced ALI.

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