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Chlamydia pneumoniae infection promotes vascular endothelial cell angiogenesis through an IQGAP1-related signaling pathway

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ABSTRACT

Chlamydia pneumoniae (*C. pneumoniae*) infection plays a potential role in angiogenesis. However, it is still an enigma how *C. pneumoniae* is involved in this process. Therefore, we investigated the effect of *C. pneumoniae* infection on angiogenesis, and then explored the roles of IQGAP1-related signaling in *C. pneumoniae* infection-induced angiogenesis. *C. pneumoniae* infection significantly enhanced angiogenesis as assessed by the tube formation assay possibly by inducing vascular endothelial cell (VEC) migration in the wound healing and Transwell migration assays. Subsequently, immunoprecipitation, Western blot and tube formation assay results showed that the phosphorylation of both IQGAP1 and N-WASP was required for the angiogenesis induced by *C. pneumoniae* infection. Our co-immunoprecipitation study revealed that IQGAP1 physically associated with N-WASP after *C. pneumoniae* infection of VECs. Actin polymerization assay further showed that in *C. pneumoniae*-infected VECs, both IQGAP1 and N-WASP were recruited to filamentous actin, and shared some common compartments localized at the leading edge of lamellipodia, which was impaired after the depletion of IQGAP1 by using the small interference RNA. Moreover, the knockdown of IQGAP1 also significantly decreased N-WASP phosphorylation at Tyr256 induced by *C. pneumoniae* infection. We conclude that *C. pneumoniae* infection promotes VEC migration and angiogenesis presumably through the IQGAP1-related signaling pathway.

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1. Introduction

Atherosclerosis is today the leading cause for stroke and coronary artery disease in the world. *Chlamydia pneumoniae* (*C. pneumoniae*) infection has been shown to be involved in atherosclerosis (Belland et al., 2004; Filardo et al., 2015). However, how *C. pneumoniae* infection leads to atherosclerosis remains unclear. Pathological angiogenesis is implicated in both early and late stages of atherosclerosis (Kwon et al., 1998; Moreno et al., 2006; Jaipersad et al., 2014). Vitreous injection of *C. pneumoniae* antigen could increase the areas of new vessel formation in a laser-induced choroidal neovascularization model (Fujimoto et al., 2010), suggesting a possible angiogenic effect of *C. pneumoniae* infection in this model. However, it is still unknown how *C. pneumoniae* infection affects angiogenesis.

Vascular endothelial cell (VEC) migration is essential to angiogenesis (Lamalice et al., 2007). Previously, we found that *C. pneumoniae* infection could promote the migration of vascular smooth muscle cells (VSMCs) (Zhang et al., 2012; Wang et al., 2013). It is, however, still unclear whether *C. pneumoniae* infection affects VEC migration, and what mechanisms are involved in this process. IQ domain GTPase-activating protein 1 (IQGAP1), an actin regulatory protein, plays a pivotal role in regulating cell migration (White et al., 2012). RNA interference-induced knockdown of IQGAP1 reduces cell migration in several cell types, including VSMCs (Zhang et al., 2012) and U87MG cells (Hu et al., 2009). In addition, IQGAP1 has also been shown to be involved in angiogenesis (Meyer et al., 2008). Our previous work showed that IQGAP1 mediated *C. pneumoniae* infection-induced migration of rat VSMCs (Zhang et al., 2012). Therefore, *C. pneumoniae* might also utilize IQGAP1 to promote VEC migration, and then angiogenesis.

IQGAP1 has been shown to stimulate actin polymerization through the Neural Wiskott-Aldrich syndrome protein (N-WASP)-related pathway (Le Clainche et al., 2007). N-WASP, another actin regulatory protein, has also been demonstrated to be able to regulate cell migration (Yamaguchi et al., 2002) and angiogenesis

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(Escudero-Esparza et al., 2012). Does N-WASP also participate in *C. pneumoniae* infection-induced VEC migration and angiogenesis? Whether IQGAP1 participates in *C. pneumoniae* infection-induced angiogenesis and VEC migration through activating N-WASP, and how IQGAP1 regulates the function of N-WASP remain unclear. Accordingly, in this study we hypothesize that *C. pneumoniae* infection promotes VEC migration and angiogenesis through the IQGAP1-related signaling pathway. We demonstrated that phosphorylation of both IQGAP1 and N-WASP was involved in *C. pneumoniae* infection-induced VEC migration and angiogenesis. Knockdown of IQGAP1 by transient transfection of its specific small interference RNA (siRNA) suppressed the infection-induced increase in the phosphorylation of N-WASP. These data suggest that the IQGAP1-related signaling pathway was involved in *C. pneumoniae* infection-induced VEC migration and angiogenesis.

2. Materials and methods

2.1. Infection of VECs with C. pneumoniae

C. pneumoniae strain AR-39 (American Type Culture Collection [ATCC] 53592) was propagated in HEp-2 cells (ATCC CCL-23) and purified by centrifugation as described previously (Wang et al., 2013). In some experiments, *C. pneumoniae* was inactivated by heat treatment (90 °C, 30 min) (Schmeck et al., 2008). The VEC line EA.hy926 (ATCC CRL-2922) was seeded at a density of 3×10^5 cells per well into 6-well plates. Once confluent, the cells were serum-starved for 12 h to achieve synchronous growth arrest. VECs were then infected with 100 μl per well of *C. pneumoniae* at a titer of 5×10^5 IFU/ml. The plates were centrifuged at $1700 \times g$ for 50 min and then incubated at 37 °C for 2 h. Subsequently, the medium was replaced with the culture medium containing Dulbecco's modified Eagle's medium, 10% fetal bovine serum, 25 mg/mL vancomycin, 10 mg/mL gentamicin, and 2 μg/mL cycloheximide (861987, A1720 and C7698, Sigma, Beijing, China). The infected cells were then authenticated by immunofluorescence with mouse monoclonal anti-*C. pneumoniae* antibody (1:500, ab155847, Abcam, Cambridge, USA) and the corresponding secondary antibodies conjugated with FITC.

2.2. RNA interference

On-TARGETplus human IQGAP1 siRNA SMARTpool and scrambled RNA (scrRNA) were purchased from Dharmacon (L-004694-00-0005 and D-001810-01-05, Thermo Fisher Scientific, Inc., Waltham, USA). VECs were transfected with siRNAs using Lipofectamine RNAiMAX (13778-075, Invitrogen, San Diego, USA) according to the manufacturer's instructions. Cells at 40% confluency were serum-starved for 1 h followed by incubation with the IQGAP1-specific siRNA (25 nmol/L) or scrRNA for 8 h in serum-free medium. The serum-containing medium (10% fetal bovine serum) was then added 40 h before experiments and/or functional assays were conducted. Transfection efficiency was confirmed by Western blot.

2.3. Tube formation assay

96-well culture plates were coated with Matrigel (356230, BD Biosciences, San Jose, USA) according to the manufacturer's instructions. VECs were respectively treated with or without a selective Src tyrosine kinase inhibitor 4-Amino-5-(4-chlorophenyl)-7-(t-butyl)pyrazolo(3,4-d)pyrimidine (PP2) (1 μmol/L, 10 min, 529573, Calbiochem, Darmstadt, Germany), PKC inhibitor GF109203X (1 μmol/L, 10 min, 0741, Tocris Bioscience, Edinburgh, United Kingdom) or chelerythrine (5 μmol/L, 20 min, Phytomarker Ltd, Tianjin, China), IQGAP1-specific siRNA (25 nmol/L, 48 h) or the N-WASP inhibitor wiskostatin (5 μmol/L, 1 h, 681525, Calbiochem, Darmstadt, Germany). Subsequently, these cells were infected with viable or heat-inactivated *C. pneumoniae* (5×10^5 IFU/ml, 100 μl per well). After infected for 18 h, a total of 2×10^4 cells per well were seeded into the 96-well plates precoated with Matrigel, and incubated at 37 °C for 6 h. Then, cells were fixed with 4% paraformaldehyde, and pictures were taken from four random microscopic fields. The tube formation was quantified by counting the number of branch points of the capillary network (Liao et al., 2013).

2.4. Cell migration assays

VEC migration was examined by the wound healing and Transwell assays as described previously (Zhang et al., 2012; Wang et al., 2013). Confluent cells were respectively treated with or without the Src tyrosine kinase inhibitor PP2, the PKC inhibitor GF109203X or chelerythrine, IQGAP1-specific siRNA or the N-WASP inhibitor wiskostatin prior to the infection with *C. pneumoniae* (at a titer of 5×10^5 IFU/ml, 100 μl per well) as described above.

In the wound healing assay, cells were allowed to migrate for 10 h or 24 h after scratch wounds. Multiple photographs of the wounds were then taken under an inverted Nikon microscope (Nikon Corporation, Tokyo, Japan). Migration velocity was evaluated by the migration index (a ratio of cell recovery area to the original wound area). In the Transwell assay, after infected with *C. pneumoniae* for 16 h, cells were seeded into the upper chambers of the Transwell inserts (1.5×10^4 cells per well, 3422, Corning, St. Lowell, USA). VECs were allowed to migrate for 8 h at 37 °C. Cells were stained with crystal violet solution after fixed with 4% paraformaldehyde. The cells from nine independent, randomly chosen visual fields were counted under an inverted Nikon microscope (200 ×) for quantification of cells.

2.5. Measurement of Src tyrosine kinase and PKC activities

VECs in 6-well plates were infected with *C. pneumoniae* (at a titer of 5×10^5 IFU/ml, 100 μl per well), and these cells were respectively cultured at 37 °C for 10 h and 24 h. Then, VECs were washed with PBS and lysed with lysis buffer (Beyotime, Haimen, China). The suspension was left on ice for 10 min. The cells were recovered with a cell scraper, and then sonicated. The lysates were clarified by centrifugation (14,000g for 15 min at 4 °C). The supernatant was recovered. The Src tyrosine kinase and PKC activities were respectively assayed with the corresponding Src tyrosine kinase and PKC kinase activity assay kits (17–131 and 17–139, Millipore, Madison, USA) according to the manufactuer's protocols.

2.6. Immunofluorescence and confocal microscopy

VECs were fixed in 4% paraformaldehyde for 30 min at room temperature, and then permeabilized with 0.1% Triton X-100 for 15 min, and washed with PBS, and blocked with 5% bovine serum albumin (BSA) for 30 min at room temperature. Cells were incubated with antibodies against IQGAP1 (1:500, 610612, BD Biosciences, San Jose, USA), N-WASP (1:200, sc-20770, Santa Cruz, San Diego, USA) or FITC-Phalloidin (ALX-350-268-MC01, Enzo, New York, USA) overnight at 4 °C and with fluorochrome-conjugated secondary antibody (Santa Cruz, San Diego, USA) for 2 h. After washing, the cell nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) (Beyotime, Haimen, China). The cover slips were mounted with Prolong antifade (P36965, Molecular Probes, Eugene, USA). The cells were analyzed with confocal microscope (LMS510, ZEISS, Germany).

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