Original Paper

Autocrine VEGF and IL-8 Promote Migration via Src/Vav2/Rac1/PAK1 Signaling in Human Umbilical Vein Endothelial Cells

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Key Words

VEGF • IL-8 • Src • Rac1 • PAK1 • Endothelial cells

Abstract

Background/Aims: Pro-angiogenic factors VEGF and IL-8 play a major role in modulating the migratory potential of endothelial cells. The goal of this study was to investigate the effect of autocrine VEGF and IL-8 in the form of self-conditioned medium (CM) on human umbilical vein endothelial cells (HUVECs). **Methods:** Enzyme-linked immunosorbent assay (ELISA) examined the automatic secretion of VEGF and IL-8 protein by HUVECs. Western blot, small interfering RNA (siRNA), pulldown and Transwell assays were used to explore the role and the mechanism of autocrine VEGF and IL-8 in migration of HUVECs. **Results:** Neutralizing VEGF and IL-8 in CM significantly abrogated CM-induced migration of HUVECs. Autocrine VEGF and IL-8 increased Src phosphorylation, Rac1 activity and PAK1 phosphorylation in a time dependent manner. Additionally, blocking Rac1 activity with Rac1 siRNA largely abolished autocrine VEGF and IL-8-induced cell migration. Vav2 siRNA suppressed autocrine VEGF and IL-8-induced Rac1 activation and cell migration. Furthermore, blocking Src signaling with PP2, a specific inhibitor for Src, markedly prevented autocrine VEGF and IL-8-induced Vav2 and Rac1 activation as well as consequently cell migration. PAK1 siRNA also significantly abolished autocrine VEGF and IL-8-induced cell migration. **Conclusions:** We demonstrated for the first time that autocrine VEGF and IL-8 promoted endothelial cell migration via the Src/Vav2/Rac1/PAK1 signaling pathway. This finding reveals the molecular mechanism in the increase of endothelial cell migration induced by autocrine growth factors and cytokines, which is expected to provide a novel therapeutic target in vascular diseases.

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Introduction

Angiogenesis is the formation of new blood vessels that sprouting from the pre-existing primitive vessels and occurs during normal development and in some disease states [1-3]. Migration of endothelial cells into the extracellular matrix towards the angiogenic factors plays a central role in angiogenesis [2]. Of the growth factors involved in angiogenesis, vascular endothelial growth factor (VEGF) acts as a key regulator of endothelial cell migration through its tyrosine kinase receptors [4-7]. Cytokines, such as interleukin-8 (IL-8), have also been recognized as angiogenic factors [8-11]. IL-8 could activate the vascular endothelial cells and induce migration behaviors [8]. VEGF and IL-8 secreted by cancer cells act as chemoattractants in promoting motility of endothelial cell during tumor angiogenesis [12-14]. It is noteworthy that VEGF and IL-8 are secreted not only by tumor cells, but also by endothelial cells, thereby enhancing endothelial cell migration and angiogenesis [15, 16]. However, the molecular mechanisms underlying the effect of autocrine VEGF and IL-8 on endothelial cell migration are not completely understood to date.

Rho GTPases transduce signals from extracellular stimuli to cause changes in cell behavior [17, 18]. Of these GTPases, active Rac1 has been shown to stimulate cell migration by inducing lamellipodia formation [19, 20]. It has been found that VEGF induce endothelial cell migration by activating Rac1 [21]. There is evidence showing that Rac1 is involved in IL-8 induced endothelial cell migration [22]. Like other Rho GTPases, Rac1 acts as a molecular switch that cycles from an inactive GDP bound state to an active GTP-bound state [23]. Rac1 is activated by guanine nucleotide exchange factors (GEFs) that promote the exchange of GDP to GTP [24]. There are 2 distinct families of Rho GEFs with 11 members in human [23]. They function upstream of Rac1 and promote Rac1-dependent cell migration [25, 26]. However, despite the abundance and importance of these GEFs in the spatio-temporal control of Rac1 activation, little is known about their implication during autocrine growth factors and cytokines induced endothelial cell migration.

The non-receptor tyrosine kinase Src regulates a number of signaling pathways that are involved in survival, adhesion, proliferation, invasion and migration [27, 28]. It has been shown that Src plays a critical role in regulating Rho GTPases such as Rac1, and one of the major functions of Src is to modulate the actin cytoskeleton that controls cell migration. A recent study reported that IL-8 up-regulated motility activity through Src signaling in human gastric cancer cells [29]. Src signaling has also been shown to be involved in VEGF-induced angiogenesis [30]. Hence, it will be interesting to clarify whether Src signaling may also be involved in autocrine VEGF and IL-8-induced endothelial cell migration.

P21-activated kinase 1 (PAK1), a direct downstream effector molecular of Rac1 and Cdc42, plays important roles in regulating cell motility, signal transduction, cell death and survival [31, 32]. It has been reported that PAK1 activity was required for endothelial cell migration and permeability, and PAK1 inhibition by autoinhibitory domain of PAK1 also suppressed angiogenesis [33, 34]. In addition, Src has been identified as a potent modulator of extracellular signal-regulated PAK1 activity [35]. Recent studies have suggested that PAK1 can be activated by growth factors and cytokines such as VEGF and IL-8 [34]. Thus, it is worthwhile to explore whether the PAK1 signaling pathway is involved in autocrine VEGF and IL-8-induced endothelial cell migration. In the present study, we investigated the signaling mechanisms underlying the effect of autocrine VEGF and IL-8 on endothelial cell migration. The Src/Vav2/Rac1/PAK1 signaling pathway for autocrine VEGF and IL-8-stimulated endothelial cell migration is identified.

Materials and Methods

Cell culture and collection of conditioned media

Human umbilical vein endothelial cells (HUVECs) were obtained from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and grown in endothelial cell growth medium according to manufacturer's instructions at 37°C and 5% CO₂. COS-7 cells were grown in Dulbecco's modified Eagles medium (Sigma,

St. Louis, MO) containing 10% fetal bovine serum (Invitrogen, Carlsbad, CA) and antibiotics (100 units/ml penicillin and 100 µg/ml streptomycin; Invitrogen). Conditioned medium (CM) was collected in sterile conditions followed by centrifugation at 1500 ×g for 10 min, and then stored at –80 °C until use. Unless otherwise mentioned, CM in this study is a self conditioned medium collected from the culture medium of HUVECs. Complete medium alone without cells was incubated under the same experimental conditions and referred as plain medium (PM) that served as control.

Cell migration assay

Cell migration was assessed in a modified Boyden chamber (Costar), in which two chambers were separated by a polycarbonate membrane (pore diameter, 8.0 µm). HUVECs were grown to subconfluence in tissue culture plates and then detached; thereafter, they were centrifuged and rendered into single cell suspensions in serum-free culture medium supplemented with 5 µg/mL BSA. The suspensions containing 2×10⁴ cells were added to wells with a membrane placed in the bottom. The cells were allowed to migrate for the indicated periods of time at 37 °C in this assay. Thereafter, the medium was discarded, stationary cells were removed with a cotton-tipped applicator and the membranes were cut out of the chamber and stained with 0.5% crystal violet. The response was evaluated in a light microscope by counting the number of cells that had migrated into the membrane.

Small interfering RNA (siRNA)

For gene knockdown, siRNA duplexes specific for Rac1 (On-Target Plus: 5'-CAC UCC CAU CAU CCU AGU GdT dT -3', 5'-CAC CGA GCA AAC UUU GdT dT -3' and 5'-CUG GAG GCC AAG AUA CUU CdT dT-3'), Vav2 (5'-GGA ACA GCG AGC UGU UUG AdT dT-3', 5'-UAC CAC CUG CUC UUG AAG GdT dT-3' and 5'-GGA GAU CAU CGA GCU GCU GdT dT -3') and PAK1 (On-Target Plus: 5'-UGA AAA CCC UCU GAG AGC CdT dT-3', 5'-GAA AAA CCC GCA GGC UGU UdT dT-3' and 5'-UGA GAU CCU GGU CAU GAG GdT dT-3') were transfected into HUVECs by using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) in serum-free OPTI-MEM according to the manufacturer's instructions. Knockdown efficiency was evaluated 48 h after transfection by measuring protein levels in cell lysates using immunoblotting assays.

Western blotting assay

Cells were washed twice with PBS on the ice, and then lysed with ice-cold RIPA lysis buffer containing 1% Phenylmethanesulfonyl fluoride (PMSF) and complete protease inhibitor cocktail (Beyotime Institute of Biotechnology, China). The lysates were then clarified by centrifugation at 12,000 g for 10 min at 4 °C. The protein extracts were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked in 5% non-fat milk at room temperature for 1 h, and then incubated with the diluted primary antibody in TBST buffer at 4°C for overnight. After incubation, membranes were washed three times for 10 min at room temperature with TBST buffer and incubated with the appropriate peroxide-conjugated secondary antibody for 1h. Membranes were then rinsed three times for 10 min with TBST buffer. Target proteins were detected using ECL reagent (Thermo Scientific, Rockford, IL) according to the manufacturer's instructions.

Pulldown assays

To perform the pulldown assay with nucleotide-free Rac1 mutant (G15ARac1), HUVECs were lysed in a buffer consisting of 1% Triton X-100, 20 mM HEPES, pH 7.4, 150 mM NaCl, 5 mM MgCl₂, and protease inhibitors. Lysates were cleared by centrifugation at 12,000 × g for 10 min. Equal amounts of protein were incubated at 4 °C for 60 min with GST or GST fusion proteins containing nucleotide-free Rac1 protein (G15ARac1) bound to glutathione- Sepharose. After 1.5 h of incubation, the beads were washed 3 times with lysis buffer. Samples were immunoblotted using antibodies against Vav2, Sos1, β-PIX and Dock180.

For detection of active Rac1, equal volumes of total cellular protein were incubated with GST-PBD beads captured on MagneGST glutathione particles (Promega, Madison, WI) at 4 °C with constant rotation for 60 min. The beads were washed three times with washing buffer (4.2 mmol/L Na₂HPO₄, 2 mmol/L KH₂PO₄, 280 mmol/L NaCl, and 10 mmol/L KCl, pH 7.2). At the end of this period, beads were captured by the magnet in a magnetic stand. After washing three times with ice-cold buffer, beads were resuspended in Laemmli buffer, boiled, and subjected to immunoblotting analysis. SDS-PAGE and immunoblotting were performed by using standard methods.

Enzyme-linked immunosorbent assay (ELISA)

At predetermined experimental time points (0, 12, 24, 48, 72 h) supernatant from HUVECs were collected and centrifuged at 1,000 g for 10 min and stored at -80°C . VEGF and IL-8 were measured by ELISA according to the kit's protocol (R&D, MN, USA).

Statistical analysis

The data were analyzed using Student's t test using the SPSS statistical software package version 13 (SPSS Inc., Chicago, IL). All data were expressed as mean $\pm$ SD. For all analyses a two-sided p value of less than 0.05 was deemed statistically significant.

Results

Autocrine VEGF and IL-8 increase the migration ability of HUVECs

To evaluate the effect of conditioned medium (CM) on migration ability of HUVECs, cells were plated on the upper cell culture inserts, with CM or plain medium (PM) in the lower chambers in the transwell assay. The results showed that the number of cells that were able to migrate the transwell membrane and came to its bottom side was ~ 3.7 fold higher in CM than in PM (Fig. 1 A), suggesting the migration potentiating effect of CM on HUVECs. In order to find out the putative mediators of CM-induced migration in HUVECs, we first examined the automatic secretion of VEGF and IL-8 protein by HUVECs. The result showed that HUVECs secreted VEGF and IL-8 protein a time-dependent manner, with the highest level at 48 h (Fig. 1 B). To investigate the role of autocrine VEGF and IL-8 in HUVECs migration, we treated CM with neutralising antibodies against VEGF and IL-8 followed by transwell assay. Our results showed inhibition of motility in HUVECs when cultured in CM treated with neutralizing antibodies against VEGF and IL-8 (Fig. 1 C). Moreover, the magnitude of this inhibition was higher in CM treated with neutralising antibodies against VEGF and IL-8 in combination than neutralizing antibodies against VEGF or IL-8 alone (Fig. 1 C), suggesting VEGF and IL-8 as major mediators of CM-induced motility in HUVECs. To further confirm our finding, we then added recombinant human VEGF (rhVEGF) and recombinant human IL-8 (rhIL-8) in PM to check whether this can increase the motility of HUVECs comparable to that in CM. Our results revealed that, addition of VEGF and IL-8 in PM increased the motility compared to PM, however, this increase was found to be further higher when they were added in combination, implying the cooperativeness between these two cytokines (Fig. 1 D).

Autocrine VEGF and IL-8-induced migration requires Rac1 activation

Rac1, as an important small GTPase, has a pivotal role in mediating cell motility in a variety of cells including endothelial cells [19, 22], we wondered whether Rac1 activation was involved in the autocrine VEGF and IL-8-induced migration of HUVECs. We hence first investigated the effect of autocrine VEGF and IL-8 on Rac1 activation using Rac1 GTPase pull-down assay. We found that Rac1 activation was significantly induced 5 minutes after CM stimulation, peaked at 15min (Fig. 2A). Compared to CM control, in HUVECs grown in CM-treated with neutralising antibodies against VEGF and IL-8, the Rac1 activation levels were significantly lower (Fig. 2B). Furthermore, in CM treated with neutralising antibodies against VEGF and IL-8 together, the Rac1 activation level was further lower (Fig. 2B). These results suggest that autocrine VEGF and IL-8 induce Rac1 activation.

Next, we examined the effect of Rac1 activation on autocrine VEGF and IL-8-induced cell migration. Depletion of Rac1 by siRNA resulted in an approximately 70% reduction in the protein level of Rac1 (Fig. 2C), which was accompanied by a remarkable inhibition of autocrine VEGF and IL-8-induced cell migration (Fig. 2D). Taken together, these experiments demonstrate that Rac1 activation is required for autocrine VEGF and IL-8-induced migration of HUVECs.

Vav2 is required for autocrine VEGF and IL-8-induced Rac1 activation and cell migration

It has been shown that activation of Rac1 is regulated by guanine nucleotide exchange factors (GEFs) [26]. To determine which exchange factors activate Rac1 downstream of

Fig. 1. Effect of autocrine VEGF and IL-8 on the migration of HUVECs. (A) Comparison of the motility of HUVECs in culture medium of CM and PM. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM relative to cultures with PM. (B) VEGF and IL-8 secreted by HUVECs in the complete media were measured by ELISA for the indicated times. (C) Effect of autocrine VEGF and IL-8 on the migration of HUVECs. HUVECs were seeded in the upper compartment of the migration chamber and CM pre-treated with neutralising antibodies against VEGF and IL-8 for 1 h in the lower chamber. Migration assay was performed after 18 h and expressed as fold increase (relative to CM control) by calculating cell number. Data were presented as mean $\pm$ SD, ** $p < 0.05$ in cultures with CM plus antibodies against VEGF or/and IL-8 relative to cultures with CM alone. (D) HUVECs were seeded in the upper compartment of the migration chamber and PM, PM added with rhVEGF or/and rhIL-8 in the lower chamber. Migration assay was performed after 18 h and expressed as fold increase (relative to PM control) by calculating cell number. Data were presented as mean $\pm$ SD, ** $p < 0.05$ in cultures with PM plus rhVEGF or/and rhIL-8 relative to cultures with PM alone.

autocrine VEGF and IL-8, we first investigated the endogenous expression of candidate Rac1 GEFs (Vav2, Tiam1, β -PIX, Sos-1 and DOCK180) in HUVECs. We found that Vav2, β -PIX, Sos-1 and DOCK180 were readily detectable in HUVECs, whereas Tiam1 was not detectable, indicating an extremely low expression level in HUVECs (Fig. 3A). To determine which GEF might be involved in Rac1 activation downstream from autocrine VEGF and IL-8, we used nucleotide-free Rac1 in a pulldown strategy. Using a GST fusion protein of the G15ARac1 mutant, we found that CM treatment increased the association of Vav2 with G15ARac1 (Fig. 3B). G15ARac1 failed to pull down Sos1 after CM treatment. Low levels of β -PIX and Dock180 were detected binding to the G15ARac1 mutant, but the level of binding was not increased by CM treatment (Fig. 3B). Furthermore, when we inhibited autocrine of VEGF and IL-8 with antibodies against VEGF and IL-8 in CM, CM treatment did not stimulate the interaction between Vav2 and G15ARac1 (Fig. 3C). These results indicate that of the GEFs examined only Vav2 increases its association with G15ARac1 after autocrine IL-8 and VEGF stimulation.

To further study the role of Vav2 in autocrine VEGF and IL-8-induced Rac1 activation and cell migration, we used siRNA against Vav2 to decrease Vav2 expression in HUVECs. Silencing Vav2 resulted in a decrease of Vav2 protein expression (Fig. 3D), and largely

Fig. 2. Effect of Rac1 activation on autocrine VEGF and IL-8-induced cell migration. (A) Effect of CM stimulation on activity of Rac1. Serum-starved HUVECs were treated with CM for the indicated time periods. After stimulation, cells were analyzed for Rac1 activation by pulldown assays. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM relative to cultures without CM. (B) Effect of autocrine VEGF and IL-8 on Rac1 activation. HUVECs treated with CM and CM neutralized for VEGF and IL-8 harvested at 15 min and lysates were prepared and processed for pulldown for Rac1-GTP. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM plus antibodies against VEGF or/and IL-8 relative to cultures with CM alone. (C) Effect of Rac1 siRNA on the expression of Rac1. HUVECs were transiently transfected with appropriate Rac1 siRNA, Rac1 siRNA pool, scrambled siren (Irr siRNA) or not for 48 h. Cells were analyzed by immunoblotted with Rac1 antibody. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures transiently transfected with Rac1 siRNA relative to non-transfected cultures. (D) Effect of Rac1 siRNA on CM-stimulated cell migration. HUVECs transfected with Rac1 siRNA as indicated were subjected to a transwell assay in the presence of CM for 18 h. ** $p < 0.05$ in cultures transiently transfected with Rac1 siRNA and treated with CM relative to non-transfected cultures and treated with CM.

inhibited autocrine VEGF and IL-8-induced Rac1 activation (Fig. 3E) and cell migration (Fig. 3F). Taken together, these experiments demonstrate that Vav2 expression is required for autocrine VEGF and IL-8-induced Rac1 activation and cell migration in HUVECs.

Src is the upstream regulator of Vav2-mediated Rac1 activation in autocrine VEGF and IL-8-induced cell migration

The non-receptor tyrosine kinase Src is widely recognized as a key mediator in Rac1 activation and is important for endothelial cell migration [36, 37]. We hypothesized that active Src might also be involved in autocrine VEGF and IL-8-induced Rac1 activation and cell migration in HUVECs. Therefore, we determined whether Src activation was required for autocrine VEGF and IL-8-induced Rac1 activation and cell migration. Immunoblotting showed a visible sign of basal active Src and a maximal effect at 5 min after CM treatment

Fig. 3. Vav2 is required for autocrine VEGF and IL-8-induced Rac1 activation and cell migration. (A) Endogenous expression of candidate Rac1 GEFs in HUVECs. Equivalent levels of protein from HUVECs or COS7 cells were probed with antibodies against other Rac1 GEFs, Vav2, β -PIX, Tiam1, Sos1 and Dock180. β -actin indicated equal amount of protein was loaded. (B) CM stimulates Vav2-Rac1 complex formation. HUVECs were serum-starved and then treated CM for 15 min, lysed, and lysates were incubated with the nucleotide-free Rac1 mutant fused to GST (GST-G15ARac1) on glutathione-Sepharose beads for 60 min at 4°C. Antibodies against Vav2, β -PIX, Sos1 and Dock180 were used to detect binding of these proteins to G15ARac1. (C) Effect of autocrine VEGF and IL-8 on Vav2-Rac1 complex formation. HUVECs treated with CM and CM neutralized for VEGF and IL-8 harvested at 15 min and lysates were prepared and processed for pull-down as described in (B). (D) Effect of Vav2 siRNA on the expression of Vav2. HUVECs were transiently transfected with appropriate Vav2 siRNA, Vav2 siRNA pool, scrambled siRNA (Irr siRNA) or not for 48 h. Cells were analyzed by immunoblotting with Rac1 antibody. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures transiently transfected with Vav2 siRNA relative to non-transfected cultures. (E) Effect of Vav2 siRNA on CM-stimulated activation of Rac1. HUVECs were transiently transfected with Vav2 siRNA or not, then stimulated with CM or not. After stimulation, cells were analyzed by pull-down with Rac1 antibody. Data were presented as mean $\pm$ SD. ** $p < 0.05$, referring to the difference between cells treated with and without CM. ## $p < 0.05$, referring to the difference between the cells transfected with Vav2 siRNA and the cells transfected with scrambled siRNA (Irr siRNA) plus CM. (F) Effect of Vav2 siRNA on CM-stimulated cell migration. HUVECs transfected with Vav2 siRNA as indicated were subjected to a transwell assay in the presence of CM for 18 h. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures transiently transfected with Vav2 siRNA and treated with CM relative to non-transfected cultures and treated with CM.

(Fig. 4A). Compared to CM control, in HUVECs grown in CM-treated with neutralising antibodies against IL-8 and VEGF, the Src activation levels were significantly lower (Fig. 4B). Importantly, the Src activation level was further lower in HUVECs grown in CM treated with neutralising antibodies against IL-8 and VEGF together (Fig. 4B). These results indicate that autocrine VEGF and IL-8 induce Src activation.

Fig. 4. The activation of Src is required for autocrine VEGF and IL-8-induced Rac1 activation and cell migration. (A) Effect of CM stimulation on phosphorylation of Src. Serum-starved HUVECs were treated with CM for the indicated time periods. After stimulation, cells were analyzed for Src activation by immunoblotted with p-Src (Tyr418) or total Src antibodies. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM relative to cultures without CM. (B) Effect of autocrine VEGF and IL-8 on Src activation. HUVECs treated with CM and CM neutralized for VEGF and IL-8 harvested at 5 min and lysates were prepared and processed for immunoblotting for p-Src or total Src. Data were presented as mean $\pm$ SD of. ** $p < 0.05$ in cultures with CM plus antibodies against VEGF or/and IL-8 relative to cultures with CM alone. (C) Effect of PP2 on phosphorylation of Vav2 at Tyr172. Cells were incubated for 15 min in the absence or presence of 30 μ M PP2 prior to CM treatment. After stimulation, cells were analyzed by immunoblotting with p-Vav2 or total Vav2 antibodies. Data were presented as mean $\pm$ SD. ** $p < 0.05$, referring to the difference between cells treated with and without CM. ## $p < 0.05$, referring to the difference between the cells treated with CM plus PP2 and the cells treated with CM alone. (D) Effect of PP2 on activation of Rac1. Cells were incubated for 15 min in the absence or presence of 30 μ M PP2 prior to CM treatment. After stimulation, cells were analyzed for Rac1 activation. Data were presented as mean $\pm$ SD. ** $p < 0.05$, referring to the difference between cells treated with and without CM. ## $p < 0.05$, referring to the difference between the cells treated with CM plus PP2 and the cells treated with CM alone. (E) Effect of PP2 on CM-stimulated cell migration. HUVECs pretreated with 30 μ M PP2 were subjected to a transwell assay in the presence of CM for 18 h. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cells treated with CM plus PP2 relative to treated with CM alone.

To investigate the role of Src in autocrine VEGF and IL-8-induced Rac1 activation and cell migration, Src kinase activity was blocked with PP2, a Src inhibitor. As shown in Fig. 4C, CM treatment induced phosphorylation of Vav2 on tyrosine 172. This phosphorylation was blocked by PP2, suggesting that this phosphorylation depended on Src kinase activity. The Src inhibitor PP2 also abolished CM-induced Rac1 activation (Fig. 4D) as well as cell migration (Fig. 4E). Taken together, these data indicate that autocrine VEGF and IL-8-induced tyrosine phosphorylation of Vav2 is regulated by Src and that Src kinase activity is required for activation of Rac1 and cell migration which induced by autocrine VEGF and IL-8 in HUVECs.

PAK1 as a downstream target of Rac1 in mediating autocrine VEGF and IL-8-induced migration

Previous reports have shown that P21-activated kinase 1 (PAK1), a main downstream effector of Rac1, is involved in regulating endothelial migration and angiogenesis [33, 34]. Therefore, we examined whether PAK1 activation also occurred in our system. Western

Fig. 5. PAK1 is a downstream target of Rac1 and its activation is required for autocrine VEGF and IL-8-induced cell migration. (A) Effect of CM stimulation on phosphorylation of PAK1. Serum-starved HUVECs were treated with CM for the indicated time periods. After stimulation, cells were analyzed for PAK1 activation by immunoblotting with p-PAK1 (Thr423) or total PAK1 antibodies. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM relative to cultures without CM. (B) Effect of autocrine VEGF and IL-8 on PAK1 activation. HUVECs treated with CM and CM neutralized for VEGF and IL-8 harvested at 30 min and lysates were prepared and processed for immunoblotting for p-PAK1 or total PAK1. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures with CM plus antibodies against VEGF and IL-8 relative to cultures with CM alone. (C) Effect of Rac1T17N on CM-stimulated activation of PAK1. HUVECs were transiently transfected with Rac1T17N or empty vector, then stimulated with CM or not. After stimulation, cells were analyzed by immunoblotting with p-PAK1 and total PAK1 antibodies. Data were presented as mean $\pm$ SD. ** $p < 0.05$, referring to the difference between cells treated with and without CM. ## $p < 0.05$, referring to the difference between the cells transfected with Rac1T17N plus CM and the cells transfected with empty vector plus CM. (D) Effect of Rac1 siRNA on CM-stimulated activation of PAK1. HUVECs were transiently transfected with Rac1 siRNA or scrambled siRNA (irr siRNA), then stimulated with CM or not. After stimulation, cells were analyzed by immunoblotting. Data were presented as mean $\pm$ SD. ** $p < 0.05$, referring to the difference between cells treated with and without CM. ## $p < 0.05$, referring to the difference between the cells transfected with Rac1 siRNA plus CM and the cells transfected with Irr siRNA plus CM. (E) Effect of PAK1 siRNA on the expression of PAK1. HUVECs were transiently transfected with appropriate PAK1 siRNA, PAK1 siRNA pool, scrambled siRNA (Irr siRNA) or not for 48 h. Cells were analyzed by immunoblotting with PAK1 antibody. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures transiently transfected with PAK1 siRNA relative to non-transfected cultures. (F) Effect of PAK1 siRNA on CM-stimulated cell migration. HUVECs transfected with PAK1 siRNA as indicated were subjected to a transwell assay in the presence of CM for 18 h. Data were presented as mean $\pm$ SD. ** $p < 0.05$ in cultures transiently transfected with PAK1 siRNA and treated with CM relative to non-transfected cultures and treated with CM.

blotting analysis showed that the amount of phosphorylated PAK1 at Thr 423 was increased significantly after CM stimulation with maximal activation at 30 min. (Fig. 5A). HUVECs grown in CM-treated with neutralising antibodies against VEGF and IL-8 showed a decreased PAK1 activation in comparison with control cells (Fig. 5B). The PAK1 activation level was further lower in HUVECs grown in CM treated with neutralising antibodies against VEGF and IL-8 together (Fig. 5B).

To determine whether autocrine VEGF and IL-8-stimulated PAK1 activity was Rac1 activation-dependent, we blocked Rac1 activity by transfecting HUVECs with Rac1 T17N (an inactive mutant of Rac1) and examined PAK1 activation. We found that, while CM treatment significantly increased PAK1 activity in cells expressing the empty vector, it failed to do so in cells expressing Rac1 T17N (Fig. 5C). We also examined the effect of Rac1 on autocrine VEGF and IL-8-induced PAK1 activation by knocking down Rac1 expression with appropriate siRNAs in HUVECs. We found that, compared with scrambled siRNA, siRNA against Rac1 noticeably attenuated CM-induced PAK1 activation (Fig. 5D).

To further determine whether autocrine VEGF and IL-8 stimulate cell migration in a PAK1-dependent manner, we investigated migration of HUVECs after transfecting these cells with the PAK1 siRNA, which reduced the protein level of PAK1 by approximately 80%, as assessed by immunoblotting (Fig. 5E) and significantly reduced CM-induced migration of HUVECs (Fig. 5F). These experiments demonstrate that PAK1 as a downstream target of Rac1 in mediating autocrine VEGF and IL-8-induced migration.

Discussion

Emerging findings indicate that VEGF and IL-8 signaling are implicated in regulating endothelial cell migration [22, 38, 39]. It has been reported that VEGF and IL-8 can promote endothelial cell migration in an autocrine manner [15, 16]. In our system, cell migration rate of HUVECs was accelerated after CM treatment compared with that of the control. At the same time, the effect of CM was inhibited by neutralising antibodies against VEGF and IL-8. Therefore, autocrine VEGF and IL-8 directly activate the migration of HUVECs, which is a critical step for angiogenesis. Based on this, the signaling mechanisms underlying the effect of autocrine VEGF and IL-8 on promoting endothelial cell migration were investigated.

The activation of Rac1, a small G-protein, mediates migration of various types of cell, including endothelial cells [40]. A primary observation in the present study is that CM induced activation of Rac1 in a time-dependent fashion in HUVECs. Moreover, we observed that CM-induced Rac1 activation was suppressed by neutralising antibodies against VEGF and IL-8, indicating that autocrine VEGF and IL-8 can activate Rac1. It has been reported that IL-8 promotes endothelial cell migration by increasing the activity of Rac1 [22]. Caron et al. have shown that activation of Rac1 mediates VEGF-stimulated endothelial cell migration [41]. Our results found that when Rac1 activation was blocked, autocrine VEGF and IL-8-stimulated cell migration was dramatically diminished. Therefore, our results suggest that Rac1 activation serves as a mediator of autocrine VEGF and IL-8-stimulate endothelial cell migration.

We next examined the potential activators for Rac1 in our system. Like all GTPases, the Rac1 is under tight spatial control, which is mediated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs) that catalyse GTP exchange and hydrolysis, respectively [23]. Specific GEF allows Rac1 to be activated in specific signal transduction pathways and coordinate more elaborate responses to specific demands at localized cellular sites [26, 42]. In this study, we identified the exchange factor Vav2 as mediating autocrine VEGF and IL-8 signaling to Rac1. Using a nucleotide-free G15ARac1 mutant, we showed that Vav2 interacts with Rac1 after CM stimulation and this association was inhibited by neutralising antibodies against VEGF and IL-8. Moreover, knockdown of Vav2 not only inhibited the increase in Rac1 activity in response to CM stimulation, but also inhibited CM stimulated migration. Together these results indicate that Vav2 plays a critical role in the response to autocrine VEGF and IL-8. A previous study showed that Vav2 activated Rac1 after VEGF stimulation through the VEGFR-2 signaling in endothelial cells [43]. On the other hand, Work from other labs has indicated that IL-8 promotes the activation of VEGF receptors in an autocrine fashion by controlling the expression of VEGF in endothelial cells [44]. Therefore, it is possible that Vav2 mediates autocrine VEGF and IL-8-induced Rac1 activation through VEGFR-2 signaling in our system. Further studies are needed to

decipher whether expression and activation of VEGFR-2 is required for Vav2-mediated Rac1 activation in response to autocrine VEGF and IL-8 in endothelial cells.

The Src tyrosine kinase has been identified as a key mediator in endothelial cell migration [45]. Signals initiated by VEGF or IL-8 are transduced into cells through the activation of Src [29, 30]. A previous study shows that Src is an upstream of Vav2 in controlling Rac1 activation [43]. The results of our present study show that CM-induced cell migration was associated with an increase in Src activity. CM-induced Src activation was suppressed by neutralising antibodies against VEGF and IL-8, indicating that autocrine VEGF and IL-8 can activate Src. Moreover, our results showed that Src inhibitor PP2 could inhibit not only autocrine VEGF and IL-8-induced phosphorylation of Vav2, but also Rac1 activation as well as migration of HUVECs. Consequently, we conclude that Src is an upstream component of Vav2/Rac1 signaling pathway in autocrine VEGF and IL-8-stimulated endothelial cell migration. Previous studies have shown that Src is critical for Rac1 activity downstream of growth factor receptors in diverse cell types [46]. Src has also been shown to be implicated in cell migration mediated by adhesion molecule and Rac1-dependent endothelial cell migration [37, 47]. Meanwhile, our data indicate that Src is capable of increasing Rac1 activity through the Vav2 signaling, which is associated the autocrine VEGF and IL-8 with endothelial cell migration. Therefore, we conclude that Src is commonly used by cells to activate Rac1 and promote migration in response to diverse signaling pathways.

We then focused on the potential downstream effector of Rac1 that is associated with autocrine VEGF and IL-8-triggered migration of HUVECs. PAK1 is widely recognized as a potential modulator of endothelial cell migration, permeability, and angiogenesis [33, 34]. Previous studies have shown that PAK1 is a downstream mediator of Rac1 in controlling tumor migration and invasion [48]. Furthermore, the expression of a constitutively active form of PAK1 in endothelial cells has been shown to induce the rapid formation of lamellipodia, filopodia, and dorsal ruffles, as well as an increase in the reorganization of actin cytoskeleton and cell migration [33]. This prompted us to investigate whether Rac1 mediates autocrine VEGF and IL-8-induced endothelial cell migration through PAK1 activation. Our results showed that CM triggers a rapid stimulation of PAK1 activity. CM-induced PAK1 activation was suppressed by neutralising antibodies against VEGF and IL-8, indicating that autocrine VEGF and IL-8 can activate PAK1. Preventing Rac1 activity by Rac1 T17N and Rac1 siRNA decreased the PAK1 activation-induced by autocrine VEGF and IL-8, suggesting that autocrine VEGF and IL-8-induced Rac1 activity was responsible for activation of PAK1 in endothelial cells. When PAK1 activity was blocked by PAK1 siRNA, autocrine VEGF and IL-8-stimulated cell migration was dramatically diminished. Therefore, our results suggest that PAK1 activation serves as a downstream mediator of Rac1 in regulating autocrine VEGF and IL-8-stimulated endothelial cell migration.

In summary, we present the first direct evidence here that autocrine VEGF and IL-8 promote cell migration of HUVECs via Src/Vav2/Rac1/PAK1 signaling. This finding reveals the molecular mechanism in the increase of endothelial cell migration induced by autocrine VEGF and IL-8, which may lead to the development of new therapies that can be applied in pathological conditions involving increase in endothelial cell migration.

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Disclosure Statement

The authors have nothing to disclose.

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