Rac1 mediates laminar shear stress-induced vascular endothelial cell migration

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The migration of endothelial cells (ECs) plays an important role in vascular remodeling and regeneration. ECs are constantly subjected to shear stress resulting from blood flow and are able to convert mechanical stimuli into intracellular signals that affect cellular behaviors and functions. The aim of this study is to elucidate the effects of Rac1, which is the member of small G protein family, on EC migration under different laminar shear stress.12,14 In the current study, ECs were exposed to laminar shear stress than that exposed to static culture. Using plasmids encoding the wild-type (WT), an activated mutant (Q61L), and a dominant-negative mutant (T17N), plasmids encoding Rac1 were transfected into EA.hy 926 cells. The average net migration distance of Rac1Q61L group increased significantly, while Rac1T17N group decreased significantly in comparison with the static controls. These results indicated that Rac1 mediated shear stress-induced EC migration. Our findings conduce to elucidate the molecular mechanisms of EC migration induced by shear stress, which is expected to understand the pathophysiologic basis of wound healing in health and diseases.

Introduction

Vascular endothelial cell (EC) migration is an essential process in angiogenesis, wound healing, vessel remodeling, and reendothelialization. During physiological and pathologic courses, many effectors, such as growth factors, chemokines, and mechanical stimulation, can regulate the migration of vascular ECs through signaling transduction pathway. The intracellular signaling pathways mediating cell migration have to respond to diverse extracellular cues and translate them into finely regulated cellular activities.13

ECs lining the interior walls of blood vessels alter the expression of “stress-sensitive” genes in response to shear flow in blood.4 There is considerable evidence that fluid shear stress functions as a significant regulatory factor in regulating EC migration through mechano-chemical transduction.5,9 Studies on intracellular signaling events have shown that shear stress activated multiple signaling molecules, including protein kinase C, focal adhesion kinases (FAK), Rho family GTPases, phosphatidylinositol 3-kinase (PI3K), and mitogen-activated protein kinases (MAPKs).10,11 Thus, shear stress can activate multiple mechano-sensing molecules and lead to initiation and propagation of signals through networks of pathways.12 These molecules lead to the activation of various signaling cascades, contributing to a highly coordinated and orchestrated mechano-chemical transduction signaling. The activated signal transduction pathway is not simply linear, but involves in cross-talk, feedback, and bidirectional communication to form a signaling network.13 Rho GTPases act as molecular switches to control signal transduction pathways by cycling between an active GTP-bound form and an inactive GDP-bound form. In the GTP-bound form, they interact with downstream targets to elicit a variety of intracellular responses.12,14 Rho family small GTPases belong to the Ras superfamily, including Cdc42, Rac, and Rho, playing important roles in regulating the actin-based cytoskeletal structure and intracellular signaling. The current studies showed that RhoA was required at the front of the migrating cell to regulate actin polymerization to form lamellipodial and membrane protrusion.15,16 In addition, the activation of Rac1 can activate RhoA to affect the formation of stress fibers and cell contraction, thereby regulates EC migration,17 which plays an important role in vascular endothelial growth factor-induced EC migration.18,19 In addition, shear stress has been proven to be an important modulator of cellular function in cell migration process.20 However, most experiments in previous research were performed under a certain shear stress.21 There is little study that compares the role of Rac1 in EC migration under different levels of shear stress.
Our previous studies suggested that Rac1 induced by interleukin-8 (IL-8) could mediate the migration of ECs, and CXCR1/2 as mechano-sensors could mediate laminar shear stress-induced endothelial cell migration. In this study, we further examine whether Rac1 mediates the migration of ECs induced by shear stress and elucidate the underlying molecular mechanisms, i.e., (1) three levels of shear stress were applied to investigate EC migration compared with static condition. (2) EA.hy 926 cells transfected with cDNA encoding the WT of Rac1 (Rac1WT), a dominant active mutant of Rac1 (Rac1Q61L), and a dominant negative mutant of Rac1 (Rac1T17N) were subjected to three levels of shear stress to evaluate the role of Rac1 in shear stress-induced EC migration. Elucidation of the molecular mechanisms of Rac1 mediating EC migration induced by shear stress would help us to understand the pathophysiological basis of wound healing in health and diseases, and to provide new ideas for the diagnosis and prevention of cardiovascular diseases and malignant tumors.

Results

Shear stress promoted EC migration

Actually, vascular ECs in vivo are under the influence of various flow conditions. To compare the effects of different shear stress on EC migration, a strip of confluent EA.hy 926 cells were denuded and subjected to shear stress of 5.56, 10.02, and 15.27 dyn/cm² (static culture condition as controls), respectively. The direction of the flow was from left to right (Fig. 1A). The left-hand side of the denuded zone was denoted as “upstream” side, correspondingly, the right-hand side denoted as “downstream.” Figure 1A showed that on the upstream side, the direction of cell migrating into the denuded zone was parallel to the direction of shear stress. The area of wound healed gradually with the duration due to EC migration. The average net migration distance at the upstream edge under each shear stress was shown in Figure 1B. It can be seen from Figure 1 that the cell migration distance under relative high shear stress (15.27 dyn/cm²) increased significantly than that under the static culture condition at each duration. Especially, there was a highly significant difference at 8 h compared with static controls (P < 0.01). Under shear stress of 5.56 dyn/cm² and 10.02 dyn/cm², the cell migration distance increased significantly than that of static control at 4 h, 6 h, and 8 h (P < 0.05). However, there was no significant difference at initial 2 h. Correspondingly, under static condition, the average net migration distance of the cells at the upstream edge increased slowly with duration (from 23.92 ± 1.63 µm at 2 h to 38.44 ± 5.64 µm at 8 h). Remarkably, under shear stress of 5.56 dyn/cm², 10.02 dyn/cm², and 15.27 dyn/cm² at 8 h, the average net migration distance of the cells at the upstream edge increased to 113.62 ± 6.06 µm, 145.31 ± 8.74 µm, and 193.13 ± 9.82 µm, respectively, compared with 38.44 ± 5.64 µm under static condition. These results suggested that EC migration was strongly depended on the magnitude of shear stress. In addition, cell migration during wound repair involves displacements both parallel and perpendicular to the direction of flow. Cell migration parallel to flow is expected to be the primary contributor to wound repair. Therefore, wound...
repair area ratio (as defined in Materials and Methods), in combination with migration distance, as another new parameter, was examined to confirm how flow impacts on the directionality of cell migration. The wound repair area of each group showed an increased trend with enhanced shear stress and duration (Fig. 1C), which was similar to results of migrated distance. However, repair area of groups showed a significant difference at initial 2 h compared with controls ($P < 0.05$). These results all demonstrated that shear stress could promote ECs migration.

Shear stress-induced vascular endothelial cells migration is dependent upon upregulation of Ra1 expression

Figure 2A showed the mRNA expression of Rac1 assessed by RT-PCR under three levels of shear stress at different durations. A trend toward increasing mRNA expression of Rac1 with shear stress and duration was observed. Under low shear stress (5.56 dyn/cm$^2$), the mRNA expression of Rac1 increased significantly only at 8 h in comparison with static controls ($P < 0.05$); while there are significant differences of Rac1 mRNA expression at initial 2 h under mid (10.02 dyn/cm$^2$) and high shear stress (15.27 dyn/cm$^2$) compared with control. It can be seen that the mRNA expression of Rac1 in EA.hy 926 cells under the different shear stress upregulated with increased duration. In addition, the mRNA expression of Rac1 also showed a time-dependent manner under shear stress, and increased significantly with duration. Correspondingly, the mRNA expression of Rac1 in static cultured EA.hy 926 cells increased slowly with duration. Furthermore, the Rac1 protein expression increased with loaded duration of laminar shear stress (under the 10.02 dyn/cm$^2$ shear stress, from 2 h to 8 h). There were significant differences of Rac1 protein expression from 4 h to 8 h compared with static controls and 2 h. Therefore, the results indicated that shear stress-induced vascular endothelial cells migration is dependent upon upregulation of mRNA and protein expression of Rac1, which is consistent with previous results of ECs migration under different shear stress.

EA.hy 926 cells migration with transfection

To further determine whether Rac1 is involved in shear stress-induced EC migration, EA.hy 926 cells transfected with respective pcDNA3-EGFP-Rac1-WT (a wild-type), pcDNA3-EGFP-Rac1-Q61L (an activated mutant), and pcDNA3-EGFP-Rac1-T17N (a dominant negative mutant) were subjected to three levels of shear stress for 8 h compared with untransfected controls. EA.hy 926 cells transfected with three plasmids were treated with or without mechanical stimulus and then fluorescence microscope was used to detect the expression of Rac1 (Fig. 3A), indicated that three plasmids had been successfully transfected into EA.hy 926 cells with transfection efficiencies were 93.2% (WT), 91% (17N), and 89.7% (Q61L), respectively. The fluorescence intensities in three transfected cells were enhanced with stimulation of low shear stress (5.56 dyn/cm$^2$) for 2 h (Fig. 3B). Figure 4B and C indicated the difference of average net migration distance and wound repair area ratio among the transfected cells and untransfected controls. Under shear stress of 5.56 dyn/cm$^2$, both the migration distance and repair area ratio of Rac1Q61L group increased significantly at every duration, and those of Rac1T17N group decreased significantly compared with control group at 4 h, 6 h, and 8 h due to inhibition of Rac1 activities. Similarly, both the migration distance and repair area ratio of Rac1Q61L group increased significantly at 4 h, 6 h, and 8 h under shear stress of 10.02 dyn/cm$^2$, and those of Rac1T17N group decreased significantly at 4 h, 6 h, and 8 h compared with control group. However, all groups had no significant difference at 2 h. In addition, under shear stress of 15.27 dyn/cm$^2$, the migration distance of Rac1Q61L group increased significantly at every duration compared with control group, and Rac1T17N group decreased significantly at 4 h, 6 h, and 8 h. The repair area ratio of Rac1Q61L group increased

Figure 2. Effects of different shear stress on the mRNA and protein expression of Rac1 in ECs. (A) The expression of Rac1 mRNA under different shear conditions for various time were semi-quantitatively detected by RT-PCR. The electrophoresis of Rac1 mRNA expression in endothelial cells when exposed to different shear stress, β-actin was used as an endogenous control. Relative quantitative values according to images analysis by Image J. (B) Representative western blots of Rac1 expression and quantification based on image analysis of the western blot bands. The expression level of β-actin in each group was used as intrinsic controls, and relative expression of Rac1 were calculated. *$P < 0.05$, **$P < 0.01$ vs. static control.
and cytoskeleton organization, while the relations of these changes to lamellipodial protrusion, cell migration, and intracellular signaling remain to be established. The process of EC migration is the cell directional motility in response to extracellular cues. EC migration is a very complex process, which is affected by many factors, including chemical factors and mechanical factors. Generally, these factors can promote cell movement and trigger intracellular signals that result in spatially and temporally coordinated reorganization of cytoskeletal and development of transient and definitive extracellular matrix contacts.

Mechanical factors play an important role in EC migration. Previous studies demonstrated that fluid shear stress could regulate EC structure and function through mechano-chemical transduction. It is found that laminar flow had more important roles in promoting EC migration than disturbed flow. Most experiments in previous research were performed under a certain shear stress. However, vascular ECs in vivo are under the influence of various flow conditions. Our results suggested that the migration distance of EA.hy 926 cells increased with shear stress and duration, and the flow could regulate the direction and speed of migration. The reasons why flow could promote cell migration were discussed. To name a few, laminar flow promotes the diffusion of intracellular chemical substances in the injured area; laminar flow can stimulate the activation of intracellular signal pathway; and laminar flow can promote physical migration of cells in the direction of flow.

So far as we know, Rho is one of the main factors that are involved in EC directional migration under shear stress. Although the exact pathway to activate small Rho GTPases is not well understood, it is widely recognized that shear stress-induced integrin-extracellular matrix signaling pathway was the main pathway of its activation. Rac1 is one member of small GTPases of Rho family that is a cycle intracellular signal pathway; and laminar flow can promote photochemical migration of cells in the direction of flow.

Our experiments also showed that, compared with control group under each level of shear stress, the high degree activation of Rac1 by Rac1Q61L transfection promoted a great degree of cell migration, while the negative mutant of Rac1 (Rac1T17N) inhibited cell migration. Both Tzima et al. and Wojciak-Stothard did high-quality research on the effects of Rac1 in EC migration, showed that Rac1 activated at 5 min and reach to peak at 30 min, and subsequently decreased at 1 h (still higher than static controls) under shear conditions. The main difference is that we applied three levels of shear stress, while 12 dyn/cm² and 3 dyn/cm² were applied in other research. Our results indicated that the level of Rac1 activation played an important role in shear stress-induced cell migration. Hu et al. reported that bovine aortic endothelial cells (BAECs) transfected with active mutant Rac1V12 increased lamellipodia in all directions, but inhibited the polarization of lamellipodial protrusion for migration. The different migration results between

**Figure 3.** The microphotograph and fluorescent images of EA.hy cell with or without transfecting. The untransfected cells and those transfected with EGFP-Rac1 WT, EGFP-Rac1 T17N, and EGFP-Rac1 Q61L were kept under static condition (A) or subjected to low shear stress (5.56 dyn/cm²) for 2 h (B) (100 ×). Fluorescence microscope showed the expression of Rac1 among EA.hy 926 cells transfected with EGFP-Rac1 WT, EGFP-Rac1 T17N, and EGFP-Rac1 Q61L. Scale bar = 100 μm.

**Discussion**

Cell migration is an essential process for normal development and homeostasis that can also contribute to important pathologies. It is well established that shear stress can modulate EC morphology significantly at 2 h, 4 h, and 6 h compared with control group, but there was no statistic difference at 8 h. The repair area ratio of Rac1T17N group decreased significantly at 4 h, 6 h, and 8 h. However, in Figure 4, under different shear stress, EC migration distance increased in EGFP-Rac1 Q61L transfected group, and decreased in EGFP-Rac1 T17N transfected group, but not changed in the Rac1 WT transfected group, suggesting that rac1 does not mediate the shear stress-induced EC migration by increasing the protein level, but by activated Rac1. Therefore, we speculated that the possible mechanisms through which activated Rac1 GTPase mediates the EC migration but not only total Rac1. It needs to be further examined to elucidate the exact mechanisms of Rac1 activity in regulating cell migrated behavior by shear stress.

Compared with control group under each level of shear stress, cells transfected with pcDNA3-EGFP-Rac1-Q61L promoted cell migration, and cells transfected with pcDNA3-EGFP-Rac1-T17N inhibited cell migration. These results further demonstrated the prominent role of Rac1 in cell migration, which is regulated by the degree of shear stress directly.

![Cell Adhesion & Migration](http://www.landesbioscience.com)
Hu et al.’s and ours may result from the different active mutant of Rac1 and magnitudes of shear stress (12 dyn/cm² in Hu et al.’s). Different active mutant of Rac1 may cause a different increased degree of Rac1 activity. The overactivation of Rac1 induced excessive lamellipodial formation, which was associated with non-polarized adhesions that are too strong for the cell to migrate. Meanwhile, our results showed that shear stress could increase the mRNA expression of Rac1. Three levels of shear stress were applied in this study, which could cause different mRNA expression of Rac1 in comparison with the shear stress of 12 dyn/cm². Consequently, Rac1 involved in signaling events play a significant role in shear stress-induced EC migration, which is expected to explore the mechanism of shear stress-induced EC migration in the future research. However, it should be noted that only total Rac1 expression was concentrated in this study, the activity of Rac1 GTPase in laminar shear stress-induced vascular endothelial cell migration should be addressed. Moreover, knockdown of Rac1 would help us to further understand how shear-stress affects the small GTPase Rac1, which is expected to be done in our near-future study.

In this study, our results confirmed that Rac1 mediated different shear stress-induced EC migration under three levels of shear stress. These findings conduce to elucidate the molecular mechanisms of EC migration induced by shear stress, which is expected to understand the pathophysiological basis of wound healing in health and disease.

**Materials and Methods**

**Cell culture**

EA.hy 926 cells (purchased from the Blood Research Institute) are hybridoma cell lines between human umbilical vein endothelial cells (HUVECs) and the epithelioma A549 cells, and retain most of the features of HUVECs, including the expression of endothelial adhesion molecules and human factor VIII, related Ag. EA.hy 926 cells were cultured in RPMI1640 medium (Invitrogen Company) supplemented with 10% fetal bovine serum (FBS, Hangzhou Sijiqing Biological Engineering Materials Co., Ltd.), 20 mmol/L HEPES (Sigma), 2% NaHCO₃, and 2% HAT (Sigma). The cells were maintained in a 5% CO₂ incubator at 37 °C.

**Shear stress experiment**

The flow chamber was designed and developed by our laboratory. The flow pattern in the experimental area of the flow chamber was laminar, two-dimensional, and fully established. The steady flow chamber is provided by a peristaltic pump (MASTER FLEX 07524-40 Cole Parmer). Three levels of shear stress were applied in this study to investigate the effects of different shear stress on EC migration, which were a relatively
“low” shear stress of 5.56 dyn/cm² that denoted shear stress levels within disturbed flow regions, a relatively "mid" shear stress of 10.02 dyn/cm² reflecting within the physiological range in human major arteries and a relatively "high" shear stress of 15.27 dyn/cm² that was typical for undisturbed flow zones within large arteries. Therefore, all experiments were performed at the flow rate of 140, 240, and 350 ml/min, corresponding to shear stress of 5.56, 10.02, and 15.27 dyn/cm², respectively.

**Scratch wound migration assay**

Cell migration was measured using a monolayer scratch injury assay as described previously. Briefly, the EA.hy 926 cells were plated onto glass slides with confluence, and then cells were washed and incubated with serum-free media overnight. At the same time, hydroxyurea (Sigma) was added to a final concentration of 5 mmol/L to further inhibit cell proliferation. A uniform straight scratch was performed in the cell monolayer using a plastic Cell Scrapper (Corning). Then, cell monolayers were washed gently, marked (for reference), and photographed using an inverted microscope (Olympus IX-71). The glass slides with wounded monolayers perpendicular to laminar shear flow direction were placed into flow chamber. Images of the wounds under both static and flow conditions were acquired at 2, 4, 6, and 8 h, respectively.

**Migration distance and wound repair area analysis**

EA.hy 926 cell migration during wound repair involves in displacements of both parallel and orthogonal to the direction of flow. Cell migration parallel to the flow direction is expected to be the primary contributor to wound repair. Therefore, the repair area ratio combined with migration distance was examined to determine whether flow impacts on the directionality of cell migration in this study. The analysis method of migrated distance and wound repair area was described in our previous study. Briefly, cell migration during the wound repair was analyzed using the image analysis software (Image Pro Plus 6.0, Media Cybernetics). The acquired images were converted from pixels to micrometers with the use of a calibration image. For each experiment, 10 cells were randomly chosen along each edge of the wound, and cell migration distance at the end of each recording period was calculated by $D_n = L_n - L_0$, where $D_n$ and $L_n$ are the net cell migration distance and the cell position at the metering point $n$ (h), respectively. $L_0$ is the original position.

Using Image Pro Plus software, the outline of wound could be traced and the area could be tabulated. The wound repair area ratio at the end of each recording period was calculated by $R_n = \frac{(A_n - A_0)}{A_0} \times 100\%$

where $R_n$ was the wound repair area ratio, $A_n$ was the area of the polygon that defines the wound's outline at the metering point $n$ (h), and $A_0$ was the origin wound repair area.

**RT-PCR**

The expression of Rac1 mRNA under laminar flow for various durations was semi-quantitatively detected by reverse transcription–polymerase chain reaction (RT-PCR). Total RNA was isolated from the cells using the TRIZol reagent (Invitrogen Company) and RT-PCR was performed by using the TaKaRa One Step RNA PCR Kit (AMV) (TaKaRa, Dalian) according to the manufacturer's instructions. To obtain the relative quantitative values for gene expression, β-actin was used as the endogenous control. Sequences of the primers used for RT-PCR listed in Table 1 were designed by Dalian Biotechnology. The PCR products were visualized by electrophoresis on a 1% agarose gel in 1 x TRIS-acetate-EDTA (TAE) buffer after staining with 0.5 µg/ml ethidium bromide.

**Western blot analysis**

EA.hy926 cells were treated with mechanical stimulus (10.02 dyn/cm²) for 2 h, and then proteins were extracted according to protein extraction kit (PMSF methods, Beyotime Biotech). Protein samples were separated on precast SDS-PAGE (10% resolving gel with 5% stacking) and semi-dry transferred to the polyvinylidene fluoride membranes (PVDF, Millipore). Membranes were blocked for 2 h at room temperature with blocking buffer containing 5% non-fat dry milk powder in Tris buffered saline containing 0.1% Tween-20 (TBS-T), and probed with 1:100 diluted primary antibodies (Rac 1 [C-1]: sc-95) specific for the target protein, and β-actin was used as the endogenous control. Membranes were incubated at 4 °C overnight, followed by incubation for 2 h at room temperature with a 1:8000 diluted HRP-linked secondary antibodies goat anti-rabbit IgG (Zhongshan Goldenbridge Biotechnology Co., Ltd.). Immunoreactive proteins were visualized using the enhanced chemiluminescence (ECL, Pierce) following the manufacturer's instructions.

**Liposome plasmid DNA transfection and immunofluorescence**

Plasmids were extracted by using plasmid mini preparation kit (Omega) according to manufacturer's instructions. Plasmids encoding the wild-type (WT), an activated mutant (Q61L), and a dominant negative mutant (T17N) were transfected into EA.hy 926 cells, respectively. To facilitate the detection of individual cells harboring the exogenously introduced GTPases, Rac1 was fused to the C-terminal of the enhanced GFP (EGFP). Plasmids encoding the GFP-tagged proteins were transfected into EA.hy 926 cells at 80% confluence using the lipofectamine method. EA.hy 926 cells transfected with three plasmids were treated with or without mechanical stimulus for various duration and then fluorescence microscope was used to detect the expression of Rac1.

**Table 1. Sequence of primers used for RT-PCR analysis**

| Gene name | Sense primer | Anti-sense primer |
|-----------|--------------|-------------------|
| β-actin   | 5’-CCAAGGCCAAC GCGGAAGA TGAC-3’ | 5’-AGGGTACATG GTGGTCCGC CAGAC-3’ |
| Rac1      | 5’-GGGGATCCCA GGGCATCAA G TGTGGTGGG-3’ | 5’-GGAATTTCTTA CAAACGACG AGGCATTTC TCTTCC-3’ |

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Data analysis
All experiments repeated three times (n = 3). Means, together with standard deviations, were calculated for the experimental and control groups. One-Way ANOVA with Duncan’s new multiple range method was performed by using data analysis software of SPSS12.0 (SPSS, Inc.). The results were expressed as means ± SD. The P values were calculated to test the level of statistical significance. The differences were considered significant at P < 0.05 and highly significant at P < 0.01.

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Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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