SPANDIDOS PUBLICATIONS

The effect of RhoA on human umbilical vein endothelial cell migration and angiogenesis *in vitro*

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Abstract. The mechanisms that control the morphologic organization of endothelial cells (ECs) into new blood vessels are not well understood. Recent studies revealed that the small G proteins of the Rho family are key regulators of cell migration, involving reorganization of the actin cytoskeleton, cell migration and the regulation of gene transcription. We hypothesized that RhoA GTPase, a member of the Rho family, may play an important role in EC organization during angiogenesis, the process of new vessel formation in pre-existing tissues. To test this hypothesis, we investigated the effects of RhoA on human umbilical vein endothelial (HUVE) cell migration and angiogenesis *in vitro*, by stably transfecting HUVE cells with sense RhoA expression plasmid through the Lipofect-2000 system. Wound assay *in vitro* and 3-dimensional cell culture were used to detect the migration and angiogenesis capacity of HUVE cells. The morphological changes of transfected cells were revealed under confocal and phase contrast microscopy. Our results demonstrated that the increased expression of RhoA in HUVE cells significantly enhanced the morphogenetic changes and cytoskeletal reorganization of the transfected cells, and also enhanced cell migration and angiogenic capacity *in vitro*, suggesting that RhoA plays an important role in the process of HUVE cell migration and angiogenesis *in vitro*.

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Abbreviatons: ECs, endothelial cells; HUVE, human umbilical vein endothelial; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; HSV TK, Herpes simplex thymidinekinase; TBS, Tris-buffered saline; FITC, fluorescein isothiocyanate; ECM, extracellular matrix

Key words: RhoA, human umbilical vein endothelial cells, migration, angiogenesis

Introduction

The formation of new capillary blood vessels through the morphologic change of endothelial or stem cells, is known as angiogenesis, which is involved in important developmental events, such as wound healing, tumor development, rheumatoid arthritis, and embryonic development (1). Angiogenesis is also involved in several rate-limiting steps during aggressive tumor growth and tumor metastases (2). The process of angiogenesis during tumor growth requires endothelial cells to branch off from pre-existing microvessels and form capillary sprout structures that will ultimately coalesce and perfuse the tumor tissue. The newly formed vessels not only provide metabolic support for expanding tumor population but also facilitate tumor cells entering systemic circulation (3). Angiogenesis is intricately regulated by a number of factors, including extracellular matrix, growth factors, membrane-bound proteinases, and integrin. These factors lead cytoskeletal rearrangement, which delicately orchestrates the various steps of angiogenesis, including endothelial cell proliferation, branching and sprouting, and lumen formation (4). Circulating tumor cells then exploit endothelial cell receptors in distal vascular beds to promote their arrest and retention in target organs (5). The significance of angiogenesis in tumor proliferation suggests that suppressing angiogenesis may be an effective way to inhibit tumor progression. This hypothesis was supported by a study, in which administration of angiogenesis inhibitors to mice with human tumors resulted in tumor regression (6). Cells migrate by rearranging the actin cytoskeleton, which occurs in five steps: forming new lamellipodia, adhering to the substratum at the front of the cell, detaching from the substratum at the tail of the cell, and retracting their tails. Formation of adhesive structures and cellular contraction are two essential steps during tumor cell migration (7). The actin cytoskeleton apparently plays a critical role in regulating the complex series of signaling events of EC shape changes (e.g. morphogenesis) during migration and angiogenesis.

The Rho family of GTPases has emerged as a key player in regulating several biological activities involving cell motility and actin cytoskeleton organization (8,9). Several

members of this family, including RhoA, Cdc42 and Rac1, are well known for their involvement in focal complex/adhesion assembly, cell polarity, gene transcription and cell-cycle progression. RhoA, in particular, mediates cell contraction during morphogenetic processes by organizing actin filaments into stress fibers. The best-characterized effector of RhoA is Rho kinase, which was shown to be involved in the formation of stress fibers and focal adhesion complexes, and to increase myosin light-chain phosphorylation (10). In addition, RhoA associates with cortical actin in focal contact sites of the cell membrane, suggesting that RhoA is a crucial player in the organization of the actin cytoskeleton as a consequence of cell locomotion, which is important in angiogenesis.

In the present study, we investigated mechanisms of RhoA involved in the cell migration and angiogenesis. Our study focused on the role of activation of RhoA in mediating cell morphology and mobility, as well as in the alteration of endothelial cell angiogenesis *in vitro*. We report that high expression of RhoA may promote endothelial cell migration and angiogenesis.

Materials and methods

Cell culture and reagents. The HUVE (human umbilical vein endothelial) cell line was purchased from Wu Han University, China. HUVE cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) at 37˚C in 5% CO_2. The mouse monoclonal antihuman RhoA antibody, rabbit monoclonal antihuman ß-actin antibody, *Bam*HI and *Eco*RI were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol reagent and G418 were purchased from Gibco (Carlsbad, CA, USA). Oligonucleotides were acquired from Shengong Biotechnology (Shanghai, China). Rabbit anti-mouse IgG alkaline phosphatase conjugate was obtained from Zhongshan Biotechnology (Beijing, China). Collagen type I and cell culture reagents and other chemical materials were purchased from Sigma (St. Louis, MO, USA), and plasmid (PEGFP-C1) was purchased from Clontech (Palo Alto, CA, USA).

Preparation of plasmid and transfection of HUVE cells. To establish a recombinant plasmid expressing the sense RhoA gene for further investigation of the effects of RhoA on human umbilical vein endothelial cell migration and angiogenesis *in vitro*, we used the PCR technique to amplify RhoA gene, and constructed the expression vector pEGFP-C1-RhoA using the clone technique. PEGFP-C1 encodes a red-shifted variant of wild-type GFP, which has been optimized for brighter fluorescence and higher expression in mammalian cells. The coding sequence of the EGFP gene contains more than 190 silent base changes that correspond to human codon usage. Sequences flanking EGFP have been converted to a Kozak consensus translation initiation site to further increase the translation efficiency in eukaryotic cells. A bacterial promoter expresses kanamycin resistance in *E. coli*. The multicloning site in pEGFP-C1 is between the EGFP coding sequences and SV40 poly A. Genes cloned into the MCS will be expressed as fusions to the C-terminus of EGFP. A neomycin resistance cassette, consisting of the SV40 early promoter, the neomycin/kanamycin resistance gene of Tn5 and polyadenylation signals from the Herpes simplex thymidine kinase (HSV TK) gene, allows stably transfected eukaryotic cells to be selected using G418. The RhoA gene was cloned into the multicloning site, as proved by analysis of restriction endonuclease (*Bam*HI and *Eco*RI) and sequence determination. The plasmid containing RhoA cDNA (PEGFP-C1-RhoA) was amplified in the *E. coli* DH 5α strain. Protocols for plasmid preparation and purification were based on the Molecular Clone-A Laboratory manual (11). HUVE cells (60% confluence/100 mm) were transfected with an empty vector (PEGFP-C1) or PEGFP-C1-RhoA. Lipofectamine (Gibco-BRL Life Technologies) was used as a carrier to transfect cells that had been serum-starved for 1 h, then incubated with vectors for 4 h at 37˚C in a humidified atmosphere of 5% CO_2. To permit cell recuperation, the mixture was replaced by complete medium containing 10% FCS and incubated overnight. Stable transfectant was selected in 800 μg/ml G418 for 10 days, then grown in medium without G418. The levels of RhoA in the transfected cells were determined in parallel experiments by immunodetection of cell lysates with an anti-RhoA epitope monoclonal antibody (Santa Cruz Biotechnology) and semi-quantitated reverse transcription-polymerase chain reaction (RT-PCR).

RNA isolation and RT-PCR. Total RNA was isolated from $1x10^7$ HUVE cells using Trizol™ reagents. RNA (2 μg) was used for cDNA synthesis by reverse transcription. The RNA samples were incubated at 70˚C for 5 min with 0.5 μg oligo-deoxythymidine primers in a final volume of 10 μl, and then incubated in 37˚C for 60 min. Reverse transcription (RT) was performed in a 25 μl reaction containing 1.25 mM deoxynucleotide triphosphate, 200 units Muloney murine leukemia virus reverse transcriptase and 1X buffer. Obtained cDNAs were amplified using specific primers. RhoA sense, 5'-CGGAATTCAGGCTGGACTCGGATTCGTT-3', and anti-sense primer, 5'-CGGGATCCCATAAGGGCTGTG CTTGCAG-3'; GAPDH sense, 5'-ACGGATTTGGTCGTA TTGGG-3', and anti-sense primer, 5'-TGATTTTGGAGG GATCTCGC-3' were used in the RT-PCR reaction. The PCR reaction system consisted of 5 μl of cDNA, 0.2 mM deoxynucleotide triphosphate, 1.25 mM $MgCl_2$, 2.5 units Taq polymerase, 1X buffer, 10 μM primers. The PCR profile was 94˚C for 30 sec, 59˚C for 1 min, 72˚C for 90 sec, for 28 cycles, followed by extension at 72˚C for 10 min. The PCR products were electrophoresed on 1.5% agarose gels (Invitrogen, Carlsbad, CA, USA), including 0.1 μg/ml ethidium bromide (EB). The amount of mRNA was semiquantitated by measuring relative intensity of the amplified GAPDH with an equal amount of cDNA.

Protein extraction and Western blot analysis. Post-transfected (PEGFP-C1 or PEGFP-C1-RhoA) HUVE cells were lysed in Laemmli buffer and centrifuged at 10,000 rpm for 5 min at 4˚C. The supernatants were saved as nuclear extracts. Samples were resolved by SDS-PAGE on 12% polyacrylamide gels and electrotransferred onto 0.2-μm nitrocellulose membranes. After a blocking step with 5% nonfat powdered milk in Tris-buffered saline (TBS) with 0.05% Tween-20 (TBS-T) at

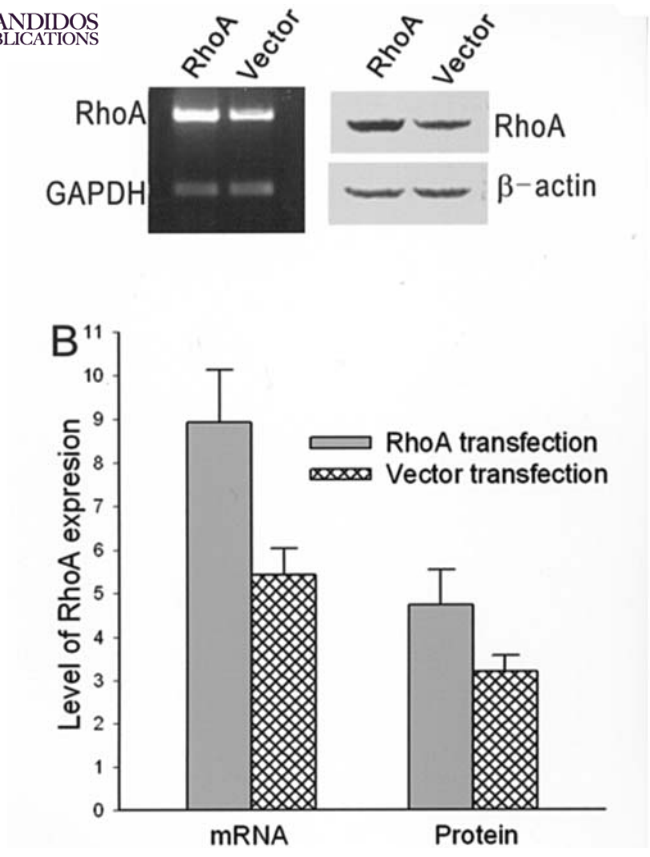

Figure 1. Expression levels of RhoA mRNA and protein of the cells transfected with RhoA (pEGFP-C1-RhoA) or vector pEGFP-C1 were detected by semiquantitated RT-PCR and Western blot analysis. (A) The expression of RhoA was compared with GAPDH (level of mRNA) and ß-actin (level of protein). The experiment was repeated 3 times. (B) Gray bars, ECs transfected with RhoA; hatched bars, ECs transfected with vector. RhoA expression in RhoA-transfected cells was significantly higher than that of vector-transfected cells (mRNA: 8.92±1.22 vs. 4.73±0.81, P=0.0038, n=3; protein: 5.42±0.61 vs. 3.21±0.36, P=0.0028, n=3).

37˚C for 1 h, the membranes were probed with the mouse monoclonal antihuman RhoA antibody (1:1000) and rabbit monoclonal antihuman ß-actin antibody (1:1000), respectively, at 4˚C overnight. After 3 washes with TBS-T, the membranes were incubated with secondary alkaline phosphatase-conjugated antibody at 37˚C for 1 h. NBT/BCIP (nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl-phosphate) was used to detect the hybridization protein. Immunoblots were exposed to Kodak image film, which was further scanned to quantify band intensities by using Biomax image analysis software. Protein extracts were loaded, equivalent to the expression level of ß-actin. Protein concentrations of extracts were determined using the Bio-Rad protein assay system with BSA as standard.

Confocal microscopy analysis of RhoA and actin filaments. Confocal microscopy was used to analyze RhoA and actin filaments of HUVE cells according to protocol described by Menager *et al* (12). RhoA was detected using a monoclonal antibody against RhoA (Santa Cruz Biotechnology) and a fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Immunotech, Marseille, France). Actin filaments were visualized by rhodamine-labeled phalloidin (Molecular Probes, Eugene, OR, USA). HUVE cells transfected with PEGFP-C1 or PEGFP-C1-RhoA, seeded on coverslips, were fixed in 3.7% formaldehyde for 10 min at room temperature. Each coverslip was placed in a glass Petri dish and incubated with a solution of 0.1% Triton X-100 in phosphate-buffered saline (PBS; pH 7.4) for 5 min. ECs were incubated with the primary monoclonal antibody against RhoA or rhodamine-labeled phalloidin for 1 h at room temperature, followed by additional incubation for 45 min with the appropriate secondary fluorescent-conjugated antibody for the primary antibody. To reduce nonspecific background staining, fixed cells with PBS containing 1% bovine serum albumin (BSA) were incubated for 30 min prior to adding the first antibody. Cells were washed 3 times during each antibody addition. Confocal laser scanning microscopy analysis was performed using a Bio-Rad MRC1024 confocal imaging system (UK), and HUVE cell morphology was observed under an Olympus phase contrast microscope. HUVE cells transfected with PEGFP-C1 or PEGFP-C1-RhoA, seeded on coverslips, were photographed after 16 h.

Wound-closure assay. Post-transfected (PEGFP-C1 or PEGFP-C1-RhoA) HUVE cells were seeded into 35-mm dishes at $1.5x10^5$ cells/cm^2 and allowed to grow to 90% confluence in their recommended media. Medium was replaced by corresponding media with 0.1% BSA for 24 h. The confluent cell monolayer was scraped with a sterile pipette tip with a constant diameter. For each dish, 3 to 5 wounds were made, and three sites of regular wounds were selected and marked. Wounded monolayers were then washed 3 times with PBS to remove cell debris. Cells were permitted to migrate into the area of clearing for 24 h. Immediate after wounding and at the end of the experiment (after 24 h), wounds were photographed and semiquantitative measurements were taken of control and treated wounds. A mean wound width was determined, and the average percent wound closure was calculated as described previously (13).

In vitro angiogenesis model. *In vitro* angiogenesis assays were performed in 3-dimensional fibrin matrixes as described previously (2 mg/ml collagen type I, 3 vol; 10X EBSS, 1 vol; 0.1 N NaOH, 1 vol; DMEM, 3 vol; and FCS, 2 vol) (14,15). The cells carrying PEGFP-C1 or PEGFP-C1-RhoA ($1x10^4$ cells/well) were suspended in the recommended medium, added to each well of Matrigel-coated 6-well plates, and the gels were incubated at 37˚C in 5% CO_2 at 100% humidity. The formation of EC tubular structures in the 3-dimensional fibrin matrix was observed and photographed with phase-contrast microscopy on the 4th day of culture, and the mean number of cordlike structures of 6 randomly chosen microscopic fields (7.3 mm^2/field) was recorded.

Statistical analysis. Values are expressed as mean ± SD of at least 3 samples. $P<0.05$ was considered statistically significant.

Results

RhoA expression on the PEGFP-C1-RhoA-transfected HUVE cell line. To study the effect of the change of RhoA on the phenotype of human umbilical vein endothelial cells, PEGFP-C1-RhoA or PEGFP-C1 was transfected. Thus, the

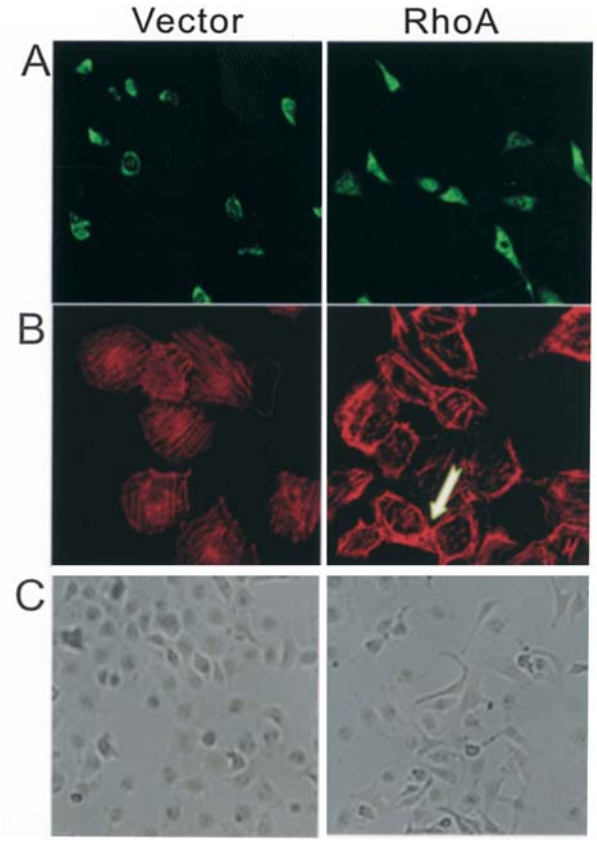

Figure 2. Effects of RhoA (PEGFP-C1-RhoA) transfection on the endothelial actin cytoskeleton and cell morphology. (A) RhoA was detected using the primary antibody against RhoA and a secondary fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG. Expression of the RhoA-transfected cells is significantly higher than that of vector (PEGFP-C1)-transfected cells. (B) F-actin was visualized by rhodamine-phalloidin staining. HUVE cells transfected with the vector had few stress fibers. In contrast, when transfected with RhoA, the number and diameter of stress fibers increased, and lamellipodia extensions occurred in the form of membrane F-actin-rich protrusions. Focal adhesion points were also detected between cells (white arrow). (C) HUVE cell morphology was observed with phase contrast microscopy. The cells transfected with RhoA showed irregular spindle shapes and higher levels of focal adhesion and lamellipodia, as well as filopodia formation.

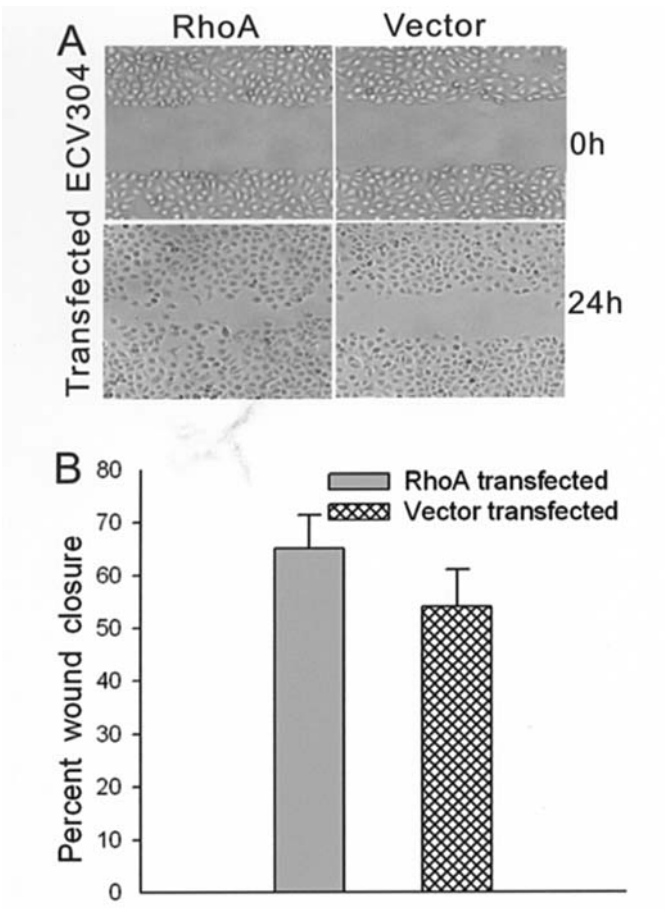

Figure 3. Effects of RhoA on EC migration *in vitro*. (A) Confluent monolayers of HUVE cells were wounded. Photographs were taken directly (t=0 h) and 24 h after wounding (t=24 h). (B) Quantification of the endothelial wound repair. EC migration was quantified 24 h after wounding. Gray bar, ECs transfected with RhoA(PEGFP-C1-RhoA); hatched bar, ECs transfected with vector (PEGFP-C1). Values are mean ± SD from 6 cultures in 3 independent experiments. The percentage of wound closure in RhoA-transfected cells was significantly higher than that of vector-transfected cells (65±6.4% vs. 54±5.5%, P=0.043, n=3).

level of RhoA mRNA was determined by using RT-PCR in HUVE cells transfected with PEGFP-C1-RhoA and PEGFP-C1. The level of RhoA mRNA was significantly higher in the PEGFP-C1-RhoA-transfected cells than that of empty PEGFP-C1-transfected cells (Fig. 1A). To determine the expression status of RhoA protein, we performed Western blot analysis. The expression level of RhoA was 5.42±0.61 and 3.21±0.36 ($P<0.05$) in PEGFP-C1-RhoA-transfected cells and empty PEGFP-C1-transfected cells, respectively (Fig. 1A), which was in accordance with RhoA mRNA expression (Fig. 1B).

Effects of RhoA on the endothelial actin cytoskeleton and cell morphology. The protein level of RhoA was evaluated by Western blot and immunofluorescence analyses. Expression of the PEGFP-C1-RhoA-transfected cells was significantly higher than that of the PEGFP-C1-transfected cells (Figs. 1A and 2A). To determine the effect of RhoA activities on the endothelial actin cytoskeleton and cell morphologies, confocal and phase contrast microscopies were employed to compare the cell lines transfected with PEGFP-C1-RhoA and PEGFP-C1. The results revealed that HUVE cells transfected with empty PEGFP-C1 had few actin stress fibers, which were visualized by staining with rhodamine-phalloidin (Fig. 2B) as described previously (16). In contrast, cells transfected with PEGFP-C1-RhoA have stronger stress fibers. It was obvious that the number and diameter of stress fibers increased, and more lamellipodia extensions occurred when RhoA was over-expressed, compared to the control cell line. At the same time, focal adhesion points were detected between cells with overexpressed RhoA (Fig. 2B). It can be demonstrated that RhoA plays an important role in regulating actin stress fiber organization. The morphological changes induced by over-expressed RhoA in HUVE cells were observed by light microscopy, and the cells transfected with PEGFP-C1-RhoA showed irregular spindle shapes and higher levels of focal adhesion and lamellipodia and filopodia formation (Fig. 2C). Taken together, these data indicate that the overexpression of RhoA can change the morphology of endothelial cells by reorganization of the actin cytoskeleton.

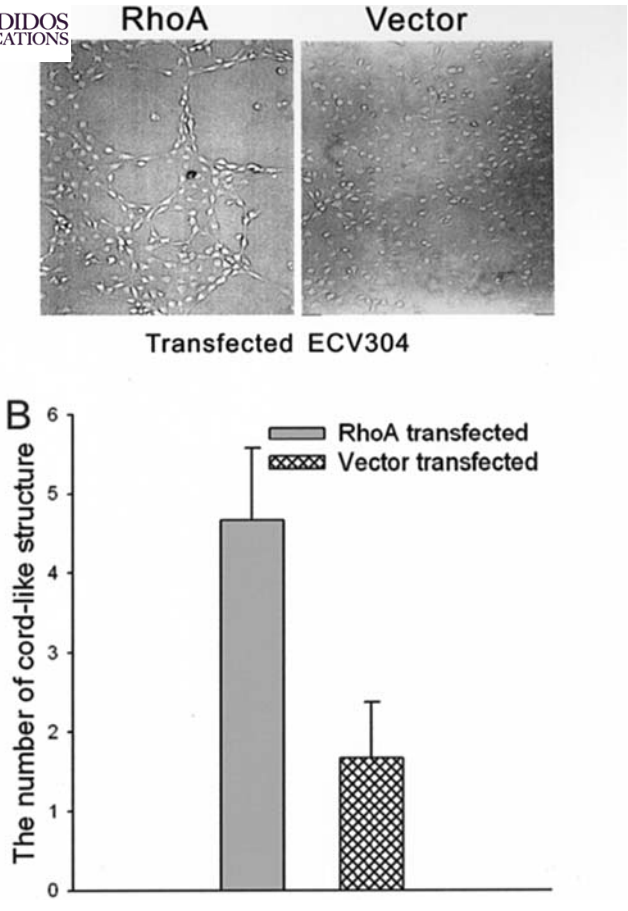

Figure 4. RhoA regulate EC organization into precapillary cords. (A) Confluent EC monolayers, consisting of equal numbers of cells, were seeded on collagen I gels and observed and photographed 4 days later. (B) Quantification of precapillary cords. Gray bar, ECs transfected with RhoA (PEGFP-C1-RhoA); hatched bar, ECs transfected with the vector (PEGFP-C1). Quantification of EC assembly into cords with morphometry indicated that RhoA significantly promoted cord formation relative to controls (4.67±1.51 vs. 1.67±1.03, P=0.001, n=6).

*Effects of RhoA overexpression on the in vitro motility of HUVE cell*s. Rho GTPases are most noted for their ability to control cell polarity and adhesion during migration by rearranging the actin cytoskeleton. To investigate whether the effector of RhoA, Rho kinase, is also involved in endothelial migration, confluent and quiescent monolayers of HUVE cells were wounded. Recovery of these monolayer cells depends on their migration during wound closure. Proliferation of ECs in response to wounding does not start in the first 24 h (17). Photographs were taken directly and 24 h after wounding to observe the changes of EC migration (Fig. 3A). The percentage of wound closure in PEGFP-C1-RhoA-transfected cells was significantly higher than that of empty PEGFP-C1-transfected cells (Fig. 3B). These results suggested that the capacity of HUVE cell migration *in vitro* can be increased by increasing the expression level of RhoA.

Effects of RhoA overexpression on forming tubular networks in 3-dimensional gels. The 3-dimensional type I collagen provokes ECs in culture to undergo marked shape changes that closely imitate precapillary cord formation *in vivo* (18). Therefore, we designed a strategy to investigate vascular morphogenesis by embedding ECs in collagen I to investigate the effects of RhoA on EC organizational behavior. The mean number of cordlike structures of 6 randomly chosen microscopic fields (7.3 mm^2/field) was computed. As shown in Fig. 4A, using quantification of organization by morphometry, the results showed that transduction with PEGFP-C1-RhoA promoted EC organization into cords, indicating that differences were highly significant (Fig. 4B). The number of cordlike structures was 4.67±1.51 and 1.67±1.03 ($P<0.05$ n=6) in PEGFP-C1-RhoA-transfected and PEGFP-C1-transfected cells, respectively. Overexpression of RhoA increased the capability of endothelial cell angiogenesis *in vitro*, suggesting that RhoA is a critical regulator of retraction-mediated assembly of ECs into precapillary cords within the 3-dimensional collagen matrix.

Discussion

Although many factors (e.g. VEGF, FGF and TGF-ß) that control the process of angiogenesis are well understood, little is known about the molecular mechanisms by which ECs physically assemble into capillary tube structures in 3-dimensional extracellular matrix (ECM) environments. During angiogenesis, proliferating ECs are organized to form new 3-dimensional capillary networks through a process involving transition of endothelial precursor cells to spindle-shape morphology in combination with alignment into solid, multicellular, precapillary, cordlike structures. Moreover, these cordlike structures are interconnected to form a polygonal network (18,19). Previous studies revealed that GTPase RhoA mediates cell contractility by organizing actin filaments into stress fibers; and active RhoA is required for a variety of complex morphogenetic processes, including embryonic gastrulation and epidermal wound healing (20,21). Thus, the importance of RhoA for these processes is consistent with a possible role in vascular morphogenesis. In the present study, we provided evidence that RhoA was a key player in the migration phenotype of endothelial cells in which acquisition was a requirement for the induction of angiogenesis (22,23).

Our data suggest that RhoA regulates actin dynamics, particularly actin-myosin-dependent cell contraction in cell migration, demonstrating that Rho GTPase proteins are able to affect actin organization by regulating gene expression necessary for the morphological changes that accompany the cell migration process. It has been demonstrated that RhoA activates the expression of metalloproteinase MMP-9, or type IV collagenase, a key proteinase governing the degradation of the basement membrane (24). In addition, as a major angiogenesis factor, in VEGF-stimulated cells, RhoA signaling is necessary for cell migration (25). Previous experiments indicated that RhoA critically regulates angiogenesis driven by VEGF and constitutively active RhoA can complement VEGF to increase angiogenesis by >100%, whereas dominant negative RhoA markedly inhibits VEGF-driven neovascularization (26). However, in this study, we demonstrate that the overexpression of RhoA is sufficient to induce migration and increase motility of human umbilical vein endothelial cells, in the absence of any exogenous stimulation, such as growth factors or cytokines. Therefore, we considered that RhoA directly promotes the organizational behavior of ECs.

EC morphogenesis is defined as the process whereby ECs assemble into tubes in 3-dimensional extracellular matrices. These events require EC interactions with ECM through integrins, and signaling events involving cytoskeletal elements that control ECs shape and cell-cell interactions that dictate the 3-dimensional structure of the tubes. The mechanisms by which these interactions lead to the ability of ECs to assemble into tubes with a fluid-filled lumen, an abluminal surface in contact with basement membrane matrix, and cell-cell junctional contacts remain unclear. In this study, we showed that RhoA is a critical regulator of retraction-mediated assembly of ECs into precapillary cords within a 3-dimensional collagen matrix, which directly links Rho activity to a morphogenetic process that imitates key aspects of EC organization during angiogenesis *in vivo*. In addition, we also suggest that Rho kinase is a key effector through which Rho regulates cord formation.

Taken together, we demonstrated that RhoA plays an important role in migration and angiogenesis of endothelial cells *in vitro* by affecting actin organization and regulating several signaling events. Our study suggests the practical importance of regulating vascular morphogenesis in combination with EC proliferation, which provides a basis for exploring new strategies to control cancer.

Acknowledgements

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