Thy-1-Induced Migration Inhibition in Vascular Endothelial Cells through Reducing the RhoA Activity

Heng-Ching Wen¹, Chieh Kao², Ruei-Chi Hsu¹, Yen-Nien Huo¹, Pei-Ching Ting¹, Li-Ching Chen¹, Sung-Po Hsu³, Shu-Hui Juan³, Wen-Sen Lee¹,3,4*

¹ Graduate Institute of Medical Sciences, Medical College, Taipei Medical University, Taipei, Taiwan, ²Graduate Institute of Cell and Molecular Biology, Medical College, Taipei Medical University, Taipei, Taiwan, ³Department of Physiology, School of Medicine, Medical College, Taipei Medical University, Taipei, Taiwan, ⁴Cancer Research Center, Taipei Medical University Hospital, Taipei, Taiwan

Abstract

Our previous study indicated that Thy-1, which is expressed on blood vessel endothelium in settings of pathological and a specific of physiological, but not during embryonic, angiogenesis, may be used as a marker for angiogenesis. However, the function of Thy-1 during angiogenesis is still not clear. Here, we demonstrate that knock-down of the endogenous Thy-1 expression by Thy-1 siRNA transfection promoted the migration of human umbilical vein endothelial cells (HUVEC). In contrast, treatment with interleukin-1β (IL-1β) or phorbol-12-myristate-13-acetate (PMA) increased the level of Thy-1 protein and reduced the migration of HUVEC. These effects were abolished by pre-transfection of HUVEC with Thy-1 siRNA to knock-down the expression of Thy-1. Moreover, over-expression of Thy-1 by transfection of HUVEC with Thy-1 pcDNA3.1 decreased the activity of RhoA and Rac-1 and inhibited the adhesion, migration and capillary-like tube formation of these cells. These effects were prevented by co-transfection of the cell with constitutively active RhoA construct (RhoA V14). On the other hand, pre-treatment with a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, Y27632, abolished the RhoA V14-induced prevention effect on the Thy-1-induced inhibition of endothelial cell migration and tube formation. Taken together, these results indicate that suppression of the RhoA-mediated pathway might participate in the Thy-1-induced migration inhibition in HUVEC. In the present study, we uncover a completely novel role of Thy-1 in endothelial cell behaviors.

Citation: Wen H-C, Kao C, Hsu R-C, Huo Y-N, Ting P-C, et al. (2013) Thy-1-Induced Migration Inhibition in Vascular Endothelial Cells through Reducing the RhoA Activity. PLoS ONE 8(4): e61506. doi:10.1371/journal.pone.0061506

Editor: Rajesh Mohanraj, UAE University, United Arab Emirates

Received November 20, 2012; Accepted March 10, 2013; Published April 17, 2013

Copyright: © 2013 Wen et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Funding: This work was supported by research grants from the National Science Council of the Republic of China (NSC 93-2320-B-038-018, NSC 94-2320-B-038-005). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

* E-mail: wslee@tmu.edu.tw

Introduction

Angiogenesis, the generation of new blood vessels from pre-existing ones, occurs during embryonic development, body growth, formation of the corpus luteum and endometrium, tissue regeneration and wound healing [1]. Abnormal angiogenesis also plays an important role in many pathological processes, including tumor growth, metastasis, diabetic retinopathy and arthritis [2,3]. Angiogenesis is a complex multi-step process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Only when the process and regulation of angiogenesis is fully understood can we begin to design a strategy for treating angiogenesis-related disorders.

In the angiogenic process, migration and proliferation of endothelial cells are two critical steps. Angiogenesis is under tight control by a balance of angiogenesis inducers and inhibitors. Activated endothelial cells reorganize their cytoskeletons, express cell surface adhesion molecules such as integrins and selectins, secrete proteolytic enzymes, and remodel their adjacent ECM [2]. Dynamic interactions between cell surface adhesive receptors (integrins) for ECM components, organization of the actin cytoskeleton and the turnover of focal adhesions are all key processes in cell locomotion and migration. Although the mechanism of angiogenesis regulation is not fully elucidated, it is generally accepted that the initiation or termination of the process is controlled by a balance between positive and negative regulators of angiogenesis. Angiogenesis may occur in the organism as a result of a number of different stimuli such as vascular injury, wounds, neoplastic growth, and/or local inflammation. While the initiation of angiogenesis has been intensive studies, little is known about the control of termination of angiogenesis. Understanding the molecular mechanism of termination of angiogenesis might provide novel strategies for therapeutic intervention of vascular growth.

Previously, we observed that Thy-1 serves as a marker for angiogenesis and demonstrated that Thy-1 was expressed during physiological and pathological angiogenesis in adult rats, but not during embryonic angiogenesis [4]. Thy-1, a 25–37 kDa glycosylphosphatidylinositol (GPI)-anchored cell surface protein, belongs to the immunoglobulin-like supergene family. Originally, Thy-1 was described as a marker for thymocyte differentiation in mice. Subsequently, Thy-1 was found to be expressed in various cell types, including neurons, fibroblasts, ovarian cancer cells, hematopoietic cells and vascular endothelial cells [5,6]. Although Thy-1 has been suggested to be involved in cellular growth, differentiation, apoptosis, adhesion, and migra-
tion, the function of Thy-1 expression during angiogenesis is still unknown. Previous in vitro studies have shown that upon stimulation such as phorbol-12-myristate-13-acetate (PMA) as well as inflammatory cytokines, interleukin-1β (IL-1β) or tumor necrosis factor-α (TNF-α), up-regulated Thy-1 mRNA in vascular endothelial cells. In the present study, we investigated the possible role of Thy-1 during angiogenesis by examining the relationship between the Thy-1 level in vascular endothelial cells and endothelial cell proliferation and migration. We found that up-regulation of Thy-1 expression by treatment of HUVEC with IL-1β or TNF-α or transfection with Thy-1 pcDNA3.1 inhibited, while knock-down the endogenous Thy-1 expression promoted, the migration in cultured human vascular endothelial cells.

**Materials and Methods**

**Cell Culture**

HUVEC or an immortalized human microvascular endothelial cell line (HMEC-1) [7] were grown in M199 (GIBCO, Grand Island, NY) containing 10% FBS (Highveld Biological, Lyndhurst, RSA), endothelial cell growth supplement (ECGS, 0.03 mg mL⁻¹) (Biomedical Technologies, Stoughton, MA) and kanamycin (GIBCO) (50 U mL⁻¹) in a humidified 37°C incubator. Cells from passages 5–10 were used.

**Plasmids**

Human Thy-1 cDNA was obtained from EST clone and cloned into LamnIffid vector via the cloning sites of Hind III and Not I. For over-expression of human Thy-1, the full length of Thy-1 was subcloned to the expression vector, pcDNA3.1(+) (Invitrogen) at the same restriction sites as noted above and the expression is driven by the CMV promoter.

**Cell Transfection**

For transient transfection of the Thy-1 or constitutively active RhoA (RhoA V14) constructs into HUVEC, jetPEI-HUVEC transfection reagent (Polyplus Transfection, Bioparc, France) was used according to the manufacturer’s protocol. Briefly, a jetPEI-HUVEC/DNA mixture was added drop-wise onto the M199M+Glutamax™ I medium (GIBCO) containing 2% FBS, mixed gently, and incubated in a humidified 37°C incubator for 4 h. The growth medium was then replaced and the cells were incubated further for 24 h. To verify the transfection efficiency, HUVEC were transfected with Thy-1-pCMS-EGFP (enhanced green fluorescent protein)-C1, and then monitored using an inverted fluorescent microscope.

**Adhesion Assay**

HUVEC or HMEC-1 transfected with vector (pcDNA3.1) or Thy-1 pcDNA3.1 were plated onto a collagen (0.1 mg/mL)-coated 24-well plate for various as indicated at 37°C, and then washed with phosphated-buffered saline (PBS). After washing, the number of adherent cells was assessed by MTT [8]. For transient transfection with vector, pcDNA3.1(+) (Invitrogen) at the same restriction sites as noted above and the expression is driven by the CMV promoter.

**Lamellipodia Assay**

HUVEC transfected with pcDNA3.1 or Thy-1 pcDNA3.1 were seeded on coverslips and incubated in growth medium for 40 h, fixed in 4% paraformaldehyde, and then permeabilized with 1% Triton X-100. To detect actin polymerization in lamellipodia, HUVEC were stained with rhodamine-phalloidin (Cytoskeleton Inc., Denver, CO). Cells were viewed under a laser confocal spectral microscope imaging system (Leica, TCS SP5; Mannheim, Germany).
Capillary-like Tube Formation Assay

Capillary-like tube formation assay was performed as described previously with minor modifications [13]. The 96-well plates were coated with 50 μL Matrigel (10 mg/mL) (BD Bioscience Pharmigen, CA, USA) by incubating at 37°C for 1 h. HUVEC were suspended in M200 (Cascade Biologics, Portland, OR, USA) supplemented with 10% FBS and endothelial cell growth supplement, and plated onto a layer of Matrigel at a density of 4 x 10^5 cells/well. The plates were then incubated for a further 4 h at 37°C, and capillary-like tube formation was observed with a microscope.

Viability Assay

Cell viability was estimated by a modified MTT [3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) assay.

Subcellular Fractionation

The cells were washed with cold PBS and lysed by Dounce homogenizer in lysis buffer (20 mM Tris, pH 8.0, 3 mM MgCl₂, 1 mM PMSF), and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected as the cytosolic fraction. Pellets were washed with cold PBS, and then homogenized in the lysis buffer (Tris 50 mM, pH 7.5, NaCl 150 mM, PMSF 1 mM, NP-40 1%, 0.1% SDS) on ice, and centrifuged at 12,000 g for 30 min at 4°C. The supernatant was collected as the membrane (particulate) fraction. Proteins of cytosolic and membrane fractions were examined for RhoA, RhoB and RhoC by Western blot analysis.

Statistical Analysis

All data were expressed as the mean value ± s.e.mean. Comparisons were subjected to one way analysis of variance (ANOVA) followed by Fisher’s least significant difference test. Significance was accepted at P<0.05.

Results

Effect of Thy-1 Over-expression on the Process of Angiogenesis

To study the role of Thy-1 in the process of angiogenesis, we transfected and over-expressed Thy-1 in HUVEC. Figures 1a and 1b show that the transfection efficiency of Thy-1-positive cells was approximately 40%. The levels of Thy-1 mRNA (Figure 1c) and protein (Figure 1d) were significantly increased in the Thy-1-transfected HUVEC as compared with vector-transfected cells. We used these Thy-1 over-expressed HUVEC to study the role of Thy-1 in the process of angiogenesis. Initially, we examined the effect of Thy-1 over-expression on the proliferation and the migration, two major events during the process of angiogenesis, in HUVEC. To examine whether over-expression of Thy-1 could affect the proliferation of HUVEC, cell number count was performed. As shown in Figure 1e, over-expression Thy-1 for 92 h did not affect the number of HUVEC. We next examined whether over-expression of Thy-1 could affect the endothelial cell migration. As illustrated in Figure 1f, transfection with Thy-1 significantly inhibited the migration of HUVEC. To confirm the effect of over-expression of Thy-1 on the endothelial cell migration, we conducted a similar study in HMEC-1. As shown in Figure 1g, transfection with Thy-1 pcDNA 3.1 also significantly inhibited the migration of HMEC-1. Since tube formation is also involved in the process of angiogenesis, we further examined the effect of Thy-1 over-expression on capillary-like tube formation. As shown in Figure 1h, over-expression of Thy-1 in HUVEC interrupted the capillary-like tube formation. To confirm the role of Thy-1 in endothelial cell migration, the following three experiments were conducted. Initially, we applied the chemicals, IL-1β and PMA, which have been demonstrated to be able to up-regulate Thy-1 expression in vascular endothelial cells, to examine whether over-expression of Thy-1 is correlated with decreased cell migration ability. The results show that treatment with IL-1β at a concentration of 10 nM (Figure 2a, top panel) or PMA at a concentration of 20 ng/mL increased the levels of Thy-1 protein (Figure 2b, top panel) and decreased the number of migrated endothelial cells (Figures 2a and 2b, bottom panel). The IL-1β- and PMA-induced increases of the Thy-1 protein level and decreases of the migrated cell number were prevented by pre-transfection of HUVEC with Thy-1 siRNA (Figures 2a and 2b). Moreover, suppression of endogenous Thy-1 expression by Thy-1 siRNA transfection induced an increase in the migrated HUVEC cell number (Figure 2c).

Effect of Thy-1 Expression on Lamellapodia Formation, Cell Survival, and Adhesion of Endothelial Cells

Since lamellapodia formation, a cytoskeletal protein projection on the mobile edge of the cell, is necessary for cell migration, the effect of Thy-1 on lamellapodia formation was also examined. As illustrated in Figure 3a, over-expression of Thy-1 reduced the formation of both stress fibers and lamellipodia in HUVEC. To confirm that the Thy-1-induced decrease in the migration of HUVEC was not due to cell death, we conducted viability assays by comparing the survival rate between vector-transfected and Thy-1-transfected cells at 24 h after transfection. MTT assays showed that there was no significant difference in cell viability between vector-transfected and Thy-1-transfected HUVEC (Figure 3b). Since the attachment and adhesion of endothelial cells to ECM are critical steps for the process of angiogenesis and would affect the endothelial cells invasion and migration, we further examined whether over-expression of Thy-1 could affect the cell adhesion on collagen. The result shows that over-expression of Thy-1 in HUVEC decreased cell attachment on the plate coated with collagen examined by MTT assay (Figure 3c) or by cell count (Figure 3d). We also tested this effect on HMEC-1. As shown in Figure 3e, over-expression of Thy-1 in HMEC-1 also decreased its adhesion on the collagen-coated plate examined by MTT assay.

Involvement of RhoA Inhibition in the Thy-1-induced Migration Suppression in HUVEC

To delineate the molecular mechanism underlying Thy-1-induced migration inhibition in HUVEC, we examined the protein levels of Rho in the Thy-1-transfected HUVEC. Western blot analysis demonstrated that the protein levels of RhoA (Figure 4a, left panel), but not RhoB and RhoC (Figure 4a, right panel), were significantly decreased in the Thy-1-transfected HUVEC as compared with the control vector-transfected cells. Since translocation of Rho GTPases from the cytosol to the cytoplasmic membrane is required for their activations and functions, we further examined the effect of over-expression Thy-1 on membrane translocation of Rho GTPases. As illustrated in Figure 4b, over-expression of Thy-1 decreased the protein levels of RhoA and Rac-1, but not RhoB and RhoC, in the membrane fraction of HUVEC. To confirm the inhibitory effect of Thy-1 over-expression on the activation of RhoA, the RhoA-GTP pull down assay was conducted. As shown in Figure 4c, over-expression of Thy-1 reduced the RhoA activity in HUVEC.
To further confirm that reduction of RhoA is associated with the Thy-1-induced inhibition of migration activity in the HUVEC, we over-expressed the constitutively active RhoA by transfection of the cells with RhoA V14. As shown in Figure 5a, transfection of HUVEC with RhoA V14 increased the protein levels of RhoA. Figure 5b shows that over-expression of RhoA V14 reduced the degree of Thy-1-induced inhibition of migration activity in the HUVEC. However, transfection of the HUVEC with Thy-1 and RhoA V14 followed by Y-27632 (5 μM), a ROCK inhibitor (a kinase associated with RhoA for transducing RhoA signaling), caused a further suppression of migration activity of the HUVEC.

Involvement of RhoA Inhibition in the Thy-1-induced Suppression in Capillary-like Tube Formation

We further examined whether the reduction of RhoA also affected the capillary-like formation. As shown in Figure 6, over-expression of Thy-1 inhibited the capillary-like tube formation. Transfection of HUVEC with RhoA V14 abolished the Thy-1-induced inhibition of capillary-like tube formation. However, transfection of the HUVEC with Thy-1 cDNA and RhoA V14 cDNA followed by Y-27632 (5 μM) treatment caused a further suppression of the capillary-like tube formation.

Discussion

Angiogenesis is a complex multi-step process involving extensive interplay between cells, soluble factors, and ECM components. The processes of angiogenesis involve four distinct sequential steps, including (1) proteolytic breakdown of the basement membrane [14,15], (2) endothelial cell proliferation, (3) migration of endothelial cells toward the angiogenic stimulus,
and (4) lumen formation [2]. Angiogenesis is under tight control by a balance of angiogenesis inducers and inhibitors.

Previously, we have proposed that Thy-1 serves as a marker for angiogenesis and demonstrated that Thy-1 was expressed during physiological and pathological angiogenesis in adult rats, but not during embryonic angiogenesis [4]. Although Thy-1 expression in human endothelial cells has been linked to the induced secretion of matrix metalloproteinase-9 and CXCL8 from neutrophil [16], and suggested to play an important role in inflammatory responses [17], what function Thy-1 could have in angiogenic capillary formation is still unclear. Based on the known properties of Thy-1, we consider that it might operate in the modulation or stimulation of endothelial cell proliferation. Surprisingly, we found that overexpression of Thy-1 did not affect the proliferation of cultured endothelial cells (Figure 1e). Instead our results showed that overexpression of Thy-1 inhibited adhesion (Figures 3c–3e), migration (Figures 1f and 1g), and capillary-like tube formation (Figure 1h) of cultured endothelial cells. To confirm the effect of Thy-1 on vascular endothelial migration, we used IL-1β and PMA to induce Thy-1 expression in HUVEC and examined their effects on endothelial cell migration. Our results demonstrated that the level of Thy-1 protein was increased (Figures 2a and 2b, top panel) and the migration of HUVEC was decreased (Figures 2a and 2b, bottom panel) in both IL-1β-treated and PMA-treated HUVEC as compared with vehicle-treated control cells. Thy-1 siRNA transfection, which knocked down the IL-1β-induced and the PMA-induced increases of the Thy-1 protein level (Figures 2a and 2b), prevented the IL-1β-induced and the PMA-induced migration inhibition in HUVEC (Figures 2a and 2b, bottom panel). Furthermore, HUVEC migration was enhanced when the endogenous Thy-1 was knocked down (Figure 2c). These findings led us to hypothesize that Thy-1 is not only a marker of adult new blood vessels, but also an indicator for newly formed blood vessels in the adult just at the cessation of the angiogenic stimulus. Moreover, the findings of the present study suggest that the Rho-mediated pathway might be involved in the signal transduction leading to the inhibition of migration and the suppression of capillary-like tube formation caused by Thy-1 over-expression in cultured HUVEC. This finding seems to be the first demonstration that Thy-1 inhibits endothelial cell migration and capillary-like tube formation through a Rho-dependent pathway.

Figure 2. Role of Thy-1 in HUVEC migration. (a) Treatment of HUVEC with IL-1β (10 nM) for 24 h significantly increased the level of Thy-1 protein and decreased the migrated cell number. Values represent the means±s.e.mean. (n = 4). *p<0.05 different from control group. #p<0.05 different from the IL-1β-treated group. (b) Treatment of HUVEC with PMA (20 ng/mL) for 24 h caused a 3.83-fold increase of the Thy-1 protein level (upper panel) and a 54% reduction of the migrated cell number (bottom panel). Values represent the means±s.e.mean. (n = 4). *p<0.05 different from control group. #p<0.05 different from the PMA-treated group. (c) Transfection with Thy-1 siRNA increased the HUVEC migration. Values represent the means±s.e.mean. (n = 3). *p<0.05. Co, control.

doi:10.1371/journal.pone.0061506.g002
Vascular endothelial cell adhesion is one of the principal requirements for cell migration and proliferation. Multiple integrins with distinct combinations of $\alpha/\beta$ subunits have been recognized at such cell sites [18]. Integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ mediate cell adhesion on collagen, which is a critical step in initiating tube formation by endothelial cells [19], whereas recruitment of integrin $\alpha_5\beta_1$, the fibronectin receptor, is required for cell migration in the process of angiogenesis. Thy-1 belongs to the immunoglobulin superfamily and has been indicated to be a regulator of cell-cell and cell-matrix interaction. Integrins are one of the major are the major trans-membrane receptors that mediate dynamic interactions between the actin cytoskeleton and the extracellular matrix (ECM) during cell motility. Integrins are $\alpha\beta$ heterodimers with an extracellular domain that binds to ECM and links to the actin cytoskeleton. In general, integrins bind to specific motifs within the matrix protein, and the binding specificity is determined by the extracellular domain of integrins that recognize diverse matrix ligands. Previously, Barker et al. demonstrated that Thy-1 expression in fibroblasts promotes focal adhesion and stress fiber formation, and inhibits migration through modulation of p190 RhoGAP and Rho GTPase activity [20]. It has been demonstrated that endothelial cell Thy-1 interacts with $\alpha_5\beta_3$, $\alpha\beta_3$, and $\alpha\beta_1$ on melanoma cells and $\alpha\beta_2$ on leukocytes and promote the migration of these cells [21–25]. However, the effect of endothelial cell Thy-1 on the migration of endothelial cells has not yet been studied. In contrast to Barker et al.’s report showing that over-expression of Thy-1 induced adhesion promotion, which might contribute to migration inhibition in rat lung fibroblasts, our results showed that over-expression of Thy-1 inhibited the adhesion of HUVEC on collagen-coated plates (Figures 3c–3e). Adhesion and protrusion are central features of cell migration. Cells exhibit a biphasic migration-velocity response to increasing adhesion strength with fast migration occurring when the strength of adhesion between the cell and the substrate is neither too strong nor too weak. At low adhesion, contraction pulls weak focal adhesions at both the cell front and rear from the substrate; at high adhesion, contraction can not overcome adhesion at the cell front or rear. Therefore, an optimum is reached at intermediate adhesion. Moreover, an optimum in migration speed is also dependent on substrate concentration. This might explain that our results are contrary to those reported by Barker et al. in fibroblasts, where they found that over-expression of Thy-1 in fibroblasts activates RhoA, increases cell adhesion and decreases cell migration [20].

It has been recognized that the post-translational modification of proteins by the addition of isoprenoids is a key physiological process for facilitating cellular protein-protein interactions and membrane-associated protein trafficking [26]. The results from the
Present study suggest that over-expression of Thy-1 could suppress the endothelial cell migration through altering the prenylation. Prenylation provides important lipid attachments for the post-translational modification of many proteins, including small GTP-binding proteins belonging to the family of Ras, Rho, Rap, and Rab GTPases. In general, Rho family proteins, which regulate cell motility, require modification with geranylgeranyl pyrophosphate [27]. Blockade of farnesyl biosynthesis leads to an inhibition in the Ras-mediated regulation of proliferation and migration in primary cultured human cells [28]. Tyrosine phosphorylation of focal adhesion kinase (FAK) triggers downstream signaling events including phosphorylation of paxillin, which is required for the regulation of Rho-family GTPases (Rho, Rac and Cdc42) and Pak (a downstream effector of Rac and Cdc42) [29]. To be functionally active, Rho proteins must be localized to the cell membrane by posttranslational modification through addition of isoprenyl groups from isoprenoid pyrophosphate substrates [30]. Rho GTPases play an important role in growth factor-stimulated cell migration and cytoskeletal organization, membrane trafficking, cell cycle control, and transcriptional activation [31–34]. To test whether Thy-1-induced HUVEC migration inhibition was mediated through regulating polyprenyl pyrophosphates synthesis, we examined the effect of over-expression of Thy-1 on the expression and activity of RhoA and Rac-1. Over-expression of Thy-1 significantly decreased the protein levels of RhoA (Figure 4a, left panel), but not RhoB and RhoC (Figure 4a, right panel). Moreover, over-expression of Thy-1 decreased the protein levels of membrane-bound RhoA and Rac-1, but not RhoB and RhoC (Figure 4b). A reduced RhoA activity was observed in the HUVEC transfected with Thy-1 pcDNA3.1 as compared with transfected with pcDNA3.1 (control vector). These data suggested that Thy-1 might interfere with cell migration via suppressing the prenylation of RhoA and Rac-1. This notion was supported by our data showing that over-expression of Thy-1 reduced the formation of stress fibers and lamellipodia in HUVEC (Figure 3a). It has been indicated that the lamellipodia is generally associated with Rac

Figure 4. Effects of Thy-1 on expression and activity of RhoA in HUVEC. HUVEC were transfected with Thy-1 pcDNA3.1 or pcDNA3.1 (control). (a) Over-expression of Thy-1 inhibited the total protein levels of RhoA. The bottom panel shows the quantified results after adjusted with their own total protein levels and expressed by fold of control (left panel). Values represent the means ± s.e.mean. (n = 3). *p<0.05. In contrast, the total protein levels of RhoB and RhoC were not significantly affected by over-expression of Thy-1 (right panel). (b) Over-expression of Thy-1 suppressed membrane-bound RhoA. Proteins were detected for RhoA, cadherin and G3PDH by Western blot analysis. Cadherin was used as a cell membrane protein marker to confirm the purities of isolation. The bottom panel shows the quantified results after adjusted with their own total protein levels and expressed by fold of control (left panel). Values represent the means ± s.e.mean. (n = 3). *p<0.05. The protein levels of membrane-bound Rac-1, but not RhoB and RhoC, were also decreased by over-expression of Thy-1 (right panel). (c) A reduced RhoA activity was observed in the HUVEC transfected with Thy-1 pcDNA3.1 as compared with transfected with pcDNA3.1 (control vector). doi:10.1371/journal.pone.0061506.g004
activation, whereas the stress fibers are with Rho activation [35]. Our data showed that over-expression inhibited the activation of both RhoA (Figure 4b, left panel and Figure 4c) and Rac-1 (Figure 4b, right panel) in HUVEC. These data confirm that the formation of stress fibers and lamellipodia in HUVEC was reduced by overexpression of Thy-1. The role of Rho-mediated pathway in Thy-1-induced inhibition of endothelial cell migration was further confirmed by the evidence that (a) over-expression of constitutive active RhoA V14 prevented the migration inhibition caused by over-expression of Thy-1 in HUVEC and (b) pretreatment of the cells with ROCK inhibitor abolished the prevention effect induced by over-expression of RhoA V14 (Figure 5b). Although the difference of migrated cell number between control group (118.89±6.02) and Thy-1 pcDNA3.1+RhoA V14 pcDNA3.1 group (90.28±24.94) did not reach statistical significance (p=0.056), the migrated cell number in the Thy-1 pcDNA3.1+RhoA V14 pcDNA3.1 group is only 76% of the control group. The reduced Rac-1 activity might account for this 24% difference. Our present data suggest that inhibition of RhoA/ROCK signaling is critical for suppressing migration activity in the Thy-1 over-expressed HUVEC. Our results are in agreement with anti-migratory effects in other cells expressing Thy-1, but differ from a previous report showing that Thy-1 inhibited the migration of rat fibroblasts through an increased focal adhesion and Rho GTPase activity [20]. It seems that Thy-1-induced migration inhibition is mediated through different mechanisms in different cell types.

Although our data strongly suggest the important role which Thy-1 might play in endothelial cell migration and capillary-like tube formation, the role of Thy-1 during angiogenesis and the issue of how Thy-1 expression in endothelial cells is regulated are still unclear. Previously, we demonstrated that the expression of Thy-1 in HUVEC is up-regulated by tumor necrosis factor-α and interleukin-1β but not by growth factors, such as basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), and transforming growth factor-β (TGF-β), suggesting that inflammatory cytokines were probably responsible for this up-regulation of Thy-1 [4]. Moreover, PMA has been shown to be able to induce up-regulation of Thy-1 in human microvascular endothelial cells, suggesting that the protein kinase C pathway might be involved in the regulation of Thy-1 expression in vascular endothelial cells [6]. However, the physiological regulators of Thy-1 expression and the role of Thy-1 during angiogenesis are still unknown and in vivo studies are needed to address these issues.

Figure 5. Involvement of the Rho/ROCK signaling pathway in the Thy-1-induced migration inhibition in HUVEC. (a) Tranfection of HUVEC with RhoA V14 increased the expression of RhoA protein in HUVEC. (b) Top panel: over-expression of Thy-1 inhibited the HUVEC migration. However, co-transfection of Thy-1-transfected HUVEC with RhoA V14 prevented the Thy-1-induced migration inhibition. Treatment with 5 μM Y-27632, a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, inhibited HUVEC migration and abolished the RhoA V14-induced prevention effect on Thy-1-mediated inhibition of HUVEC migration. Bottom panel: representative photographs demonstrated the results of Figure 5b. Values represent the means ± s.e.mean. (n = 3). *p<0.05.
doi:10.1371/journal.pone.0061506.g005
In conclusion, this study provides the evidence that over-expression of Thy-1 caused inactivation of RhoA, which in turn resulted in the inhibition of endothelial cell migration and capillary-like tube formation. These findings suggest that expression of Thy-1 in endothelial cells during pathological angiogenesis might be important for angiogenesis.

Acknowledgments

We thank Professor Meei-Jyh Jiang for providing RhoA V14 cDNA.

References

1. Carmeliet P (2003) Angiogenesis in health and disease. Nat Med 9: 653–660.
2. Gupta MK, Qin RY (2003) Mechanism and its regulation of tumor-induced angiogenesis. World J Gastroenterol 9: 1144–1155.
3. Munoz-Chapuli R, Quesada AR, Angel Medina M (2004) Angiogenesis and signal transduction in endothelial cells. Cell Mol Life Sci 61: 2224–2243.
4. Lee WS, Jain MK, Arkoae BM, Zhang D, Shaw SY, et al. (1998) Thy-1, a novel marker for angiogenesis upregulated by inflammatory cytokines. Cere Res 02: 845–851.
5. Craig W, Kay R, Cates RL, Lansdorp PM (1993) Expression of Thy-1 on human hematopoietic progenitor cells. J Exp Med. 177: 1331–1342.
6. Saalbach A, Wetzig T, Haustein UF, Anderegg U (1999) Detection of human soluble Thy-1 in serum by ELISA. Fibroblasts and activated endothelial cells are a possible source of soluble Thy-1 in serum. Cell Tissue Res 298: 307–315.
7. Ades EW, Candal FJ, Sverlick RA, George VG, Summers S, et al. (1992) HEMC-1: establishment of an immortalized human microvascular endothelial cell line. J Invest Dermatol 99: 683–690.
8. Liu HJ, Lin PY, Lee JW, Hsu HY, Shih WL (2005) Retardation of cell growth by avian reovirus p13 through the activation of p33 pathway. Biochem Biophys Res Commun 336: 709–715.

Figure 6. Involvement of Rho/ROCK signaling pathway in the Thy-1-induced inhibition in capillary-like tube formation. Over-expression of Thy-1 inhibited the capillary-like tube formation. However, co-transfection of Thy-1 transfected HUVEC with RhoA V14 prevented the Thy-1-induced inhibition of capillary-like tube formation. Treatment with 5 μM Y-27632 inhibited capillary-like formation and abolished the RhoA V14-induced prevention effect on Thy-1-mediated inhibition of capillary-like tube formation.

doi:10.1371/journal.pone.0061506.g006

Author Contributions

Conceived and designed the experiments: HCW CK RCH WSL. Performed the experiments: HCW CK RCH YNH PCT LCC SPH. Analyzed the data: HCW CK RCH YNH PCT LCC SPH SHJ WSL. Contributed reagents/materials/analysis tools: SHJ WSL. Wrote the paper: WSL.
9. Uchima Y, Sawada T, Nishihara T, Maeda K, Obiha M, et al. (2004) Inhibition and mechanism of action of a protease inhibitor in human pancreatic cancer cells. Pancreas 29: 123–131.
10. Tse AK, Wan CK, Shen XL, Yang M, Fong WF (2005) Honokiol inhibits TNF-alpha-stimulated NF-kappaB activation and NF-kappaB-regulated gene expression through suppression of IKK activation. Biochem Pharmacol 70: 1443–1457.
11. Ho PY, Liang YC, Ho YS, Chen CT, Lee WS (2004) Inhibition of human vascular endothelial cells proliferation by terbinafine. Int J Cancer 111: 51–59.
12. Pilorget A, Annabi B, Bouzeghrane F, Marvaldi J, Luis J, et al. (2005) Inhibition of angiogenic properties of brain endothelial cells by platelet-derived sphingosine-1-phosphate. J Cereb Blood Flow Metab 25: 1171–1182.
13. Grant DS, Kimura JL, Fridman R, Aubert B, Placek BA, et al. (1992) Interaction of endothelial cells with a laminin A chain peptide (SIRVAV) in vitro and induction of angiogenic behavior in vivo. J Cell Physiol 153: 614–625.
14. Kalebić T, Garbisa S, Glaser B, Løetza LA (1983) Basement membrane collagen degradation by migrating endothelial cells. Science 221: 281–283.
15. Beltong G, Martini L, Robinet A (2004) Matrix metalloproteinases and matrikines in angiogenesis. Crit Rev Oncol Hematol 49: 203–220.
16. Saalbach A, Arndhold J, Lessig J, Simon J C, Anderegg U (2008) Human Thy-1 induces secretion of matrix metalloproteinase-9 and CXCL8 from human neutrophils. Eur J Immunol 38: 359–366.
17. Mason JC, Yarwood H, Tänzok A, Sugars K, Harrison AA, et al. (1996) Human Thy-1 is cytokine-inducible on vascular endothelial cells and is a signaling molecule regulated by protein kinase C. J Immunol 157: 874–883.
18. Wu MH (2005) Endothelial focal adhesions and barrier function. J Physiol 569: 359–366.
19. Merovitich K, Bergeron F, Lebelond L, Grouss B, Pouier C, et al. (2005) A novel RGD antagonist that targets both alpha5beta3 and alpha6beta1 induces apoptosis of angiogenic endothelial cells on type I collagen. Vascular Pharmacol 44: 77–89.
20. Barker TH, Grenett HE, MacEwen MW, Tilden SG, Fuller GM, et al. (2004) Thy-1 regulates fibroblast focal adhesions, cytoskeletal organization and migration through modulation of p190 RhoGAP and Rho GTPase activity. Exp Cell Res 295: 488–496.
21. Choi J, Leyton L, Nham SU (2005) Characterization of alphaX I-domain binding to Thy-1. Biochem Biophys Res Commun 331: 557–561.
22. Wetzel A, Chavakis T, Preisser, KT, Sticherling M, Haustein UF, et al. (2004) Human Thy-1 (CD90) on activated endothelial cells is a counterreceptor for the leukocyte integrin Mac-1 (CD11b/CD18). J Immunol 172: 3850–3859.
23. Saalbach A, Wetzel A, Haustein UF, Sticherling M, Simon JC, et al. (2005) Interaction of human Thy-1 (CD90) with the integrin alphabeta3 (CD51/CD61): an important mechanism mediating melanoma cell adhesion to activated endothelium. Oncogene 24: 4710–4720.
24. Saalbach A, Hildebrandt G, Haustein UF, Anderegg U (2002) The Thy-1/Thy-1 ligand interaction is involved in binding of melanoma cells to activated Thy-1-positive microvascular endothelial cells. Microsc Res 64: 86–93.
25. Saalbach A, Haustein UF, Anderegg U (2000) A ligand of human thy-1 is localized on polymorphonuclear leukocytes and monocytes and mediates the binding to activated thy-1-positive microvascular endothelial cells and fibroblasts. J Invest Dermatol 115: 882–888.
26. McTaggart SJ (2006) Isoprenylated proteins. Cell Mol Life Sci 63: 255–267.
27. Rikitake Y, Liao JK (2005) Rho GTPases, statins, and nitric oxide. Circ Res 97: 1232–1235.
28. Bouterf HL, Sattelmeyer V, Czud S, Vordermark D, Rosenk K, et al. (2000) Inhibition of Ras farnesylation by lovastatin leads to downregulation of proliferation and migration in primary cultured human glioblastoma cells. Anticancer Res 20: 2761–2771.
29. Abedi H, Zachary I (1995) Signalling mechanisms in the regulation of vascular cell migration. Cardiovasc Res 30: 544–556.
30. Seabra MC (1998) Membrane association and targeting of prenylated Ras-like GTPases. Cell Signal 10: 167–172.
31. Ridley AJ, Hall A (1992) The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70: 389–399.
32. Ridley AJ, Schwartz MA, Burridge K, Firtel RA, Ginsberg MH, et al. (2003) Cell migration: integrating signals from front to back. Science 302: 1704–1709.
33. Van Arsd L, D’Souza-Schorey C (1997) Rho GTPases and signaling networks. Genes Dev 11: 2295–2322.
34. Fryer BH, Field J (2005) Rho, Rac, Pak and angiogenic old roles and newly identified responsibilities in endothelial cells. Cancer Lett 229: 15–23.
35. Hall A (1998) Rho GTPases and the actin cytoskeleton. Science 279: 509–514.