Involvement of RhoA/Rho Kinase Signaling in VEGF-Induced Endothelial Cell Migration and Angiogenesis In Vitro

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Objective—Growth factor–induced angiogenesis involves migration of endothelial cells (ECs) into perivascular areas and requires active remodeling of the endothelial F-actin cytoskeleton. The small GTPase RhoA previously has been implicated in vascular endothelial growth factor (VEGF)–induced signaling pathways, but its role has not been clarified.

Methods and Results—VEGF induced the activation of RhoA and recruited RhoA to the cell membrane of human ECs. This increase in RhoA activity is necessary for the VEGF-induced reorganization of the F-actin cytoskeleton, as demonstrated by adenoviral transfection of dominant-negative RhoA. Rho kinase mediated this effect of RhoA, as was demonstrated by the use of Y-27632, a specific inhibitor of Rho kinase. Inhibition of Rho kinase prevented the VEGF-enhanced EC migration in response to mechanical wounding but had no effect on basal EC migration. Furthermore, in an in vitro model for angiogenesis, inhibition of either RhoA or Rho kinase attenuated the VEGF-mediated ingrowth of ECs in a 3-dimensional fibrin matrix.

Conclusions—VEGF-induced cytoskeletal changes in ECs require RhoA and Rho kinase, and activation of RhoA/Rho kinase signaling is involved in the VEGF-induced in vitro EC migration and angiogenesis. **(*Arterioscler Thromb Vasc Biol*. 2003;23:211-217.)**

Key Words: endothelial cells ■ migration ■ angiogenesis ■ cytoskeleton ■ stress fibers

Angiogenesis, the formation of new blood vessels from existing ones, is involved in tissue repair and a variety of diseases, including tumor development, diabetic retinopathy, and rheumatoid arthritis.[1–3] Vascular endothelial growth factor-A (VEGF) is a major angiogenic factor. VEGF induces endothelial permeability,[4] migration, proliferation, and angiogenesis[5] by multiple mechanisms, including alteration of the F-actin cytoskeleton[6] and induction of gene expression.[2,3]

Reorganization of the F-actin cytoskeleton and cell-matrix adhesion play crucial roles in endothelial cell (EC) migration in angiogenesis and the repair of injuries along the endothelium. For cells to migrate, they must form new lamellipodia, adhere to the substratum at the front of the cell, detach from the substratum at the tail of the cell, and retract their tail.[7] Formation of adhesive structures and cellular contraction are essential in this process. ECs contain cytoskeletal "cables" of F-actin and nonmuscle myosin filaments that can contract and exert tension.[8,9] A prominent group of these F-actin cables are the stress fibers (SFs), which are linked to the cell membrane at focal adhesions (FAs). VEGF is known to induce the formation of SFs and FAs in vitro.[6] In vivo, SFs occur mainly in ECs of large arteries[10] and, to a lesser extent, in those of the entire microvasculature,[11] but they are largely absent from the venous system.[10,12] Many studies have shown that SFs develop during EC adaptation to unfavorable or pathological situations, including wound healing, atherosclerosis, and hypertension.[12,13]

It has become well established that the formation of SFs and FAs is induced by Ras-related GTPases of the Rho family.[14] Indeed, it was found that Rho-like small GTPases regulate cell motility[15] in a variety of cell types. Using C3 transferase, a specific inhibitor of the small GTPase RhoA, Aepfelbacher et al[16] showed that Rho is involved in wound-induced formation of SFs and endothelial migration in an in vitro wound-healing assay.

Numerous effectors have been identified for RhoA. The best-characterized effector is Rho kinase.[17] Rho kinase was shown to be involved in the formation of SFs and FA complexes[18] and to increase myosin light-chain phosphorylation.[19] Uehata et al[20] and Ishizaki et al[21] reported a synthetic pyridine analogue that inhibits Rho kinase with high specificity compared with myosin light-chain kinase. This cell-permeant inhibitor, Y-27632, was able to prevent RhoA-mediated SF formation in smooth muscle cells.

RhoA is coupled to $G\alpha_{13}$ via p115RhoGEF, a guanine nucleotide exchange factor that activates RhoA.[22] Mice lacking $G\alpha_{13}$ failed to develop an organized vascular system at E8.5, even though ECs had been differentiated in these embryos.[23] These results indicate that $G\alpha_{13}$ is required for angiogenesis. Angiogenesis depends on cell migration. Cell

Received October 7, 2002; revision accepted November 25, 2002.

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Arterioscler Thromb Vasc Biol. **is available at http://www.atvbaha.org** **DOI: 10.1161/01.ATV.0000054198.68894.88**

migration may be defective in these mice, because fibroblasts generated from these embryos do not migrate in response to $G\alpha_{13}$-coupled receptor agonists such as thrombin.[23] These results suggested that Rho signaling might play a role in angiogenesis. Indeed, Hla and coworkers (Lee et al[24]) found evidence for the role of RhoA in sphingosine-1-phosphate–induced in vitro angiogenesis, which was completely blocked by inhibition of RhoA by C3 transferase. Spontaneous angiogenesis in vitro (Matrigel assay) and in vivo (chick chorioallantoic membrane [CAM] assay) was also dependent on RhoA.[25] Other studies pointed to a more prominent role for the related small GTPase Rac.[26–28] Recent interest in the proangiogenic and antiangiogenic effects of the widely used group of cholesterol-lowering drugs called statins suggested that inactivation of RhoA accounts for the antiangiogenic effects of statins.[29,30]

In the present study, we investigated whether RhoA/Rho kinase signaling is involved in VEGF-induced in vitro EC migration and angiogenesis and whether RhoA/Rho kinase signaling might play a role in the accompanying cytoskeletal changes.

Methods

Materials

Medium 199 supplemented with 20 mmol/L HEPES, L-glutamine, and penicillin/streptomycin were obtained from BioWhittaker; newborn calf serum was obtained from Gibco. Tissue-culture plastic ware was from Costar. A crude preparation of EC growth factor was prepared from bovine brain. Human serum was obtained from a local blood bank and was prepared from 10 to 20 healthy donors, pooled, and stored at 4°C. Human serum albumin (HSA) was from Sanguin CLB (Amsterdam, the Netherlands). Trypsin was purchased from Gibco, heparin and thrombin were from Leo Pharmaceutical Products, and human fibrinogen was from Chromogenix. Factor XIII was generously provided by Drs H. Boeder and P. Kappus (Centeon Pharma, Marburg, Germany). Human recombinant tumor necrosis factor (TNF-α) was a gift from Dr J. Travernier (Biogent, Gent, Belgium). VEGF was a gift from Dr H.A. Weich (Braunschweig, Germany). C3 transferase and Ad-N19RhoA were kind gifts from Dr A.J. Ridley (Ludwig Cancer Institute, London, UK).[31] Rhodamine phalloidin was from Molecular Probes. Y-27632 was supplied by Yoshitomi Pharmaceutical Industries (Saitama, Japan).

Cell Culture and Adenoviral Transfection

Human foreskin microvascular endothelial cells (hMVECs) and human umbilical vein endothelial cells (HUVECs) were isolated, cultured, and characterized as previously described.[32,33] ECs were cultured on fibronectin-coated dishes in medium 199 supplemented with 20 mmol/L HEPES (pH 7.3), 10% heat-inactivated human serum, 10% heat-inactivated newborn calf serum, 150 mg/mL crude EC growth factor, 2 mmol/L L-glutamine, 5 U/mL heparin, 100 IU/mL penicillin, and 100 mg/mL streptomycin at 37°C under a 5% CO_2/95% air atmosphere. Twenty-four hours before the experiments were started, growth factor was withdrawn from hMVEC cultures. Adenoviral transfection was performed as described.[31]

Assay of RhoA Activity and Membrane-Bound RhoA

Rhotekin-binding assays were performed essentially as described.[34,35] In brief, 30-cm^2 confluent HUVECs were preincubated for 1 hour in medium 199 plus 1% HSA. Cells were stimulated and lysed. Lysates were cleared by centrifugation and incubated with bacterially produced GST-RBD (where RBD stands for the Rho-binding domain of Rhotekin and GST for gluthathione-*S*-transferase) immobilized on glutathione-coupled Sepharose beads for 30 minutes at 4°C. Beads were washed, eluted in Laemmli sample buffer, and analyzed by Western blotting with a rabbit polyclonal anti-RhoA antibody. Membrane-bound RhoA was separated from cytosolic RhoA as before.[36]

Migration Assay

Three- to 5-day postconfluent hMVECs with the typical cobblestone morphology were used. Wounds with a constant diameter were made with a sterile pipette tip. Before being wounded, EC monolayers were pretreated for 30 minutes with 10 μmol/L Y-27632, and Y-27632 remained present for the next 24 hours. Immediately after wounding and at the end of the experiment (after 24 hours), wounds were photographed and semiquantitative measurements were made of control and treated wounds. A mean wound width was determined, and an average percent wound closure was calculated.

In Vitro Angiogenesis Model

In vitro angiogenesis assays were performed in 3-dimensional fibrin matrixes as described previously.[33] The formation of tubular structures of ECs in the 3-dimensional fibrin matrix was analyzed by phase-contrast microscopy, and the mean length of tubelike structures of 6 randomly chosen microscopic fields (7.3 mm^2/field) was measured with an Olympus CK2 microscope equipped with a monochrome CCD camera (MX5) connected to a computer with Optimas image analysis software and expressed as a percentage of control.

F-Actin Staining

The presence of F-actin was visualized by direct staining with rhodamine-phalloidin (1:100) in ECs grown on glass coverslips.[37] 4′,6-diamidino-2-phenylindole (DAPI) was present in the mounting medium (Vectashield from Vector) as a nuclear counterstain.

Statistical Analysis

Data are reported as mean±SD. Comparisons between >2 groups were made by 1-way ANOVA, followed by a Bonferroni-adjusted χ^2 test. Differences were considered significant at the $P<0.05$ level.

Results

VEGF-Induced Changes in the EC Cytoskeleton Require Activation of RhoA and Rho essentially

To study the effects of VEGF on the endothelial cytoskeleton, hMVECs were stained for F-actin with rhodamine-phalloidin. Nonstimulated EC monolayers appeared as tightly connected cells with a characteristic peripheral rim of F-actin (Figure 1). Stimulation of the cells with 10 ng/mL VEGF caused an increase in cytoplasmic F-actin staining visible after 10 minutes (Figure 1), accompanied by the loss of peripheral F-actin. Many SFs were formed, which remained present for at least 3 hours. Occasionally, VEGF also induced the formation of small gaps between neighboring cells (indicated with an arrow in Figure 1).

To evaluate whether VEGF induces RhoA activity, we used a pulldown assay with the fusion protein GST-RBD, which recognizes only RhoA-GTP, the active form of RhoA. Thrombin induced a robust increase in RhoA-GTP, thus providing a positive control.[35] A modest but consistent increase in RhoA-GTP was observed in cells treated for 1 minute with VEGF (Figure 2A); it returned to basal levels after 30 minutes. The increase in RhoA activity was accompanied by a more sustained recruitment of RhoA to the cell membrane (Figure 2B).

To assess the role of RhoA in regulating VEGF-induced changes in the F-actin cytoskeleton, confluent HUVECs were infected with recombinant adenoviruses that express the

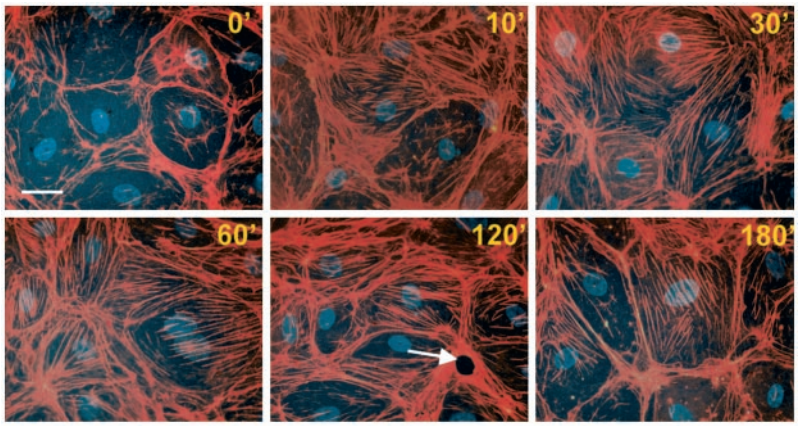

Figure 1. VEGF induces the formation of F-actin stress fibers in hMVECs. hMVECs were preincubated for 1 hour in medium 199 plus 1% HSA and stimulated with 10 ng/mL VEGF-A for the indicated times. Arrow indicates the presence of a gap between neighboring ECs. F-actin was visualized by rhodamine-phalloidin staining. Nuclei were counterstained by DAPI. Bar=10 μm. Similar results were observed in 5 different cultures.

dominant-negative mutant N19RhoA.[31] Infection with Ad-LacZ slightly increased cortical F-actin in control cells but had no effect on VEGF-induced SF formation. Infection with Ad-N19RhoA prevented VEGF-mediated increases in SFs to a large extent (Figure 3A). Similarly, preincubation for 24 hours with 5 μg/mL of the RhoA inhibitor C3 transferase abolished the VEGF-induced cytoskeletal reorganization (not shown). In cells pretreated with the Rho kinase inhibitor Y-27632, F-actin was strictly located at the peripheral rim (Figure 3B). Cells developed a wrinkled appearance but remained tightly connected. VEGF was no longer able to induce SFs in these cells. Taken together, these data indicate that VEGF-induced changes in the endothelial F-actin cytoskeleton require activation of RhoA and Rho kinase.

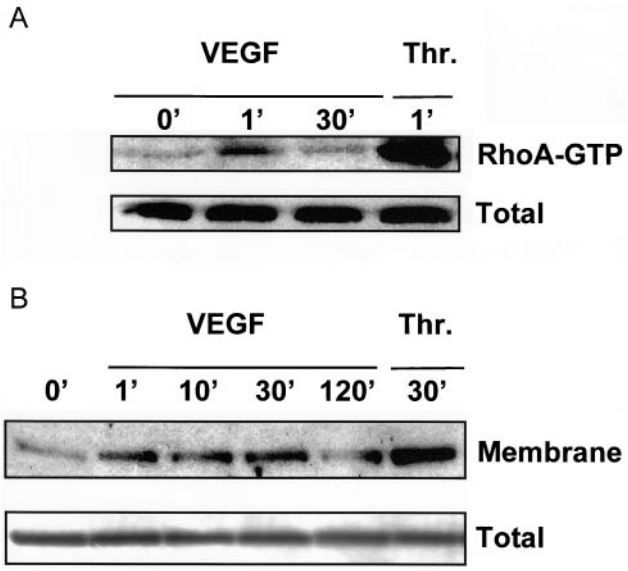

Figure 2. VEGF and thrombin (Thr.) activate RhoA and recruit RhoA to the cell membrane. ECs were stimulated for the indicated times with VEGF-A (25 ng/mL) or thrombin (1 U/mL). A, Active RhoA (RhoA-GTP) was affinity precipitated with GST-RBD and quantified by immunoblot analysis. A fraction of the original extracts was taken and also studied by immunoblot analysis (total), showing equal amounts of total RhoA protein in all samples. B, Membrane fractions (upper panel) were isolated from total lysates (lower panel) by ultracentrifugation at 100 000*g* for 1 hour. RhoA in each fraction was determined by immunoblot analysis. Different exposure times for total and membrane-bound RhoA are shown.

Inhibition of Rho Kinase Reduces VEGF-Induced Cell Migration

To investigate whether Rho kinase is involved in the VEGF-enhanced endothelial migration, confluent and quiescent monolayers of hMVECs were wounded. Recovery of these monolayers depends on migration only, because proliferation of ECs in response to wounding does not start before 24 hours.[38] Twenty-four hours after wounding, the percentage of wound closure under nonstimulated conditions was 52±9%, so that both stimulation and inhibition of EC migration could be determined (Figure 4B).

Treatment with 10 ng/mL VEGF enhanced endothelial migration significantly (Figure 4B). In the presence of 10 μmol/L Y-27632, basal EC migration was slightly but not statistically significantly inhibited when compared with control conditions. However, coincubation of VEGF with Y-27632 abolished the VEGF-enhanced migration completely (Figure 4B). These data indicate that inhibition of Rho kinase does not alter basal EC migration but prevents the VEGF-induced EC migration.

Role of Rho Kinase in the Formation of Tubular Structures by hMVECs in a Fibrin Matrix

Stimulation with a combination of VEGF and TNF-α induced the formation of tubular structures that invaded a fibrin matrix when hMVECs were cultured on top of the fibrin matrix (Figure 5A and 5B and Koolwijk et al[33]). The confluent monolayer remained unaltered in the absence of either growth factor or cytokine (cf Figure 5A, a).

Simultaneous incubation of the VEGF/TNF-α–stimulated monolayers for 7 days with the Rho kinase inhibitor Y-27632 did not alter the morphology of the EC monolayers on top of the fibrin matrix or the attachment of cells to the fibrin matrix, indicating that Y-27632 had no toxic effects on the ECs (Figure 5A, c and d). Treatment with Y-27632 reduced the mean tube length of the capillary-like tubular structures formed in response to VEGF/TNF-α in a dose-dependent manner (Figure 5B). However, at a maximally inhibiting concentration of 10 μmol/L Y-27632, the number of onsets of tubelike structures that started to form in response to VEGF/TNF-α increased (Figure 5A and 5B). In hMVECs treated with 5 μg/mL C3 transferase (renewed every other day), mean tube length was reduced by 40.2±13.6% (mean±SD, 8 determinations, P=0.01). Similarly, transfection of Ad-

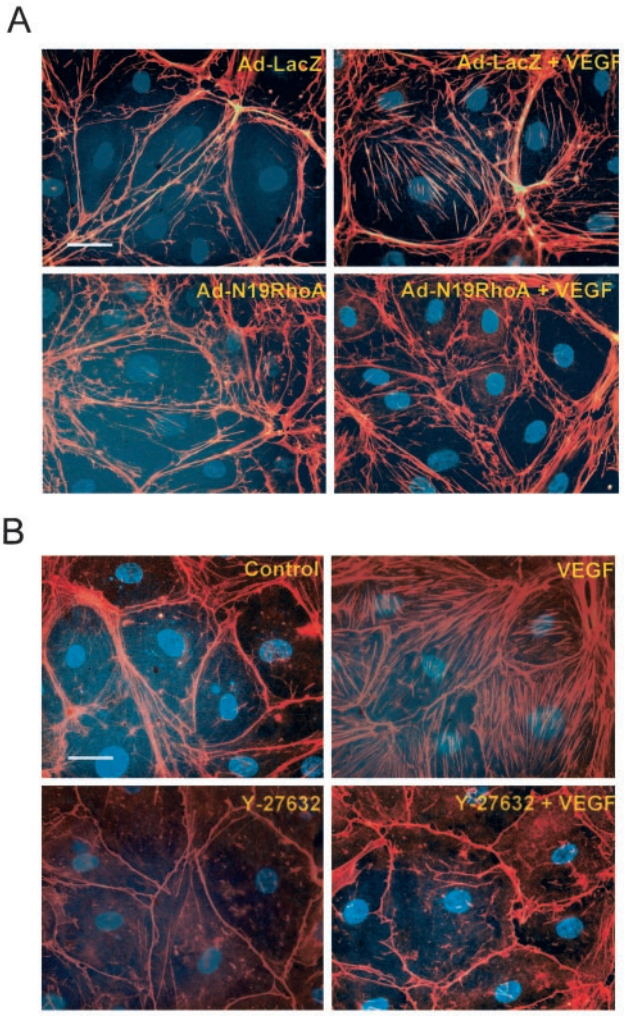

Figure 3. VEGF-induced changes in the EC cytoskeleton depend on RhoA and Rho kinase. A, hMVECs were infected with Ad-LacZ or Ad-N19RhoA. Two days after transfection, cells were incubated for 1 hour in medium 199 plus 1% HSA and subsequently stimulated with 10 ng/mL VEGF for 60 minutes or treated with vehicle (Control). B, hMVECs, pretreated for 1 hour with 10 μmol/L Y-27632 or untreated, were stimulated with 10 ng/mL VEGF for 60 minutes or treated with vehicle (Control). F-actin was visualized by rhodamine-phalloidin staining. Nuclei were counterstained by DAPI. Bar=10 μm.

N19RhoA reduced mean tube length by 48.5±17.6% (mean±SD, 10 determinations, $P=0.001$) compared with Ad-LacZ–transfected cells. Thus, RhoA and Rho kinase activity are necessary for the proper ingrowth of ECs in a fibrin matrix.

Discussion

The major finding of this study is that inhibition of either RhoA or Rho kinase reduced VEGF-induced endothelial migration and angiogenesis in vitro and abolished the accompanying growth factor–induced changes in the endothelial cytoskeleton. Furthermore, we show that VEGF induces a modest RhoA activation.

Stimulation of endothelial monolayers with VEGF induced the formation of SFs in accordance with other reports.[6,39] These SFs were formed rapidly and were maintained for at least 3 hours. They probably exist much longer. Cohen et al[40] reported the expression of SFs in ECs 45 hours after VEGF treatment. In Swiss 3T3 cells, transfection of constitutive active Rho kinase results in the formation of SFs and is prevented by inhibition of Rho kinase.[20,41] In the present study, we show that Rho kinase is also involved in VEGF-induced cytoskeletal changes in ECs.

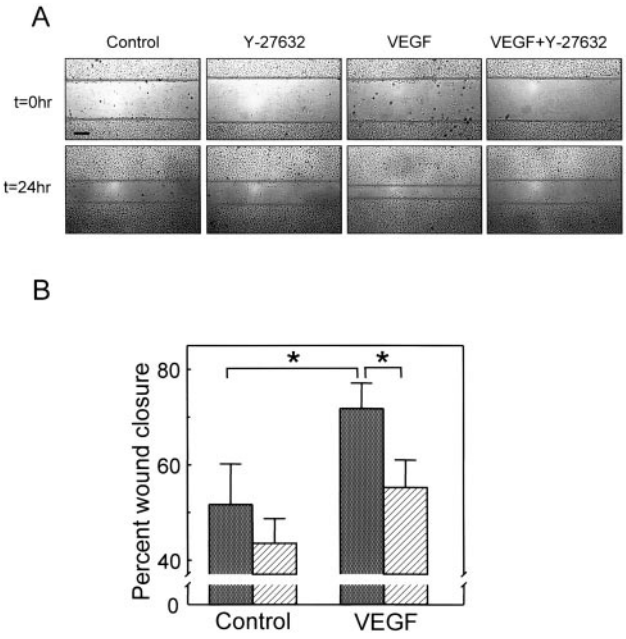

Figure 4. Effects of Y-27632 on EC migration in vitro. A, Confluent monolayers of hMVECs were pretreated with 10 μmol/L Y-27632 for 1 hour, when indicated, and wounded. Subsequently, the ECs were stimulated with 10 ng/mL VEGF or treated with vehicle (Control). Photographs were taken directly after wounding (t=0 hours) and 24 hours after wounding (t=24 hours). Bar=300 μm. B, Quantification of the endothelial wound repair. EC migration was quantified 24 hours after addition of VEGF or vehicle (Control). Hatched bars indicate hMVECs pretreated with 10 μmol/L Y-27632 for 1 hour; filled bars, sham-treated hMVECs. Values are mean±SD from 6 cultures in 2 independent experiments. *$P<0.05$.

Our study provides evidence that VEGF induces RhoA activity. This enhanced RhoA activation is accompanied by recruitment of RhoA to the cell membrane. The mechanisms by which VEGF activates RhoA in ECs remain unclear. Recently, some evidence was provided that VEGFR-2, Gq/11, and phospholipase C are involved in the signaling pathway.[42] Gingras et al[43] have shown involvement of RhoA in tyrosine phosphorylation of VEGF-activated signaling intermediates, including FAK, paxillin, and phospholipase C-γ. Interestingly, activation of RhoA resulted in an increase in tyrosine phosphorylation of the primary VEGF receptor VEGFR-2 and its kinase activity, thus providing a potential mechanism for the role of RhoA in VEGF-induced angiogenesis.

A marked difference in the level of RhoA activation by VEGF and thrombin was observed. Interestingly, Jo et al[44] observed similar differences in the level of RhoA activation by urokinase plasminogen activator (uPA; modest)- and lysophosphatidic acid (robust)-stimulated cells. They proposed a model in which the Ras–extracellular-related kinase

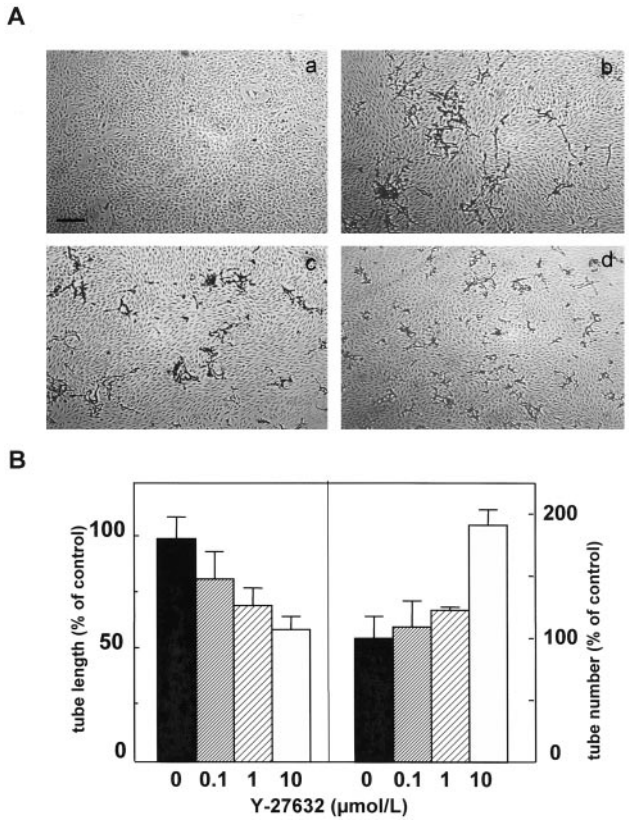

Figure 5. VEGF-induced capillary-like tube formation is reduced by the Rho kinase inhibitor Y-27632. A, hMVECs were cultured on top of a 3-dimensional fibrin network and were either left unstimulated (a) or were stimulated with a combination of 25 ng/mL VEGF/1 ng/mL and TNF-α (b, c, and d) either in the absence (b) or presence of 1 μmol/L Y-27632 (c) or 10 μmol/L Y-27632 (d). After 7 days of incubation, photomicrographs were taken. Bar=300 μm. B, Dose-dependent effect of Y-27632 on the mean tube length (left panel) and mean tube number (right panel) formed after 7 days by VEGF/TNF-α–stimulated hMVEC monolayers. Values are mean±SD of 6 cultures.

(ERK) and RhoA/Rho kinase pathways cooperate to promote uPA-induced cell migration. Neutralizing either pathway was sufficient to block the uPA response, indicating an important contribution of the modest RhoA activation to the uPA response. The different levels in RhoA activation between thrombin- and VEGF-stimulated cells that we observed are likely to underlie functional differences between these 2 mediators. Thrombin stimulates a generalized contraction of ECs mediated by RhoA.[35] F-actin staining did not reveal such an effect in VEGF-stimulated cells. However, we observed that in VEGF-stimulated cells, RhoA signaling is necessary for migration. Under those conditions, RhoA is likely to mediate retraction of the trailing edge of migrating cells, analogous to its role in leukocyte (trans)migration.[45] Migration is crucial for the repair of injured blood vessels, angiogenesis, and atherogenesis and is accompanied by the formation of SFs in vivo.[46] Conflicting results have been reported with regard to the role of RhoA and Rho kinase in cell migration. Aepfelbacher et al[16] found that inhibition of RhoA with a high concentration of C3 transferase attenuated HUVEC migration. Nobes and Hall[47] showed that treatment of fibroblast monolayers with C3 transferase had no effect on wound closure, and Y-27632 even slightly enhanced wound closure. Here, we show that these apparently contradictory data probably result from differences in the type of stimulus for migration. In our experience, Y-27632 did not significantly affect basal cell migration, in accordance with the data of Nobes and Hall. However, VEGF-induced endothelial migration was completely blocked by Y-27632. This fits with the data from Aepfelbacher et al, as their evidence for a role of RhoA in migration was derived from experiments performed in the presence of an endothelial growth supplement.

Inhibition of Rho kinase did not prevent onset of the in vitro angiogenesis process. At a maximal effective concentration of 10 μmol/L, treatment with Y27632 even increased the number of capillary-like tubes. This means that the initial cell movement is not affected by Y-27632 but that the ingrowth in the fibrin matrix is inhibited. This is likely to be the result of the disturbed migratory capability of the ECs. The increase in tube number probably reflects a lower adhesive capability that allows endothelial remodeling, because treatment with Y-27632 resulted in the loss of FAs (vinculin staining; data not shown), the anchoring structures of the cytoskeleton to the matrix.

The cholesterol-lowering statins interfere with RhoA activation in ECs.[36,48] Thus, the involvement of Rho proteins in angiogenesis described in this study may be 1 of the factors that contribute to the overall effect of statins on angiogenesis. Both proangiogenic[49,50] and angiostatic[29] effects of statins have recently been reported. Those opposite effects of statins might be partly explained by distinct mechanisms of angiogenesis associated with cancer, tissue ischemia, or inflammation.[51] Other investigators reported proangiogenic effects of statins at low- and angiostatic effects at high-dose statin treatment[52,53]: the former via stimulation of the phosphatidylinositol-3-kinase pathway[53] and the latter via inhibition of protein geranylation, most likely geranylation of RhoA.[29,52] This latter inhibition may become more important in conditions in which the activation of the phosphatidylinositol-3-kinase/AKT pathway is compromised. Finally, statins might promote reendothelialization of injured areas in the vasculature.[54] No clinical evidence is currently available demonstrating that statins increase morbidity as a consequence of increased tumor vascularization.

The process of angiogenesis in vivo is, already in its initial phase, accompanied by an increase in endothelial permeability. This increase in permeability results in the formation of a fibrinous exudate and the deposition of a provisional matrix, providing an excellent situation for the ingrowth of ECs. We and other investigators have previously shown that an increase in endothelial permeability, such as can be induced by thrombin, is mediated by activation of RhoA and Rho kinase.[31,35,55] The effects of VEGF are complex and involve both the formation of transendothelial pores and intercellular gaps.[56] The VEGF-induced changes in the EC cytoskeleton observed under our experimental conditions were accompanied by the formation of small gaps between neighboring ECs, indicative of endothelial barrier dysfunction. These data support the idea that the changes that occur in microvascular

ECs, which contribute to increased permeability, also facilitate generation of a proangiogenic state of the endothelium.

Acknowledgments

This study was financially supported by the Netherlands Heart Foundation (grant 94.048) and the Dutch Cancer Society (grant TNOP 97 to 1511). We would like to thank Jos Grimbergen, Erna Peters, and Bea van der Vecht for excellent technical assistance.

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