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Terbinafine inhibits endothelial cell migration through suppression of the Rho-mediated pathway

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Abstract

We showed previously that terbinafine, an allylamine with fungicidal activity, could inhibit angiogenesis by suppressing the endothelial cell proliferation. In the present study, we further showed that terbinafine (0–120 μmol/L) dose dependently inhibited the adhesion and migration of human umbilical vascular endothelial cells (HUVEC). Western blot analysis showed that terbinafine decreased the levels of Ras protein and membrane-bound RhoA protein. Moreover, the terbinafine-induced migration inhibition in HUVEC was prevented by pretreatment with farnesol or geranylgeraniol. Pretreatment of HUVEC with Ras inhibitor peptide or a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, Y27632, abolished the farnesol- or geranylgeraniol-induced prevention effect on the terbinafine-induced migration inhibition, respectively. These data suggest that the consuming or depletion of geranylgeranyl pyrophosphate and consequent suppression of protein geranylgeranylation and farnesylation, which is essential for activation of Rho GTPases and Ras, respectively, might account for the terbinafine-induced inhibition of HUVEC migration. The levels of phosphorylated focal adhesion kinase and paxillin protein and the mRNA levels of matrix metalloproteinase-2 and matrix metalloproteinase-9 were also decreased by terbinafine treatment. Taken together, these results indicate that suppression of Rho-mediated pathway might be involved in the signal transduction leading to the inhibition of cell migration caused by terbinafine in HUVEC. [Mol Cancer Ther 2006;5(12):3130–8]

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Introduction

Angiogenesis, the formation of blood vessels from preexisting 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 pathologic processes, including tumor growth, metastasis, diabetic retinopathy, and arthritis (2, 3). The processes of angiogenesis involve four distinct sequential steps, including the following: (*a*) proteolytic breakdown of the basement membrane (4, 5), (*b*) migration of endothelial cells toward the angiogenic stimulus, (*c*) endothelial cell proliferation, and (4) lumen formation (2, 6).

Angiogenesis is a complex multistep process involving extensive interplay between cells, soluble factors, and extracellular matrix (ECM) components. Migration of endothelial cells is a critical step in the angiogenic process. Activated endothelial cells reorganize their cytoskeleton, 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.

Previously, we showed that the antifungal drug, terbinafine, which is an inhibitor of ergosterol synthesis at the stage of squalene epoxidation and has been used for treating the toenail onychomycosis, could suppress proliferation of various tumor cells *in vitro* and *in vivo* by inhibiting DNA synthesis and activating apoptosis (7). We also found that terbinafine could inhibit the proliferation of cultured human umbilical vascular endothelial cell (HUVEC), the capillary-like tube formation, and sprouting of capillary. The terbinafine-induced cell cycle arrest in HUVEC occurred when the activity of cyclin-dependent kinase 2 was inhibited just as the protein level of p21 was increased and cyclin A was decreased (8). In the present study, we continued to study the mechanism of terbinafine-induced antiangiogenic activity by examining the effect of terbinafine on HUVEC adhesion and migration.

Materials and Methods

Cell Cultures

HUVEC were cultured in gelatin-coated plates with M199 medium containing fetal bovine serum (10%), sodium heparin (100 units/mL), endothelial cell growth supplement (0.03 mg/mL), and kanamycin (10 mg/mL) and incubated at 37°C in 5% CO_2 in air. Cells from passages 5 to 10 were used.

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Adhesion Assay

Adhesion assays were done as described previously (9). Briefly, HUVEC were plated onto a collagen-coated (0.1 mg/mL) or fibronectin-coated (0.1 mg/mL) 24-well plate, treated with various concentrations of terbinafine (0–120 μmol/L) for 1, 4, or 6 h at 37°C, and then washed with PBS. After washing, the adherent cells were allowed to grow in cultured medium for the other 18 h without terbinafine before cell counting. The cell number was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (10).

Migration Assay

Migration assay was done as described previously with minor modifications (9, 11). To assess the migration potential of HUVEC, the lower face of Transwells (8-μm pore size) was precoated with type 1 collagen at a concentration of 1 mg/mL for 1 h at 37°C. The Transwells were then assembled in a 24-well plate, and the lower chambers were filled with 600 μL M199 containing 10% fetal bovine serum and endothelial cell growth-stimulating factors. Two hundred microliter of cells (10^5 per mL) were inoculated onto the upper chamber of each Transwells. Terbinafine (0–120 μmol/L) was added into the medium of lower and upper chambers. The plate was then placed at 37°C in 5% CO_2/95% air for 18 h. After removing the nonmigrating cells with a cotton swab, cells that had migrated to the lower surface of the filters were fixed and stained with 0.1% crystal violet/20% (v/v) methanol. All assays were done in triplicate. Three random fields were chosen in each insert, and the cells were counted and photographed under a light microscope (×200).

Subcellular Fractionation

The cells were washed with cold PBS, lysed by Dounce homogenizer in lysis buffer [20 mmol/L Tris (pH 8.0), 3 mmol/L $MgCl_2$, 1 mmol/L phenylmethylsulfonyl fluoride], 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, then homogenized in the lysis buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1% NP40, 0.1% SDS] on ice, and centrifuged at 12,000 × *g* for 30 min at 4°C. The supernatant was collected as the particulate (membrane) fraction. Proteins of cytosolic and particulate fractions were detected for RhoA, RhoB, and RhoC by Western blot analysis.

Western Blot Analysis

To determine the expression levels of focal adhesion kinase (FAK), paxillin, Ras, RhoA, RhoB, RhoC, and glyceraldehyde-3-phosphate dehydrogenase (G3PDH) in HUVEC, the total proteins were extracted, and Western blot analyses were done as described previously (8). Briefly, HUVEC were cultured in 15-cm Petri dishes. After reaching subconfluence, the cells were rendered quiescent by incubation for 24 h in M199 containing 2% fetal bovine serum. The cells were released from quiescence with culture medium containing 10% fetal bovine serum, treated with various concentrations of terbinafine (0–120 μmol/L), and then incubated at 37°C. At different time points, the cells were washed with PBS (pH 7.4), incubated with extraction buffer [50 mmol/L Tris (pH 7.5), 150 mmol/L NaCl, 1 mmol/L phenylmethylsulfonyl fluoride, 1% NP40, 0.1% SDS] on ice, and then centrifuged at 12,000 × *g* for 30 min. The cell extract was then boiled in a ratio of 3:1 with sample buffer [250 mmol/L Tris-HCl (pH 6.8), 40% glycerol, 400 mmol/L DTT, 8% SDS, 0.2% bromphenol blue]. Electrophoresis was carried out using a 12% SDS-polyacrylamide gel (2 h, 110 V, 40 mA, and 50 μg protein per lane). Separated proteins were transferred to polyvinylidene difluoride membranes (1 h and 400 mA), treated with 5% fat-free milk powder (Anchor, Auckland, New Zealand) to block the nonspecific IgGs, and incubated for 1 h with specific antibody for FAK, phosphorylated FAK, paxillin, phosphorylated paxillin, Ras, RhoA, RhoB, RhoC, or G3PDH at a concentration of 0.2 μg/mL (Jackson ImmunoResearch Laboratories, West Grove, PA). The blot was then incubated with anti-mouse, anti-rabbit, or anti-goat IgG (Jackson ImmunoResearch Laboratories) linked to horseradish peroxidase (1:10,000) for 1 h. Subsequently, the blot was developed using the enhanced chemiluminescence system (Amersham, Buckinghamshire, England). The intensity of each band was quantified by densitometry analysis using Image Pro Plus 4.5 software.

Reverse Transcription-PCR

Total cellular RNA was extracted from HUVEC with Trizol (Life Technologies, Carlsbad, CA) according to the manufacturer's protocol (12). The RNA pellet was washed with 70% cold ethanol, air dried, and redissolved in 20 μL diethyl pyrocarbonate–treated water (13). Two microliter of total RNA were used in a total of 20 μL reaction volume as a template for PCR amplification. PCR was done under standard conditions in 20 μL of 10 mmol/L Tris (pH 8.3), 40 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 250 μmol/L dNTP, 10 pmol/L of each primer (sense and antisense), and 1 unit Taq DNA polymerase (14). The experimental conditions were as follows: 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The PCR regimen was as follows: matrix metalloproteinase (MMP)-2, 5′-TTCAAGGACCGGTTCATTTGGCGGACTGTG-3′ and 5′-TTCCAAACTTCACGCTCTTCAGACTTTGGTT-3′ (15); MMP-9, 5′-GGAGACCTGAGAACCAATCTC-3′ and 5′-TCCAATAGGTGATGTTGTCGT-3′; and G3PDH, 5′-TGAAGGTCGGAGTCAACGGATTTGGT-3′ and 5′-CATGTGGGCCATGAGGTCCACCAC-3′ (16). The PCR products were electrophoresed on a 1.8% agarose in 1× Tris-acetate/EDTA buffer and stained with ethidium bromide solution.

Statistics

All data were expressed as the mean value ± SE. Comparisons were subjected to one-way ANOVA followed by Fisher's least significant difference test. Significance was accepted at $P < 0.05$.

Results

Effect of Terbinafine on Migration of HUVEC

Previously, we showed that terbinafine exerts an anti-angiogenic activity through inhibiting the growth of

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endothelial cells (8). To examine whether inhibition of endothelial cell migration is also involved in the terbinafine-induced inhibition of angiogenesis, a Transwell migration assay was done in HUVEC. As shown in Fig. 1A, terbinafine at a range of concentrations (30–120 μmol/L) dose dependently inhibited the migration of HUVEC, and the migration activity was completely stopped when the terbinafine concentration reached 120 μmol/L.

Inhibition of Endothelial Cell Attachment to Matrix Caused by Terbinafine

Because the attachment and adhesion of endothelial cells to ECM are critical steps for the process of angiogenesis and would affect the endothelial cell migration, we further examined whether terbinafine could affect the cell adhesion on different matrices. Pretreatment of HUVEC for 24 h with terbinafine at a concentration of 120 μmol/L, but not 60 μmol/L or below, caused a significant decrease of cell number attached on the plate coated with collagen (Fig. 1B, *left*) or fibronectin (Fig. 1B, *right*).

Effect of Terbinafine on the Levels of Phosphorylated FAK and Paxillin

Because the focal adhesion complex is one of the important components promoting cell motility, the adhesive interactions between cells and ECM can influence the attachment and transmigration across the surrounding cells (17). We evaluated the effects of terbinafine on the formation of focal adhesion complexes by examining the levels of tyrosine phosphorylations of FAK and paxillin, two elementary proteins for forming stable adhesion complexes and transducing the survival/motility signals, in the terbinafine-treated HUVEC by using Western blot analysis. After 24 h of pretreatment of the cells with terbinafine (0–120 μmol/L) followed by 1 h challenged with 10% FBS, the ratios of both phosphorylated FAK/FAK (Fig. 2A) and phosphorylated paxillin/paxillin (Fig. 2B) were decreased in the terbinafine-treated HUVEC compared with vehicle-treated HUVEC (control). These terbinafine-induced inhibitions were in a dose-dependent manner.

Effect of Terbinafine on the Levels of Rho and Ras

To further delineate the molecular mechanisms underlying terbinafine-induced migration inhibition in HUVEC, we examined the protein levels of Rho and Ras in the terbinafine-treated HUVEC. As shown in Fig. 3A, terbinafine at a range of concentrations (0–120 μmol/L) dose dependently decreased the total protein levels of RhoB, but not Rho A and RhoC, in HUVEC. Because translocation of Rho GTPases from the cytosol to the cytoplasmic membrane is required for their activations and functions, we further examined the effect of terbinafine treatment on membrane translocation of Rho GTPases. As shown in Fig. 3B, terbinafine (0–120 μmol/L) dose

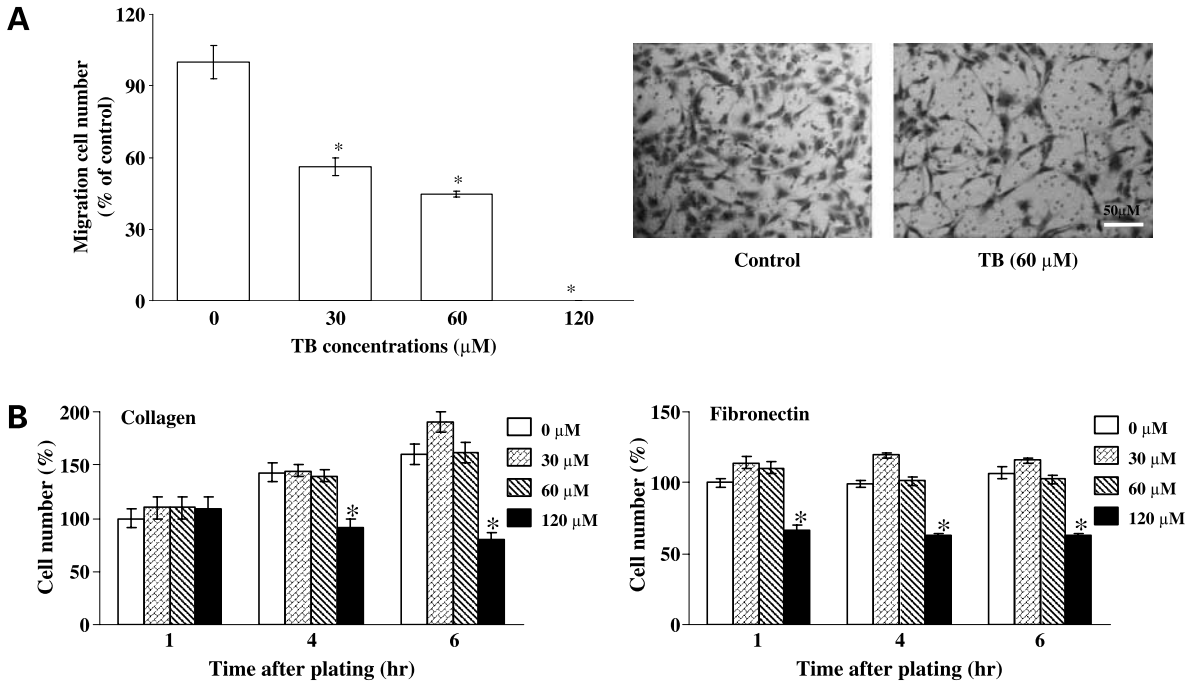

Figure 1. Inhibitory effects of terbinafine on HUVEC migration and adhesion. **A,** *left,* terbinafine (*TB*) dose dependently inhibited HUVEC migration through collagen (1 mg/mL); *right,* representative photographs show that terbinafine at a concentration of 60 μmol/L inhibited migration of HUVEC compared with control without terbinafine treatment. **B,** terbinafine inhibits HUVEC adhesion to collagen (*left*) and fibronectin (*right*). Wells were coated with collagen (0.1 mg/mL) or fibronectin (0.1 mg/mL). HUVEC were preincubated with terbinafine (0, 30, 60, or 120 μmol/L) for 24 h. The terbinafine-treated HUVEC were seeded on the precoated plates and allowed to attach for 1, 4, or 6 h. Cell numbers were assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The number of cells treated with vehicle without terbinafine for 1 h is 100%. *Columns,* mean ($n = 4$); *bars,* SE. *, $P < 0.05$ different from control.

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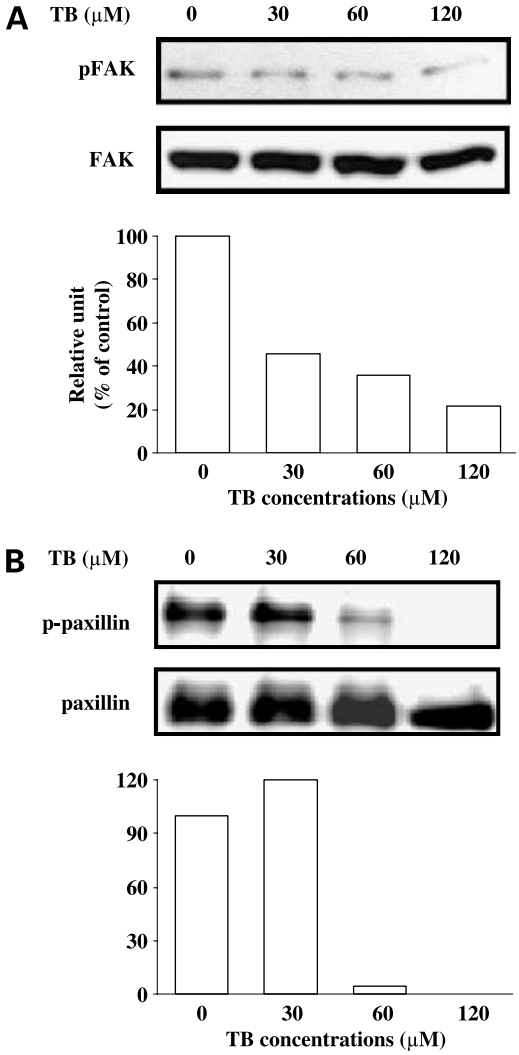

Figure 2. Terbinafine inhibits focal adhesion complex formation. HUVEC were treated with or without various concentrations of terbinafine. After treatment with terbinafine for 24 h, the cells were stimulated with serum for an additional 1 h followed by total protein extraction and Western blot analysis. Terbinafine dose dependently decreased the levels of phosphorylated FAK (*pFAK*; **A**) and paxillin (*p-paxillin*; **B**). Quantification was made by densitometry analysis using Image Pro Plus 4.5 software. *Bottom,* quantified results after being adjusted with their own total protein levels and expressed by percentage of control.

dependently increased the protein levels of RhoA in the cytosolic fraction of HUVEC. In contrast, RhoB is mainly located in the membrane fraction, and terbinafine did not affect the subcellular distribution of RhoB. The membrane translocation of RhoC was increased by terbinafine treatment. The absence of G3PDH immunoreactivity in the membrane fraction indicated the success of subcellular fractionation. Terbinafine also dose dependently inhibited the protein levels of Ras (Fig. 3C). These results indicate that the interference of prenylation might be involved in the inhibition of HUVEC migration caused by terbinafine.

Involvement of Suppressions of Farnesylation and Geranylgeranylation in Terbinafine-Induced Migration Inhibition in HUVEC

Because farnesylation and geranylgeranylation are important for subcellular distribution and functions of Ras and Rho, we further examined the involvement of farnesylation of the Ras proteins and geranylgeranylation of the Rho proteins in regulating the HUVEC migration. To examine whether prenylation was involved in the inhibition of migration induced by terbinafine, we pretreated HUVEC with isoprenoid pyrophosphate precursors (farnesol and geranylgeraniol) followed by terbinafine treatment. As shown in Fig. 4, pretreatment of HUVEC with farnesol (30 μmol/L) partially prevented the terbinafine-induced migration inhibition in HUVEC. This prevention effect caused by farnesol treatment was abolished by Ras inhibitor peptide (VPPPVPPRRR). Ras inhibitor alone caused a slight decrease in HUVEC migration. On the other hand, pretreatment of HUVEC with geranylgeraniol (30 μmol/L) completely prevented the terbinafine-induced migration inhibition in HUVEC (Fig. 5). This prevention effect caused by geranylgeraniol was abolished by 5 μmol/L Y27632, a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor. Moreover, treatment of HUVEC with Y27632 without terbinafine caused migration inhibition of HUVEC. Taken together, these data suggest that suppressions of farnesylation and geranylgeranylation might be involved in the terbinafine-induced migration inhibition in HUVEC.

Effects of Terbinafine on MMP Expression

The activity of MMPs is also associated with the process of angiogenesis. To evaluate the effects of terbinafine on the expression of MMP-2 and MMP-9, total RNA was isolated from HUVEC and reverse transcription-PCR analysis was conducted. The reverse transcription-PCR products of G3PDH were used as an internal control. Terbinafine at a range of concentrations (30–120 μmol/L) dose dependently inhibited the mRNA levels of MMP-2 (Fig. 6A) and MMP-9 (Fig. 6B), suggesting that inhibition of MMP-2 and MMP-9 might be involved in the terbinafine-induced antiangiogenic activity.

Discussion

Previously, we showed that terbinafine at a range of concentrations (30–120 μmol/L) dose dependently inhibited angiogenesis by suppressing the proliferation of HUVEC and capillary-like tube formation *in vitro* and sprouting angiogenesis *in vivo* (8). Here, we further showed that terbinafine (30–120 μmol/L) dose dependently inhibited HUVEC adhesion and migration. The findings of the present study suggest that the Rho-mediated pathway might be involved in the signal transduction leading to the migration inhibition caused by terbinafine in cultured HUVEC. To our knowledge, this is the first demonstration that terbinafine inhibits HUVEC migration through a Rho-dependent pathway.

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Vascular endothelial cell adhesion (on the substratum by integrins) is one of the principal requirements for cell migration and proliferation (18) and by which multiple integrins with distinct combinations of α/β subunits have been recognized (19). Integrin $\alpha_1\beta_1$ and $\alpha_2\beta_1$ mediate cell adhesion on collagen, which is a critical step in initiating tube formation of endothelial cells (20), whereas recruitment of integrin $\alpha_5\beta_1$, the fibronectin receptor, is required for cell migration in the process of angiogenesis. In the present study, we showed that terbinafine at a concentration of 120 μmol/L caused a 50% inhibition in the adhesion of endothelial cells on collagen- or fibronectin-coated plates (Fig. 1B). In examination of the effect of terbinafine on cell migration, we found that terbinafine at a concentration of 120 μmol/L completely inhibited HUVEC migration (Fig. 1A). These findings suggest that inhibition of cell adhesion could contribute only partially to endothelial migration inhibition induced by terbinafine treatment. Moreover, our previous study showed that terbinafine at a concentration of 120 μmol/L did not cause any cell death in HUVEC (8), suggesting that the terbinafine-induced endothelial migration inhibition was not due to cytotoxicity.

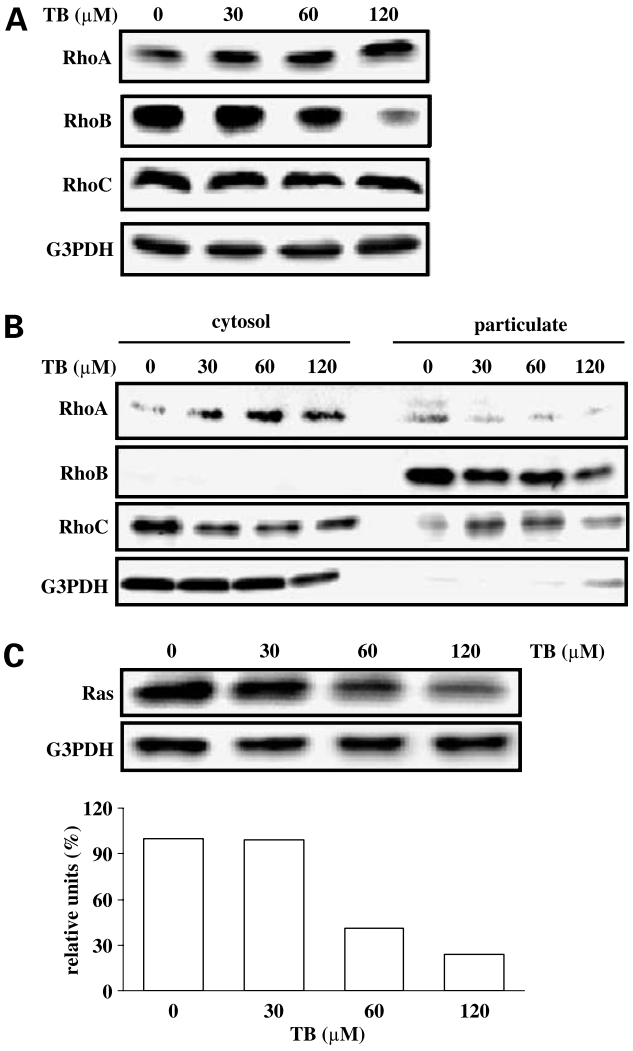

Figure 3. Effects of terbinafine on Rho expression and membrane translocation in HUVEC. HUVEC were incubated with or without various concentrations of terbinafine. **A,** terbinafine dose dependently inhibited the total protein levels of Rho B, but not RhoA and RhoC. **B,** terbinafine suppressed membrane-bound RhoA. Proteins were detected for RhoA, RhoB, and RhoC by Western blot analysis. In this experiment, 12% of SDS-acrylamide gels were used. **C,** terbinafine dose dependently inhibited the Ras protein levels.

In cholesterol synthesis pathway, squalene epoxidase converts squalene to 2,3-oxidosqualene. As an inhibitor of squalene epoxidase, terbinafine might alter the protein prenylation by changing the expression of farnesyl pyrophosphate and geranylgeranyl pyrophosphate (GGPP), which are the upstream molecules of squalene, and regulate protein post-translational modification. The importance of the protein prenylation is underscored by the nature of the ~300 prenylated proteins in the human proteome; many of them participate in a multitude of signal transduction pathways related to cell growth, differentiation, and migration (21). Here, we showed that farnesol (a precursor of farnesyl pyrophosphate) partially and geranylgeraniol (a precursor of GGPP) completely reversed the terbinafine-induced migration inhibition in HUVEC (Figs. 4 and 5), suggesting that synthesis inhibition or depletion of farnesyl pyrophosphate and GGPP, which is essential for protein farnesylation and geranylgeranylation, respectively, occurred in terbinafine-induced migration inhibition in HUVEC. It has been shown that inhibition of squalene epoxidase could induce the accumulation of squalene (22). Surprisingly, we found that terbinafine, an inhibitor of squalene epoxidase, caused a decrease of the farnesyl pyrophosphate and GGPP, which in turn inhibited endothelial cell migration. One of the possible explanations is that terbinafine might directly inhibit the synthesis of farnesyl pyrophosphate and GGPP or promote their degradation. However, the exact mechanisms underlying terbinafine-induced inhibition of polyisoprenyl pyrophosphates deserve further investigation.

It has been recognized that the post-translational modification of proteins by the addition of isoprenoids is a key physiologic process for facilitating cellular protein-protein interactions and membrane-associated protein trafficking (21). The results from the present study suggest that terbinafine could suppress the cell migration through altering the prenylation. Prenylation serves as important lipid attachments for the post-translational modification of a variety of 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 GGPP (23). Blockade of farnesyl biosynthesis leads to an inhibition in the Ras-mediated regulation of proliferation and migration in primary cultured human cells (24). Tyrosine phosphorylation of 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; ref. 25). To be functionally active, Rho proteins must be localized to

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the cell membrane by post-translational modification through addition of isoprenyl groups from isoprenoid pyrophosphate substrates (26). Rho GTPases play an important role in growth factor-stimulated cell migration and cytoskeletal organization, membrane trafficking, cell cycle control, and transcriptional activation (27–31). To test whether terbinafine suppressed HUVEC migration through regulating polyisoprenyl pyrophosphates synthesis, we examined the effect of terbinafine on the expression of isoprenylated protein. Terbinafine at a range of concentrations (30–120 μmol/L) dose dependently decreased the protein levels of Rho B, but not RhoA and RhoC protein (Fig. 3A). On the other hand, terbinafine dose dependently decreased the levels of membrane-bound RhoA and accumulated the RhoA in the cytosolic compartment (Fig. 3B). In contrast, terbinafine increased the levels of membrane-bound of RhoC and did not affect the levels of membrane bound of RhoB. These data suggested that terbinafine might interfere with cell migration via suppressing the prenylation of RhoA. Although various Rho GTPase family members (such as RhoA, RhoB, and RhoC) are highly homologous, our present data suggest that inhibition of RhoA/ROCK signaling is critical for suppressing migration activity in the terbinafine-treated HUVEC. Unlike RhoA protein, which is located in plasma membrane for regulating actin stress fiber formation and integrin signaling, RhoB is located in the endosome and nuclear membranes with a unique function in intracellular trafficking of growth factor receptors, such as the epidermal growth factor receptor (32). RhoB plays an inhibitory role during cell cycle regulation and is up-regulated in response to stress stimuli (33). It has been indicated that RhoA and RhoC have overlapping functions, including promotion of cell motility, cytoskeletal alterations, and metastasis (34). Treatment of HUVEC with terbinafine induced an increase instead of a decrease of RhoC translocation from cytosol fraction to particulate fraction, suggesting that RhoC might not be involved in the terbinafine-induced inhibition of HUVEC migration. In the present study, we also showed that terbinafine (30–120 μmol/L) dose dependently inhibited the levels of Ras protein (Fig. 3C). Pretreatment of HUVEC with farnesol partially prevented the terbinafine-induced migration inhibition in HUVEC. This prevention effect mediated by farnesol was abolished by pretreated HUVEC with Ras inhibitor (Fig. 4A), suggesting that Ras-mediated pathway was involved in the terbinafine-induced migration inhibition in HUVEC.

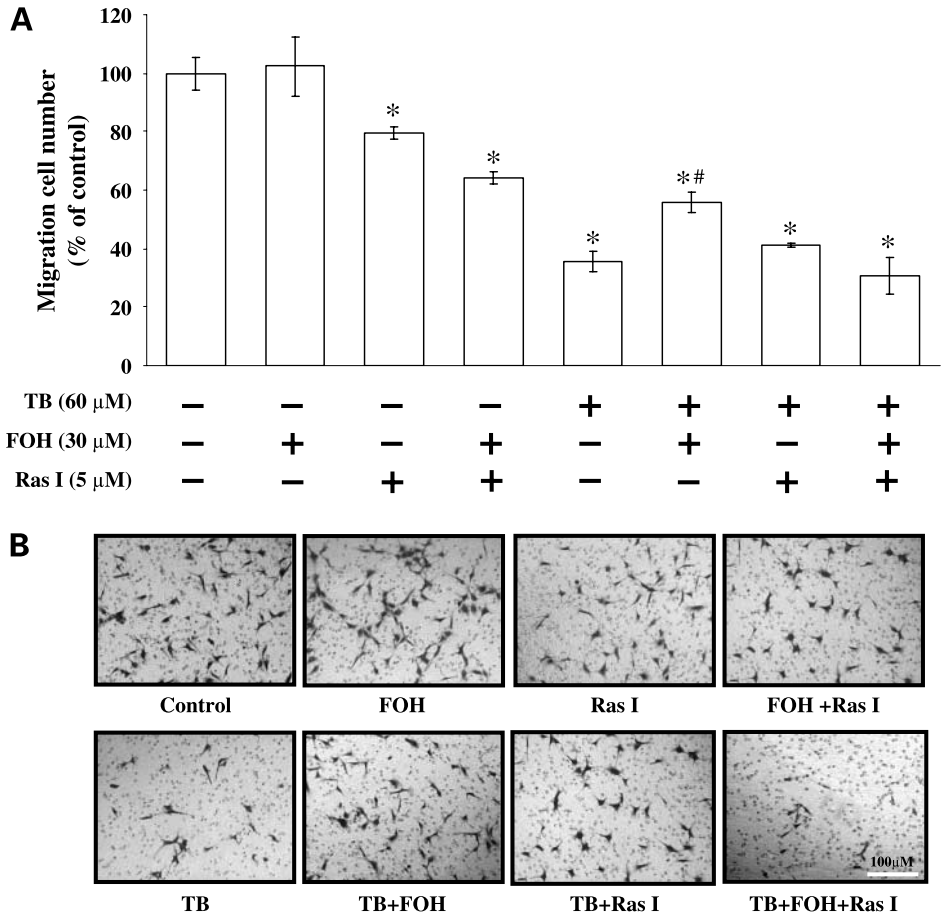

Figure 4. Involvement of Ras signaling pathway in the terbinafine-induced inhibition in HUVEC migration. **A,** treatment with farnesol (30 μmol/L) alone did not affect the HUVEC migration. However, pretreatment of HUVEC with farnesol partially reversed the terbinafine-induced migration inhibition. Treatment with Ras inhibitor peptide (VPPPVPPRRR; 5 μmol/L) inhibited HUVEC migration and abolished the 30 μmol/L farnesol (*FOH*) – induced prevention effect on 60 μmol/L terbinafine-mediated inhibition of HUVEC migration. **B,** representative photographs showed the results of Fig. 5A. *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$ different from control; #, $P < 0.05$ different from terbinafine-treated group.

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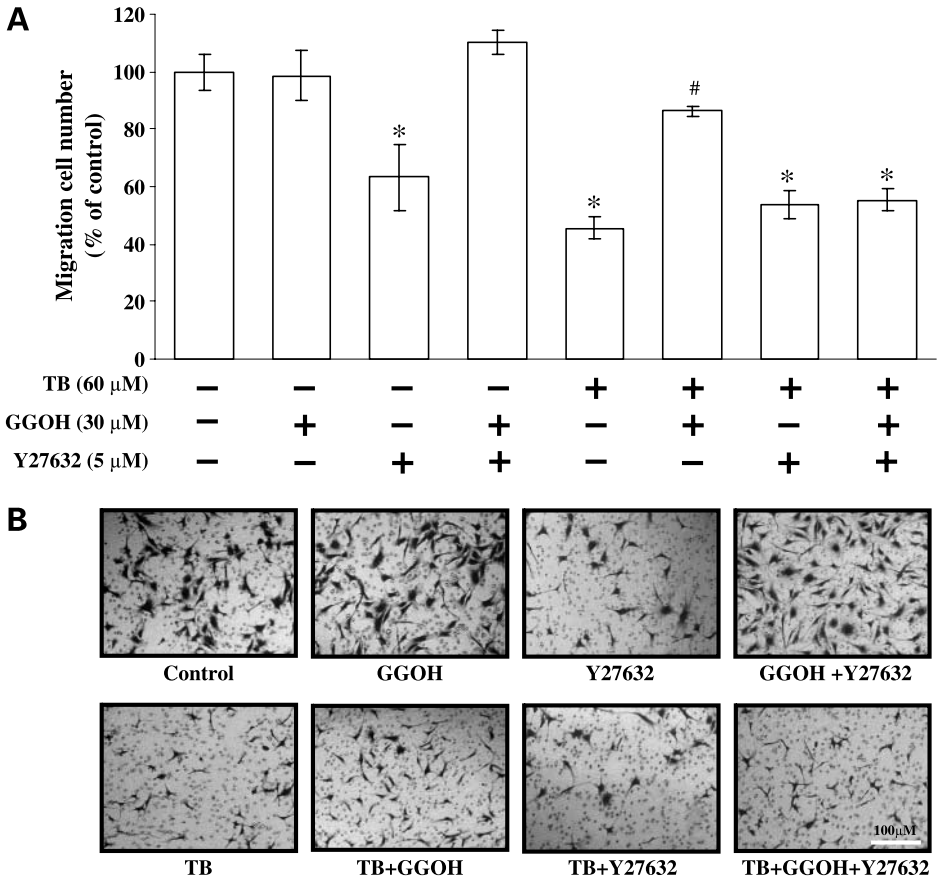

Figure 5. Involvement of Rho/ROCK signaling pathway in the terbinafine-induced inhibition in HUVEC migration. **A,** treatment with 30 μmol/L geranylgeraniol (*GGOH*) alone did not affect the HUVEC migration. However, pretreatment of HUVEC with geranylgeraniol completely reversed the terbinafine-induced migration inhibition. Treatment with 5 μmol/L Y27632, a ROCK (a kinase associated with RhoA for transducing RhoA signaling) inhibitor, inhibited HUVEC migration and abolished the 30 μmol/L geranylgeraniol-induced prevention effect on 60 μmol/L terbinafine-mediated inhibition of HUVEC migration. **B,** representative photographs showed the results of Fig. 6A. *Columns,* mean ($n = 3$); *bars,* SE. *, $P < 0.05$ different from control; #, $P < 0.05$ different from terbinafine-treated group.

Proteolytic breakdown of the basement membrane, which results from secretion and activation of MMPs in response to exogenous signals, such as cytokines, growth factors, and cell-matrix interactions, is also essential for endothelial cell migration (5). During angiogenesis, the extracelluar proteolytic activity in the endothelial pericellular environment was significantly up-regulated. The phenomenon of a temporal sequence of matrix dissolution and assembly is replaced by a more mature collagenous matrix with time, in which fibrin provided a temporary matrix scaffold for migrating endothelial cells. It was shown that the MMPs might mediate the basement membrane degradation. The zinc-dependent enzymes MMPs can be divided into secreted MMPs and membrane-type MMPs by their structural difference. Secreted MMPs include collagenases (e.g., MMP-1), gelatinases (MMP-2 and MMP-9), stromelysins (e.g., MMP-3), and other MMPs (e.g., MMP-7). The catalytic activity of secreted MMPs is tightly regulated. The secreted inactive proenzymes (zymogens) are later activated in the extracellular compartment. MT1-MMP is endowed with two characteristics important for cell migration: association with the plasma membrane, which focuses matrix digestion close to the cell surface, and the capacity to catalytically activate the precursor of MMP-2, which allows for amplification of the degradative process (35). It has been shown that Rho family can regulate the expression and activation of MMP-2 and MMP-9. RhoA triggers signaling pathways, which up-regulate expression of MMP-9 at specific membrane localizations and may confer a highly invasive phenotype to endothelial cells (36). Rac1 mediates MMP-2 activation during the encounter between invading tumor cells and collagen-rich stroma and thereby facilitated collagenolysis and cell invasion (37, 38). Treatment of HUVEC with terbinafine caused a decrease in the mRNA levels of MMP-2 and MMP-9 (Fig. 6). We presumed that the reduced expressions of MMP-2 and MMP-9 might also contribute to the inhibition of cell migration in terbinafine-treated HUVEC, and Rho might be one of the key targets of terbinafine for down-regulating the expression or inactivation of MMP-2 and MMP-9.

In the present study, we showed that terbinafine suppressed the phosphorylation of FAK and paxillin (Fig. 3). The phosphorylation state and activity of FAK are tightly related to cell adhesion to the ECM through integrin receptors (39). Paxillin is a downstream target of FAK. It has been suggested that tyrosine phosphorylation of FAK and paxillin is able to influence cellular events either that are dependent on cell adhesion (such as cell proliferation and survival) or that require modulation of cell adhesion (such as cell migration; ref. 40). The tyrosine

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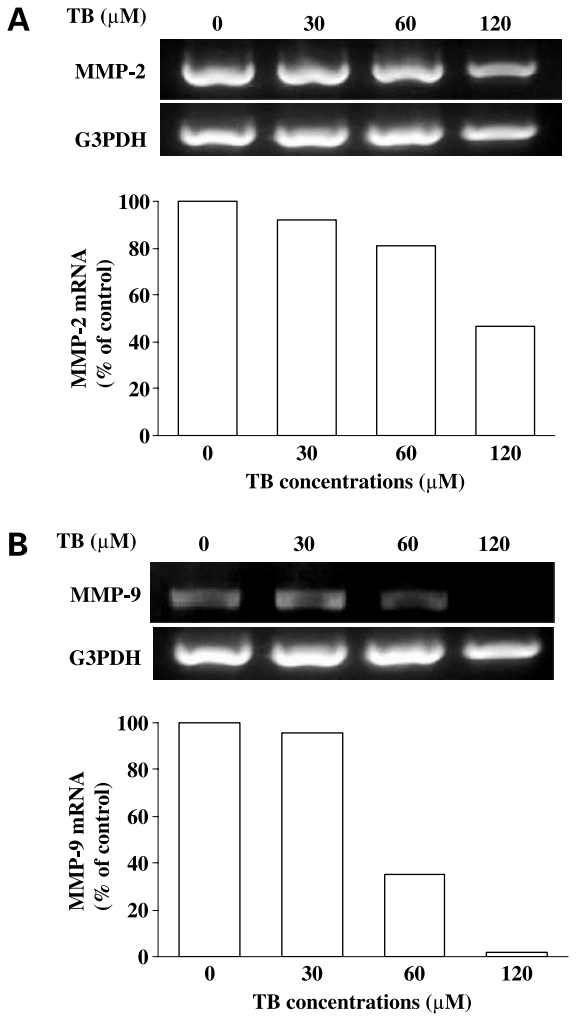

Figure 6. Effects of terbinafine on expression of MMPs in HUVEC. Expression of MMP-2 (**A**) and MMP-9 (**B**) mRNA in HUVEC was inhibited by terbinafine dose dependently. Expression of *G3PDH* housekeeping gene was used to verify adjustment of samples to equal cDNA amounts. Quantification was made by densitometry analysis using Image Pro Plus 4.5 software. *Bottom,* quantified results after being adjusted with their own G3PDH levels and expressed by percentage of control.

phosphorylation of FAK and paxillin is necessarily and sufficiently mediated by Rho (25, 41). Our results suggest that terbinafine might inhibit endothelial cell migration through inhibiting the activation of FAK and paxillin via Rho-mediated pathway.

In conclusion, this study provides the evidence that terbinafine suppressed the prenylation of Rho, which in turn caused inactivation of FAK and paxillin, and eventually resulted in the inhibition of endothelial cell adhesion and migration. Taken together, our previous and current findings strongly suggest the potential applications of terbinafine as an antiangiogenic agent because it is capable of retarding the cell cycle of endothelial cells, reducing the adhesion and migration of endothelial cells, interrupting the tube formation, and inhibiting sprouting angiogenesis. This study also provides new insights into the regulation of endothelial cell behaviors by medical products, which regulate the cholesterol synthesis process.

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