Antiangiogenic vinflunine affects EB1 localization and microtubule targeting to adhesion sites

Stéphane Honoré,[1] Alessandra Pagano,[1] Géraldine Gauthier,[1] Véronique Bourgarel-Rey,[1] Pascal Verdier-Pinard,[1] Karine Civiletti,[1] Anna Kruczynski,[2] and Diane Braguer[1]

[1]Institut National de la Sante et de la Recherche Medicale UMR 911, Centre de Recherche en Oncologie Biologique et en Oncopharmacologie Aix-Marseille Université, Marseilles, France; and [2]Pierre Fabre Oncology Research Institute, Toulouse, France

Abstract

The motile behavior of endothelial cells is a crucial event for neoangiogenesis. We previously showed that noncytotoxic concentrations of vinflunine inhibit capillary-like tube formation on Matrigel and endothelial cell migration with a concomitant increase in interphase microtubule dynamic instability. In this article, we further investigated the effects of vinflunine on migration and cytoskeleton interaction dynamics in HMEC-1 endothelial cells. We confirmed that vinflunine, at low and noncytotoxic concentrations (0.01 – 1 nmol/L), inhibited endothelial cell random motility by 54%. This effect was associated with a decrease in the percentage of stable microtubules and in the mean duration of pauses for dynamic ones. Moreover, we found that vinflunine altered adhesion site targeting by microtubules and suppressed the microtubule (+) end pause that occurs at adhesion sites during cell migration (from 151 $\pm$ 20 seconds in control cells to 38 $\pm$ 7 seconds in vinflunine-treated cells, $P < 0.001$). This effect was associated with the inhibition of adhesion site dynamics and the formation of long-lived stress fibers. Importantly, we found that vinflunine altered EB1 localization at microtubule (+) ends. These results highlight a new mechanism of action of vinflunine, which act by disrupting the mutual control between microtubule and adhesion site dynamics and strengthen the role of +TIPs proteins such as EB1 as key regulators of endothelial cell motility. [Mol Cancer Ther 2008;7(7):2080 – 9]

Received 2/12/08; revised 4/7/08; accepted 4/23/08.

Grant support: INCa and Cancéropole PACA (RS019), and by European Commission FP6 specific targeted project Nano4drugs (LSHB-CT-2005-019102).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Diane Braguer, UFR Pharmacie, Institut National de la Sante et de la Recherche Medicale UMR 911, 27 Boulevard Jean Moulin, 13005 Marseilles, France. Phone: 33-4918-35635; Fax: 33-4918-35635. E-mail: diane.braguer@univmed.fr

Copyright © 2008 American Association for Cancer Research.

doi:10.1158/1535-7163.MCT-08-0156

Introduction

The formation of a functional vascular network through angiogenesis is a key event associated with tumor growth, cancer progression, and metastasis (1, 2). Microtubule-targeting agents (MTAs), which can be classically subdivided into microtubule-stabilizing (e.g., Taxanes) and microtubule-destabilizing agents (e.g., *Vinca* alkaloids), exert an antiangiogenic activity both *in vitro* and *in vivo* at very low and noncytotoxic concentrations (3–5). Vinflunine, the newest MTA of the *Vinca* alkaloid class in clinical development, shows antivascular effects (6) and a strong *in vivo* antiangiogenic activity at doses 40- to 20-fold lower than its maximal therapeutic dose (7). At noncytotoxic concentrations, MTAs affect angiogenesis by disturbing endothelial cell migration and differentiation into the capillary network, two crucial events during tumor angiogenesis (8, 9). Cell migration can be viewed as a periodically repeating sequence of events that includes the formation of pseudopodial protrusions, attachment and translocation of the cell body in the direction of new adhesion sites (10). Microtubules and actin filaments play a critical role in intracellular trafficking, cell polarity, and motility. These functions involve the ability of these polarized polymers to assemble and disassemble rapidly, and to interact with proteins that regulate their dynamic properties both temporally and spatially. Although the central role of the actin cytoskeleton in migration is largely known, more recent data supports a significant role for microtubules in this process (11). In fact, a coordinated regulation of microtubules, actin cytoskeleton, and adhesion sites is likely to be essential throughout the migration process (12). Several putative mechanisms by which MTAs could inhibit cell migration have been proposed. These include (*a*) the impairment of microtubule-organizing center reorientation toward the leading edge, (*b*) the inhibition of Rho GTPases and their downstream effectors by altering the cycle of microtubule polymerization and depolymerization, (*c*) the blockade of lamellipodia formation and cell polarization as a consequence of the inhibition of intracellular protein trafficking and vesicle transport, (*d*) the inhibition of microtubule-mediated integrin clustering and increased integrin avidity, and (*e*) HSP90 degradation (13–19). Within cells, most microtubule (+) ends are oriented toward and interact with the cell cortex, whereas the (−) ends are usually stably capped and often anchored at the centrosome, which is the main organelle responsible for the nucleation of new microtubules. Microtubule (+) ends exhibit a behavior called dynamic instability which is the switch between phase of growth and rapid shortening or pause (reviewed in ref. 20). We previously showed that low antiangiogenic and antimigratory concentrations of MTAs such as paclitaxel or vinflunine significantly increase interphase microtubule dynamic instability (21–23).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

However, even if microtubule involvement in cell migration is now evident and require interactions with both adhesion sites and actin cytoskeleton (12), the relationship between microtubule dynamics and cell migration remains poorly understood (20, 24). In this view, a growing list of proteins that interact with the microtubule (+) ends called (+) end-tracking proteins (+ TIPs), are critically important for many microtubule-regulated processes including cell migration. Among them, EB1 regulate microtubule dynamic instability (25), the guidance of microtubules toward the leading edge through interaction with a microtubule-actin cross-linker such as ACF7, and the selective microtubule stabilization at the cell cortex during cell migration (26).

In order to fully understand the functional implication of microtubule dynamics in the antiangiogenic activity of MTAs, we investigated the effect of vinflunine on the microtubule (+) end interaction with other cytoskeleton structures during cell migration. We showed that vinflunine, at subnanomolar and noncytotoxic concentrations, inhibited endothelial cell motility. Such an effect was associated with a decrease in microtubule pause at the cell cortex, and an inhibition of microtubule targeting to adhesion sites. This lack of adhesion site targeting by microtubules led to an inhibition of adhesion site dynamics and to the formation of long-lived stress fibers. Importantly, we found that these effects were associated with EB1 mislocalization at microtubule (+) ends. Therefore, our results reveal a new mechanism of action of a MTA, vinflunine, and highlight the role of microtubule (+) end complexes in the microtubule–actin–adhesion site crosstalk during cell migration. These findings open a new avenue of research, particularly in the identification and characterization of proteins that are involved in such crosstalk that can constitute new and specific targets for antiangiogenic therapy.

Materials and Methods

Drug and Cell Culture

Human dermal microvascular endothelial cell line (HMEC-1) were routinely maintained at 37°C and 5% CO_2 in MCDB-131 medium (Life Technologies) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L of glutamine, 1% penicillin and streptomycin, 1 μg/mL of hydrocortisone (OHC; Pharmacia and Upjohn), and 10 ng/mL of epithelial growth factor (R&D Systems). HMEC-1 cells were used between passages 3 and 12. For all experiments, exponentially growing cells in endothelial growth medium 2 were seeded on 10 μg/mL of fibronectin-coated (Sigma-Aldrich) culture plates (8×10^3 cells/cm^2) and the ratio of drug molar concentration/cell number was strictly identical for all experiments. A stock solution of vinflunine (Pierre Fabre Oncology) was prepared in distilled water and maintained frozen at −20°C.

Random Motility Measurements

Cells were seeded in 24-well culture plates. One hour later, the cells were treated with vinflunine at various concentrations or were untreated and subjected to time-lapse videomicroscopy for 10 h. Random motility measurements were done as previously described (23, 27). Briefly, 90 cells from three independent experiments were tracked for 10 h. The average cell speed (S) and the coordinates of the nuclei centroids were computed by the Metamorph software (Universal Imaging Corporation). For each cell, the mean square displacement $<D^2>$ was determined from the formula:

$$<D^2> = \sum_{i=1}^{M} d_{i^2}$$

where d_i is the displacement of the cell from its initial position at time level $t = i\Delta t$.

From this value, we determined, as follows, the random motility coefficient (μ) that reflects the cell ability to migrate into a new area and the persistence time (P) that constitutes the average time between significant direction changes:

$$<D^2> = 2n\mu t = nS^2Pt$$

where t is time, S the average cell speed and $n = 2$ in our two-dimensional walk model.

Transfection of HMEC-1 with GFP-α Tubulin, GFP-Actin, GFP-EB1, mcherry-Tubulin and DsRed-Paxillin Plasmids

The transfection of HMEC-1 cells was done as previously described (23). Briefly, 8×10^5 cells were resuspended in 100 μL of the specific electroporation R buffer (Amaxa). Four to eight micrograms of plasmid DNA (GFP-actin, mcherry-tubulin, GFP-EB1, and pEGFP-α-tubulin; ref. 23), and DsRed-paxillin provided by Alan Rick Horwitz (University of Virginia, Charlottesville, VA; ref. 28) were added to the cell suspension, transferred to a 2.0 mm electroporation cuvette, and nucleofected with Amaxa Nucleofector (Amaxa) using program number T-016. DNA quantity, cell concentration, and buffer volume were kept constant throughout all experiments. After transfection, cells were immediately transferred in endothelial growth medium 2. Twenty-four hours later, cells were then treated for 4 h with vinflunine at various concentrations. Time-lapse microscopy and measurements were then done.

Time-lapse Microscopy and Image Acquisition

Transfected cells were placed in endothelial growth medium 2 containing the same concentration of vinflunine and supplemented with ascorbic acid (0.1 μg/mL) to reduce photodamage, in a double coverslip chamber maintained at 37 ± 1°C. Time-lapse acquisitions for microtubule dynamics experiments were done on a Leica DM-IRBE fluorescence microscope (Leica), 100× objective lens. Thirty-one images per cell were acquired at 4-s intervals using a digital camera (CCD camera coolsnap FX; Princeton Instruments) driven by Metamorph software (Universal Imaging Corporation) as previously described (29). Double fluorescence time-lapse acquisitions were done on a Nikon TE 2000 microscope (Nikon) equipped for multifluorescence time-lapse analysis at 60× objective lens. Cells were imaged every 10 s for 5 to 45 min using a

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

digital camera (CCD camera coolsnap HQ; Princeton Instruments).

Analysis of Microtubule Dynamics

Analysis of microtubule dynamics was done as described previously using the track point function of the Metamorph software (22). Briefly, changes in length of ≥0.5 μm were considered as growth or shortening events. Changes in length of <0.5 μm were considered as phases of attenuated dynamics or pauses. The percentage of paused microtubules in the cortex zone at the leading edge (i.e., 5 μm behind the cell periphery) was calculated on the basis of the total number of microtubule ends found in this zone in 30 cells from three independent experiments. Paused microtubules were defined as microtubules completely stabilized (nondynamic) in the cortex during the entire time course of the experiment (2 min). For dynamic microtubules in the cortex zone, the mean duration of pause was determined for 90 microtubules for each condition from three independent experiments.

Analysis of Microtubule – Adhesion Site Targeting

The percentage of adhesion site targeting by microtubule (+) ends was analyzed by direct observation of time-lapse video sequences. All peripheral microtubules of nine cells from three independent experiments were analyzed for each condition. Microtubules that targeted an adhesion site at least once during the time course of the experiment (10 min) were considered as adhesion site–targeting microtubules. The duration of microtubule pauses at adhesion sites was analyzed on 90 microtubule–adhesion site interactions in each condition from three independent experiments by using the track point function of the Metamorph software.

Indirect Immunofluorescence Analysis

Cells were grown on 4-well Labtek II chamber slides (Labtek) precoated with fibronectin (10 μg/mL) and incubated with various vinflunine concentrations for 4 h. For actin staining, cells were fixed with 3.7% formaldehyde, permeabilized with 1% saponin, and incubated with phalloidin-TRITC (1/1,000; Sigma) for 30 min. Cells were then observed using a Leica DM-IRBE microscope, 100× objective lens, coupled with a digital camera (Coolsnap FX). For microtubule and endogenous EB1 staining, cells were fixed for 5 min with methanol (−20°C) and incubated with anti-EB1 antibody (clone 5; BD Biosciences) for 1 h, and then with a TRITC-coupled antimouse secondary antibody (Jackson Laboratories). Cells were then labeled for tubulin using an FITC-coupled anti–α-tubulin antibody (clone DM1A; Sigma). Cells were then observed using a Nikon TE 2000 microscope (Nikon).

Results

Antiangiogenic Concentrations of Vinflunine Inhibit Endothelial Cell Random Motility

The cell motility behaviors of HMEC-1 cells were analyzed by time-lapse videomicroscopy by determining four variables: the average cell speed of migration, the percentage of time spent in a stationary state, the persistence time, and the random motility coefficient. The average migration speed of HMEC-1 cells was 0.42 ± 0.07 μm/min. HMEC-1 cells spent ~43% of the time in a nonmigrating phase that we termed "stationary state." The mean persistence time, which is a measure of the average time between two significant direction changes, was 16 ± 2.7 minutes. Finally, the random motility coefficient, which reflects the capacity of a cell population to migrate into a new area was 1.32 ± 0.34 μm^2/min.

We previously showed that vinflunine inhibited capillary-like tube formation on Matrigel at noncytotoxic concentrations of ≤2 nmol/L (23). Antiangiogenic concentrations of vinflunine (0.01–2 nmol/L) decreased by 50%, the mean migration speed of HMEC-1 cells (Fig. 1A). For concentrations >2 nmol/L, the migration speed was reduced to a lesser extent. Importantly, the decrease in the average migration speed was mainly due to an increase in the percentage of time that the cells spent in a stationary state, from 43% to a maximum of 70% for control cells and cells treated with 1 nmol/L of vinflunine, respectively (Fig. 1B). In addition, the persistence time was increased by up to 100%, reaching 35 minutes at vinflunine concentrations ranging from 0.01 to 1 nmol/L ($P < 0.05$; Fig. 1C). Finally, the random motility coefficient was decreased by 54% in this range of concentrations (Fig. 1D). The increase in persistence time and the decrease in random motility were lower at concentrations ≥2 nmol/L of vinflunine. These results indicate that the antimigratory effect of vinflunine optimally occurs at noncytotoxic concentrations (0.01–1 nmol/L).

Vinflunine Inhibits Microtubule Pauses at the Cell Periphery

One of the most sensitive effects of MTAs in cells is the alteration of microtubule dynamics. We previously showed that antiangiogenic concentrations of vinflunine increase microtubule dynamic instability (i.e., growth and shortening rates and overall dynamicity) of dynamic microtubules in living HMEC-1 cells (23). In this study, we further examined the effect of vinflunine on microtubule pauses at the cell periphery. At antiangiogenic concentrations (0.01–2 nmol/L), the percentage of microtubules that were paused at the cell periphery during the time course of the experiment (2 minutes) was decreased from 49% in control cells to <15% in vinflunine-treated cells (Fig. 2A). In addition, the mean duration of pauses of dynamic microtubules was decreased by ~30% (Fig. 2B). These results show that vinflunine significantly decreases microtubule pauses at the cell periphery in living HMEC-1 cells.

Vinflunine Disturbs Microtubule – Adhesion Site Crosstalk

One of the major interphase functions of microtubules during cell migration is to regulate adhesion site dynamics and/or turnover. This process involves microtubule (+) end targeting of adhesion sites and microtubule pauses at adhesion sites (30). Because vinflunine inhibits microtubule pauses at the cell cortex, we investigated the effect of vinflunine on the targeting of adhesion sites by microtubule (+) ends. For this purpose, HMEC-1 cells were

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

cotransfected with DsRed-paxillin and GFP-tubulin to visualize both microtubules and adhesion sites. Double fluorescence time-lapse acquisition every 10 seconds over a period of 10 minutes clearly showed that microtubule (+) ends specifically target paxillin-containing adhesion sites at the cell periphery in control cells (Fig. 3A). In contrast, in vinflunine-treated cells, the microtubule network was disorganized with curved microtubules which did not reach the peripheral adhesion sites (Fig. 3B). Indeed, the percentage of microtubules that targeted adhesion sites during the time course of the experiment (10 minutes) was significantly decreased by vinflunine (1 nmol/L) from 88% to 62% ($P < 0.05$; Fig. 3C). Moreover, in control cells, microtubule (+) ends were paused at peripheral adhesion sites for a long period of time (Fig. 4A; Supplementary Movie 1).[3] In contrast, in vinflunine-treated cells, microtubule pause at adhesion sites lasted for a shorter time when it occurred (Fig. 4B; Supplementary Movie 2).[3] Indeed,

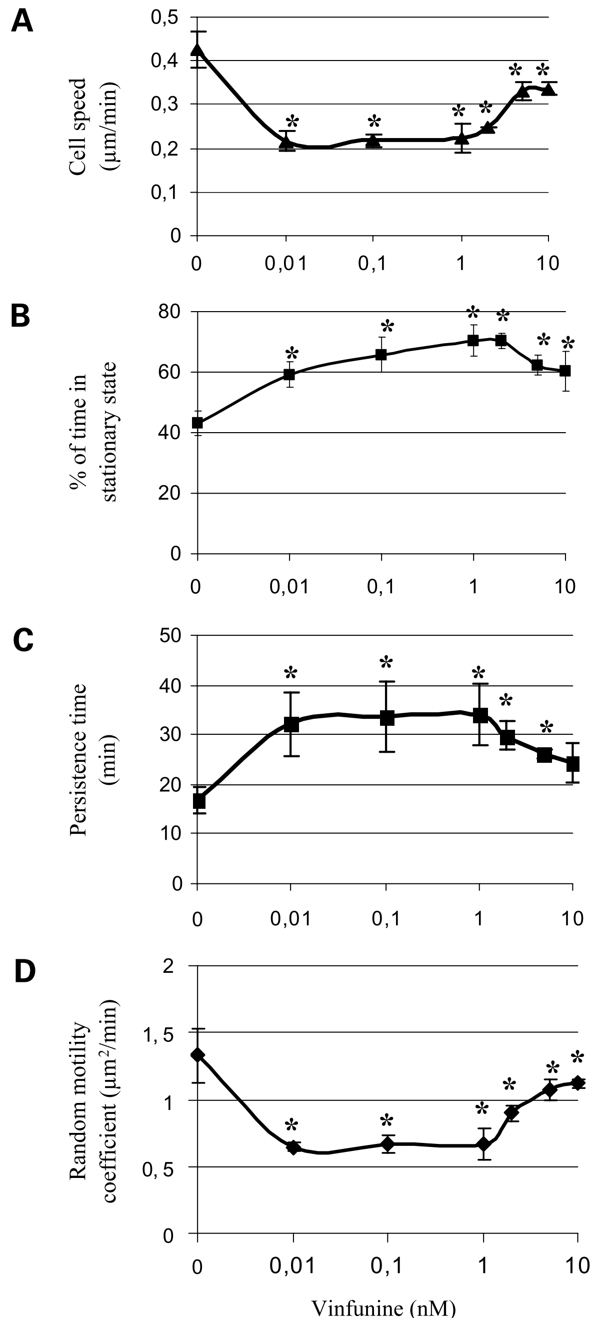

Figure 1. Vinflunine inhibited HMEC-1 random motility. HMEC-1 cells were seeded on fibronectin-coated plates (10 µg/mL) and incubated with various concentrations of vinflunine 1 h later. Cells were then tracked for 10 h and random motility variables were calculated using the random-walk model. Motility variables were the cell speed (**A**), the percentage of time spent in a stationary state (**B**), the persistence time (**C**), and the random motility coefficient (**D**). *Points,* mean; *bars,* SE (*, $P < 0.05$; Student's *t* test).

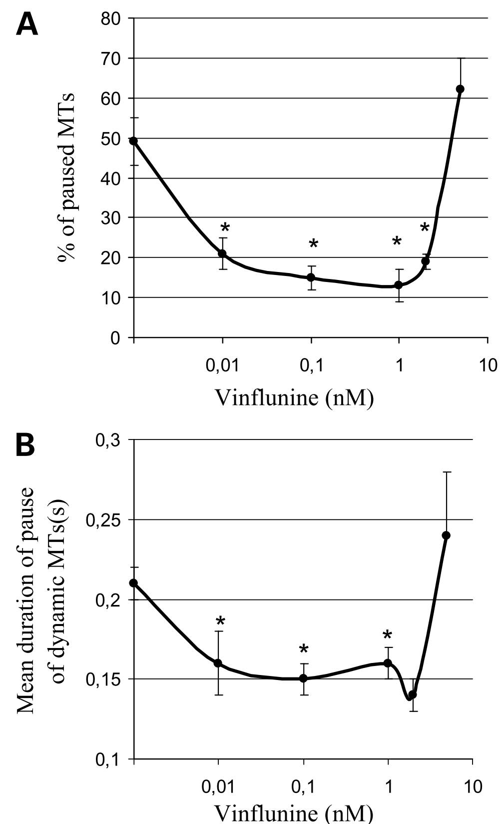

Figure 2. Vinflunine suppressed microtubule pauses at the cell periphery. HMEC-1 cells transiently transfected with GFP-tubulin were incubated with various concentrations of vinflunine for 4 h. Microtubule dynamics were then analyzed by time-lapse video microscopy. The percentage of microtubules that were paused at the cell periphery during the time course of the experiment (2 min; **A**) and the microtubule mean duration of pauses of dynamic microtubules (**B**) were determined by tracking peripheral microtubule (+) ends over time. Note that 90% and 60% of cellular microtubules targeted the cell periphery in control cells and in vinflunine-treated cells, respectively. *Points,* mean; *bars,* SE (*, $P < 0.05$; Student's *t* test).

[3] Supplementary materials for this article are available at Molecular Cancer Therapeutics Online (http://mct.aacrjournals.org/).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

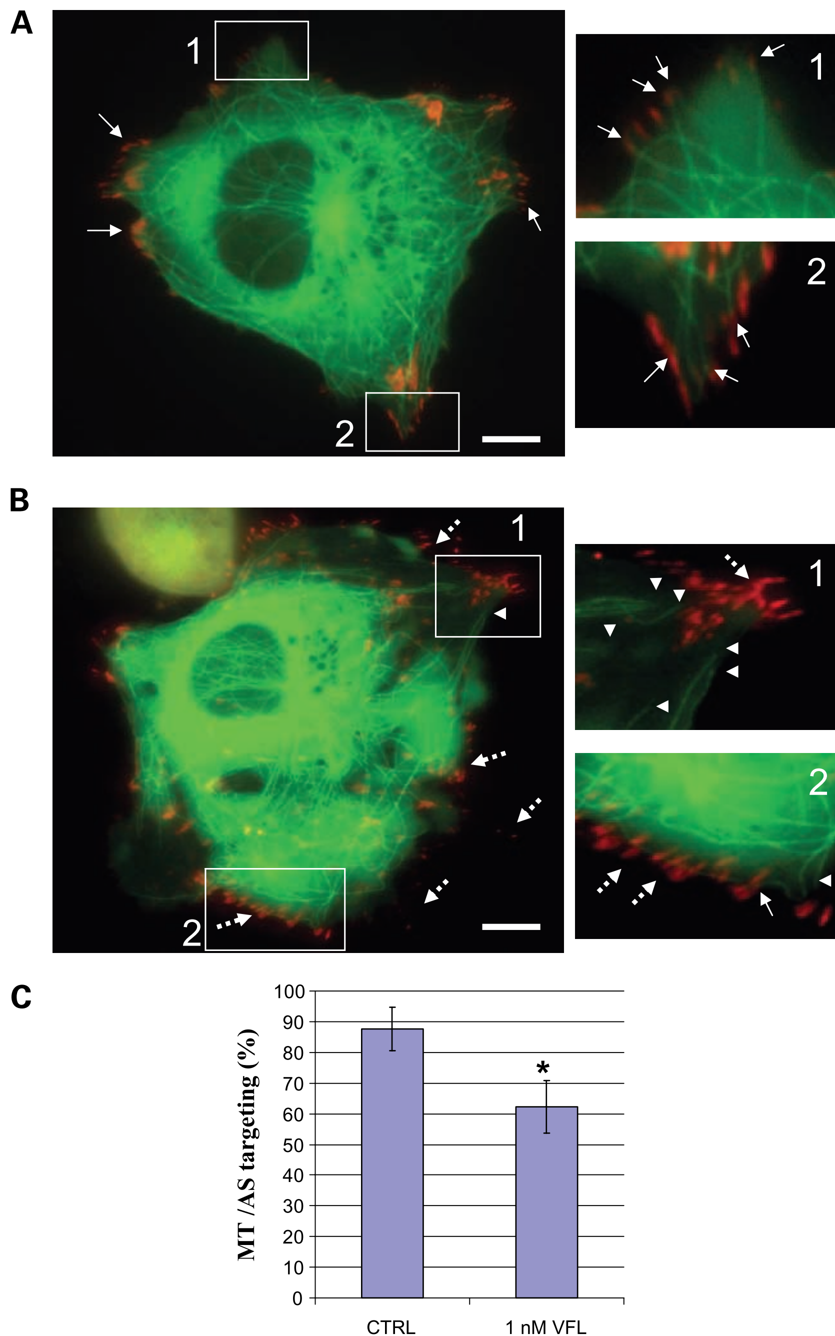

Figure 3. Vinflunine suppressed microtubule adhesion site targeting. **A** and **B,** video frames of HMEC-1 cells cotransfected with GFP-tubulin and DsRed-paxillin, untreated (**A**) or treated (**B**) with 1 nmol/L of vinflunine for 4 h. *Right,* higher magnifications of insets 1 and 2. In the control cell, many peripheral microtubule (+) ends targeted adhesion sites at the cell periphery (*arrows*). In contrast, in the vinflunine-treated cell, many microtubule (+) ends (*arrowheads*) did not target any adhesion site (*dashed arrows*). *Bars,* 10 μm. **C,** percentage of microtubules (*MT*) that target an adhesion site (*AS*) during the time course of the experiment (10 min) in control HMEC-1 cells or after incubation with 1 nmol/L of vinflunine (*bars,* SD; *, $P < 0.05$; Student's *t* test).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

vinflunine strongly decreased the mean duration of microtubule pauses at adhesion sites from 151 ± 20 seconds in control cells to 38 ± 7 minutes in vinflunine-treated cells ($P < 0.001$; Fig. 4C). Altogether, these results show that vinflunine strongly inhibits the microtubule–adhesion site crosstalk that occurs during endothelial cell migration.

Vinflunine Inhibits Adhesion Site Dynamics and Induces Stress Fiber Formation

To better understand the consequences of the alteration of the microtubule–adhesion site crosstalk by vinflunine, we analyzed its effect on actin and adhesion site organization and dynamics, which are crucial for cell migration. For this purpose, HMEC-1 cells were cotransfected with DsRed-paxillin and GFP-actin plasmids. Migrating control cells displayed large lamellipodia with more intense actin staining at the leading edge, where new adhesion sites were formed, concomitantly with the appearance of actin-rich membrane ruffles. Cells also displayed adhesion site sliding at the retracting edge (Fig. 5A; Supplementary Movie 3).[3] In contrast, in vinflunine-treated cells, the cortical actin network and membrane ruffles disappeared, and many thick and long-lived stress fibers were formed

between static focal adhesions (Fig. 5B; Supplementary Movie 4).[3] These effects on adhesion site dynamics were more readable on longer time course experiments (Supplementary Movies 5 and 6).[3] Vinflunine-induced stress fiber formation was further confirmed by direct immunofluorescence staining on fixed cells using Phalloidin-TRITC (Supplementary Fig. S1).[3] These results show that vinflunine alters adhesion site and actin dynamics in endothelial cells at antiangiogenic concentrations.

Vinflunine Alters EB1 Localization at Microtubule (+) Ends

Several lines of evidence indicate that microtubule + TIPs, including EB1, are critically important for microtubule ''search and capture'' mechanism and for the stabilization of

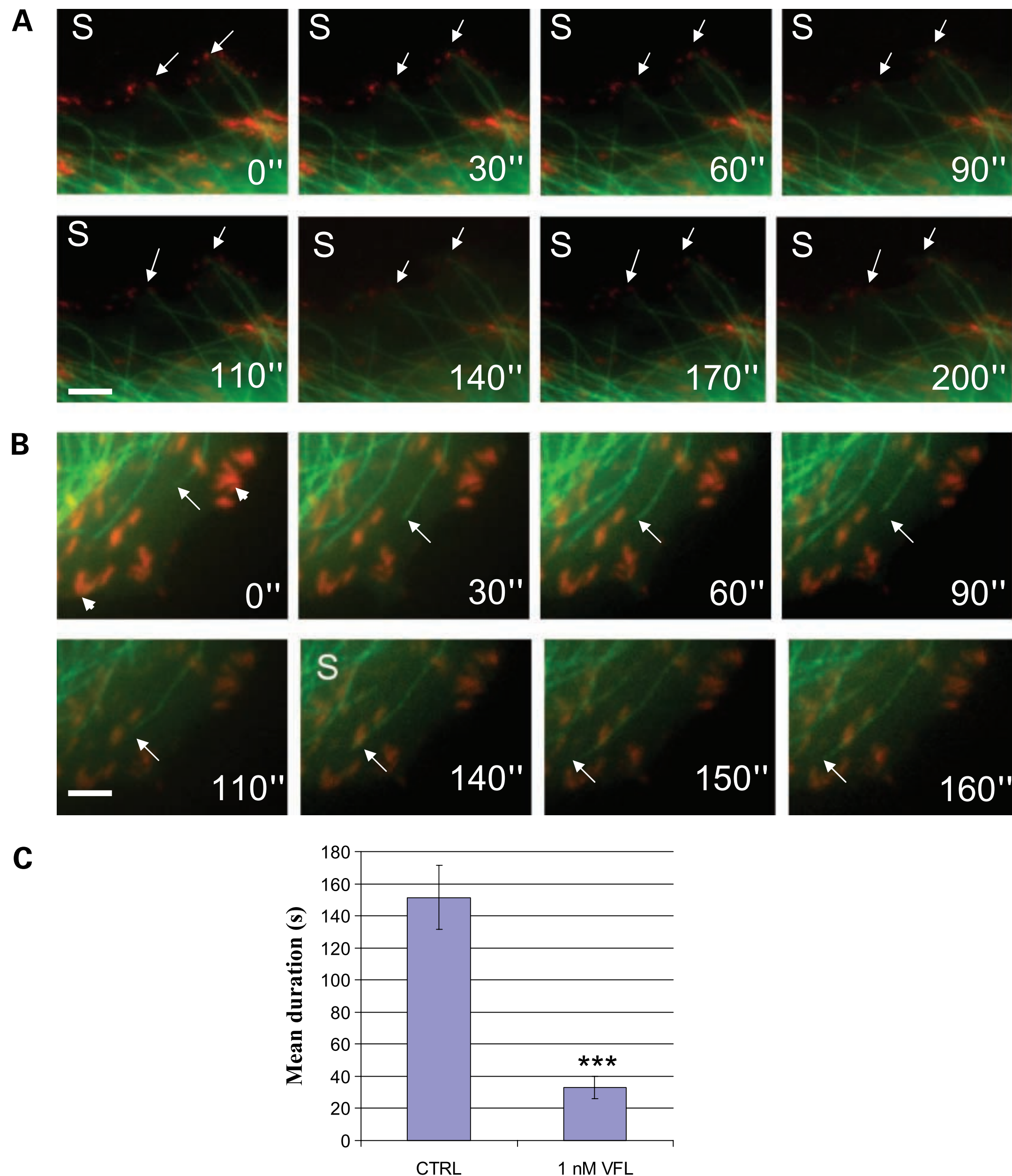

Figure 4. Vinflunine inhibited microtubule pauses at adhesion sites. Time-lapse video sequence of the peripheral region of HMEC-1 cells cotransfected with GFP-tubulin and DsRed-paxillin (**A**) and treated with 1 nmol/L of vinflunine for 4 h (**B**). Time is indicated in seconds (″). In the control cell (**A**), microtubule (+) ends specifically targeted the peripheral adhesion sites and remained stabilized to these adhesion sites for long periods of time (>200 s). Video frames showing the indicated microtubule (+) ends stabilized at adhesion sites are labeled with an ''*S*''. Note that most of these adhesion sites were small in size (focal complexes). In sharp contrast, in the vinflunine-treated cells (**B**), peripheral adhesions were not targeted and were larger in size (focal adhesions; *arrowheads*). In this condition, when a microtubule – adhesion site targeting event occurred (*arrows* on the time-lapse sequence), the duration of the microtubule stabilization at the adhesion site was much shorter than in control cells (<30 s; frames labeled with an ''*S*''). *Bars,* 3 μm. **C,** mean microtubule duration of pauses at adhesion sites (*bars,* SD; ***, $P < 0.001$; Student's *t* test).

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

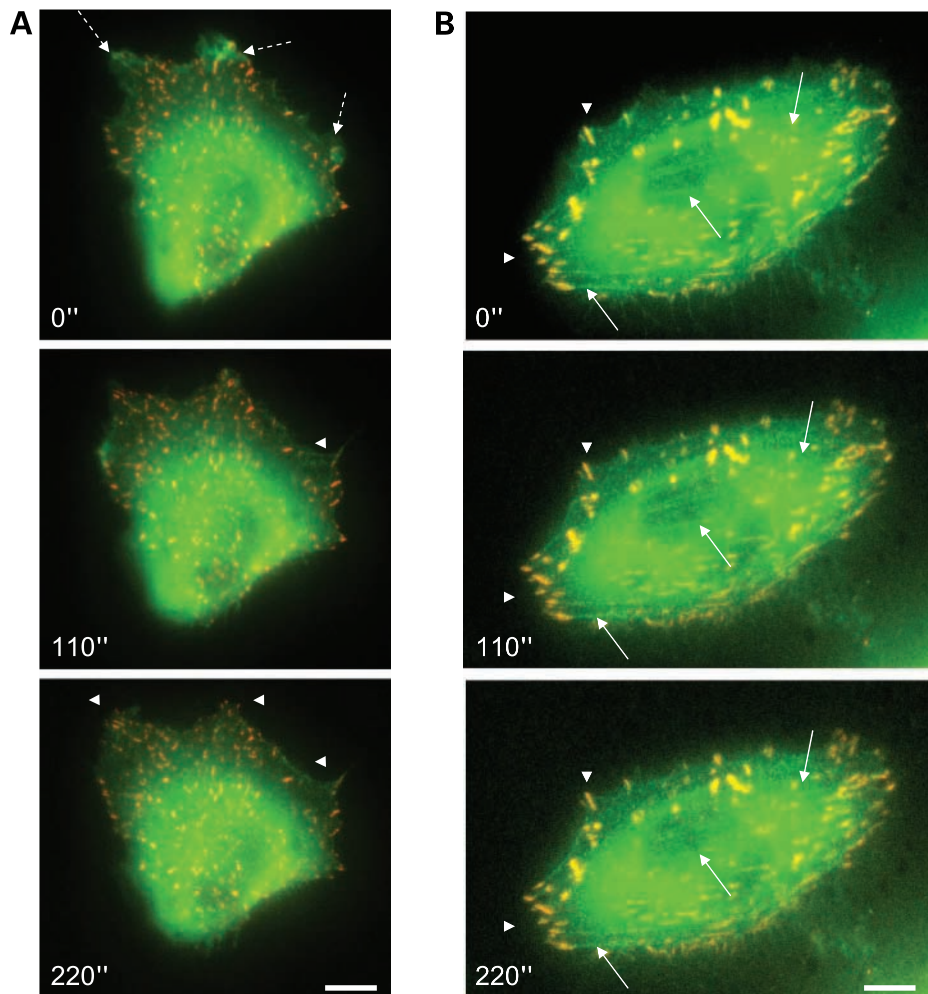

Figure 5. Vinflunine inhibited adhesion site dynamics and induced stress fiber formation. HMEC-1 cells transiently transfected with GFP-actin and DsRed-paxillin were analyzed by time-lapse video microscopy. **A,** in control cells, more intense cortical actin staining occurred at the leading edge of migrating cells at the zone of membrane ruffles. Paxillin-containing adhesion sites were very dynamic with the formation of new adhesion sites at the leading edge and sliding adhesion at the retracting edge. The formation of new adhesion sites (*arrowheads*) occurred in the zone of actin-rich membrane ruffles (*dashed arrows*). **B,** in cells incubated with 1 nmol/L of vinflunine, cortical actin staining was not observed whereas many long-lived stress fibers (*arrows*) were formed between static focal adhesions (*arrowheads*). *Bars,* 10 μm.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

microtubules within the cell cortex (31). Thus, we examined the effect of vinflunine on EB1 localization. For this purpose, HMEC-1 cells were cotransfected with GFP-EB1 and mcherry-tubulin to visualize both microtubules and EB1 dynamics. In control cells, video microscopy showed that GFP-EB1 concentrated at growing microtubule (+) ends and appeared as comets moving towards the cell periphery (Supplementary Movie 7).[3] In contrast, no comets were visible in vinflunine-treated cells (1 nmol/L; Supplementary Movie 8).[3] To further confirm this effect of vinflunine on EB1 localization, HMEC-1 cells were immunostained for endogenous EB1 and α-tubulin. In control cells, endogenous EB1 displayed a comet-like distribution at microtubule (+) ends. Upon vinflunine treatment, EB1 comets were strongly altered and more likely appeared as dots at microtubule (+) ends (Fig. 6).

Discussion

In this article, we revealed how microtubules participate in the mechanism responsible for the antimigratory effect of vinflunine on endothelial cells that we described previously (23). Inhibition of endothelial cell motility by vinflunine was associated with (*a*) a decrease in the percentage of stable microtubules and in the mean duration of pauses for dynamic microtubules; (*b*) a decrease in adhesion site targeting by microtubule (+) ends, and in microtubule pauses at adhesion sites; (*c*) a decrease in adhesion site dynamics and an increase in stress fibers; (*d*) an alteration of EB1 localization at microtubule (+) ends.

One of the most critical functions of interphase microtubules during cell migration is to target adhesion sites, a process guided by the actin cytoskeleton, thus regulating adhesion site dynamics and turnover (30, 32–35). The mechanism of targeting includes the growth of microtubules toward the adhesion site followed by a short association of the microtubule (+) end with the adhesion site. Then, the microtubule can either continue to grow, undergo catastrophe and shorten, or be stabilized at the adhesion site. Thus, the pause of microtubule (+) ends in interphase cells seems to be dependent on their capture and transient stabilization by adhesion sites (30, 33, 35). Such a process is recognized to promote cell relaxation by

inhibiting focal complex maturation into focal adhesions and the subsequent actomyosin contractility, thus decreasing the adhesion forces to the substrate to promote cell migration (32, 34). Altogether, our results suggest that the antimigratory effect of vinflunine results from an inhibition of microtubule–adhesion site crosstalk, thus inhibiting adhesion site dynamics and leading to the formation of long-lived stress fibers. Alternatively, the formation of stable stress fibers and the lack of adhesion site dynamics could be a downstream secondary effect of inhibition of migration. However, because vinflunine is a MTA, we expect that the primary mechanism of its antimigratory action is related to disturbance in microtubule function. This is underscored by the fact that vinflunine significantly reduced the localization of EB1 at microtubule (+) ends.

How does vinflunine reduce the size of EB1 comets? As EB1 localize at growing microtubule (+) ends, the inhibition of microtubule dynamics by vinflunine, and particularly growing events, which is the main mechanism of action of MTAs *in vitro* and at cytotoxic concentrations in cells (36), may be a cause of the loss of EB1 at microtubule (+) ends. However, in our experiments, vinflunine was used at low noncytotoxic and antiangiogenic concentrations that do not suppress microtubule dynamic instability but rather

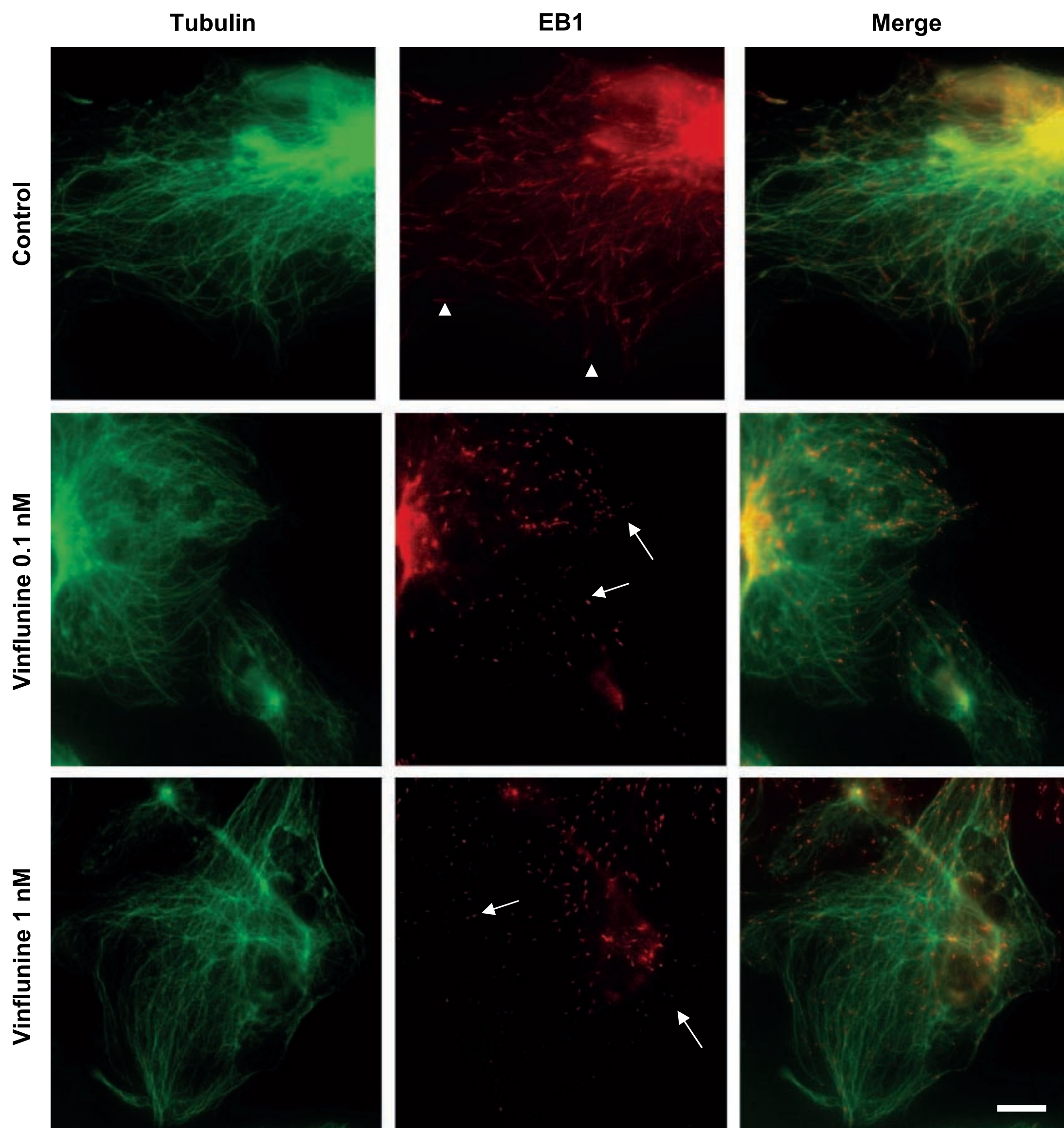

Figure 6. Vinflunine altered EB1 localization at microtubule (+) ends. HMEC-1 cells were incubated with various concentrations of vinflunine for 4 h and then immunostained for α-tubulin and EB1. In control cells, EB1 appeared as long comets at the peripheral microtubule (+) ends (*arrowheads*). In contrast, in cells incubated with vinflunine, the length of the EB1 comets was significantly reduced and more likely appeared as dots at the (+) end of microtubules (*arrows*). *Bars,* 10 μm.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

increase it (23). In these conditions, we did not observe a complete loss of endogenous EB1 at the tip of the microtubules. Instead, a significant reduction in the size of EB1 comets was evidenced such that EB1 appeared as dots at the (+) end of microtubules. This is consistent with the fact that in vinflunine-treated endothelial cells, microtubules continued to grow beyond adhesion sites with similar rates compared with noncaptured microtubules in untreated cells (data not shown). Thereby, tubulin and/or microtubule conformational changes induced by vinflunine, which like other *Vinca* alkaloids preferentially binds to the microtubule (+) end (36), may alter EB1 interaction and/or decrease the number of EB1 binding sites at the microtubule (+) end. Thus, the reduction of EB1 at microtubule (+) ends is more likely a consequence of a direct inhibition of its binding than a consequence of any changes in microtubule dynamic instability. Besides these direct effects of vinflunine on EB1-microtubule interaction, we cannot exclude the activation of a signaling pathway that posttranslationally modifies EB1. The transition from tip-specific binding to microtubule decoration can be mimicked for p150^{glued}, which directly binds to EB1 by using kinase inhibitors and site-directed mutants (37, 38). This suggests that kinase activity is responsible for the (+) tip specificity of tip trackers. Interestingly, EB1 phosphorylation by the COP9 signalosome protects it from degradation by the ubiquitin system and the EB1 COOH terminus is sufficient for interaction with COP9 (39); therefore, vinflunine may inhibit EB1-COP9 interaction and accelerate EB1 degradation which would occur at the rear of EB1 comets when EB1 dissociates from microtubules and would result in dot-like EB1 distribution at microtubule tips.

How does the reduction of EB1 comets affect endothelial cell migration? EB1 has been proposed as the core regulator of microtubule (+) end functions because all +TIPs shown to interact with microtubules directly bind to EB1 (26). Microtubule targeting and it subsequent capture at adhesion sites would involve proteins for which a minimum steady state concentration of EB1 is necessary for direct binding. Several proteins compete for binding to the COOH terminus of EB1, such as those containing a CAP-Gly domain similar to p150^{glued}, and are known to participate in microtubule targeting at the cell cortex. Moreover, the possible lower affinity of protein complexes involved in microtubule anchoring/stabilization at adhesion sites and/or steric hindrance at microtubule tips may require a sufficient set of free EB1 at the rear of the comet to interact for a minimum amount of time. Overexpression of the EB1 COOH terminus in fibroblasts inhibits their migration (40), which would be consistent with a buffering of such low-affinity protein complexes. Moreover, the model of force generation by cytoskeleton end-tracking proteins developed by Dickinson et al. (41) suggests that regulated cycles of EB1 interaction with tubulin are critical. Interestingly, it has been recently shown that EB1 suppresses microtubule dynamic instability and increases microtubule pause *in vitro* (25). Thus, the effect of vinflunine on EB1 at microtubule (+) ends may be directly involved in the decrease in microtubule pauses and in the increase in microtubule dynamic instability we previously evidenced (23).

Altogether, our observations suggest that low antiangiogenic concentrations of MTAs inhibit endothelial cell migration by preventing microtubule targeting of adhesion sites through the disruption of normal EB1 turnover at microtubule +ends. A proteomic approach is in progress to identify and characterize microtubule (+) end protein complexes that are involved in endothelial cell migration. These complexes may lead to biomarkers with potential diagnostic values and to new therapeutic targets.

Disclosure of Potential Conflicts of Interest

A. Kruczynski: Pierre Fabre Medicadent employee. The other authors reported no potential conflicts of interest.

Acknowledgments

We greatly acknowledge Dr. Rick Horwitz for the gift of DsRed-paxillin plasmid. We also thank Drs. Eddy Pasquier, Bertrand Pourroy, and Ali Badache for reading the manuscript and helpful discussions.

References

1. Folkman J. Tumor angiogenesis: therapeutic implications. N Engl J Med 1971;285:1182 – 6.

2. Folkman J. Angiogenesis. Annu Rev Med 2006;57:1 – 18.

3. Miller KD, Sweeney CJ, Sledge GW, Jr. Redefining the target: chemotherapeutics as antiangiogenics. J Clin Oncol 2001;19:1195 – 206.

4. Hayot C, Farinelle S, De Decker R, et al. *In vitro* pharmacological characterizations of the antiangiogenic and anti-tumor cell migration properties mediated by microtubule-affecting drugs, with special emphasis on the organization of the actin cytoskeleton. Int J Oncol 2002; 21:417 – 25.

5. Wang J, Lou P, Lesniewski R, Henkin J. Paclitaxel at ultra low concentrations inhibits angiogenesis without affecting cellular microtubule assembly. Anticancer Drugs 2003;14:13 – 9.

6. Holwell SE, Hill BT, Bibby MC. Anti-vascular effects of vinflunine in the MAC 15A transplantable adenocarcinoma model. Br J Cancer 2001;84: 290 – 5.

7. Kruczynski A, Poli M, Dossi R, et al. Antiangiogenic, vascular-disrupting and anti-metastatic activities of vinflunine, the latest vinca alkaloid in clinical development. Eur J Cancer 2006;42:2821 – 32.

8. Pasquier E, Honore S, Braguer D. Microtubule-targeting agents in angiogenesis: where do we stand? Drug Resist Updat 2006;9:74 – 86.

9. Pasquier E, Andre N, Braguer D. Targeting microtubules to inhibit angiogenesis and disrupt tumour vasculature: implications for cancer treatment. Curr Cancer Drug Targets 2007;7:325 – 34.

10. Lauffenburger DA, Horwitz AF. Cell migration: a physically integrated molecular process. Cell 1996;84:359 – 69.

11. Watanabe T, Noritake J, Kaibuchi K. Regulation of microtubules in cell migration. Trends Cell Biol 2005;15:76 – 83.

12. Ballestrem C, Magid N, Zonis J, Shtutman M, Bershadsky A. Interplay between the actin cytoskeleton, focal adhesions, and microtubules. In: Ridley AP, Peckham M, Clark P, editors. Cell motility: from molecules to organisms. John Wiley and Sons, Ltd.; 2004. p.75 – 99.

13. Liu SM, Magnusson KE, Sundqvist T. Microtubules are involved in transport of macromolecules by vesicles in cultured bovine aortic endothelial cells. J Cell Physiol 1993;156:311 – 6.

14. Hamm-Alvarez SF, Alayof BE, Himmel HM, et al. Coordinate depression of bradykinin receptor recycling and microtubule-dependent transport by taxol. Proc Natl Acad Sci U S A 1994;91:7812 – 6.

15. Waterman-Storer CM, Salmon E. Positive feedback interactions between microtubule and actin dynamics during cell motility. Curr Opin Cell Biol 1999;11:61 – 7.

16. Zhou X, Li J, Kucik DF. The microtubule cytoskeleton participates in control of β2 integrin avidity. J Biol Chem 2001;76:44762 – 9.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024

17. Hotchkiss KA, Ashton AW, Mahmood R, Russell RG, Sparano JA, Schwartz EL. Inhibition of endothelial cell function *in vitro* and angiogenesis *in vivo* by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol Cancer Ther 2002; 1:1191 – 200.

18. Bijman MN, van Nieuw Amerongen GP, Laurens N, van Hinsbergh VW, Boven E. Microtubule-targeting agents inhibit angiogenesis at subtoxic concentrations, a process associated with inhibition of Rac1 and Cdc42 activity and changes in the endothelial cytoskeleton. Mol Cancer Ther 2006;5:2348 – 57.

19. Murtagh J, Lu H, Schwartz EL. Taxotere-induced inhibition of human endothelial cell migration is a result of heat shock protein 90 degradation. Cancer Res 2006;66:8192 – 9.

20. Honore S, Pasquier E, Braguer D. Understanding microtubule dynamics for improved cancer therapy. Cell Mol Life Sci 2005;62: 3039 – 56.

21. Pasquier E, Carre M, Pourroy B, et al. Antiangiogenic activity of paclitaxel is associated with its cytostatic effect, mediated by the initiation but not completion of a mitochondrial apoptotic signaling pathway. Mol Cancer Ther 2004;3:1301 – 10.

22. Pasquier E, Honore S, Pourroy B, et al. Antiangiogenic concentrations of paclitaxel induce an increase in microtubule dynamics in endothelial cells but not in cancer cells. Cancer Res 2005;65:2433 – 40.

23. Pourroy B, Honore S, Pasquier E, et al. Antiangiogenic concentrations of vinflunine increase the interphase microtubule dynamics and decrease the motility of endothelial cells. Cancer Res 2006;66:3256 – 63.

24. Wadsworth P. Regional regulation of microtubule dynamics in polarized, motile cells. Cell Motil Cytoskeleton 1999;42:48 – 59.

25. Manna T, Honnappa S, Steinmetz MO, Wilson L. Suppression of microtubule dynamic instability by the +TIP protein EB1 and its modulation by the CAP-Gly domain of p150(Glued). Biochemistry 2008; 47:779 – 86.

26. Lansbergen G,. and Akhmanova A. Microtubule plus end: a hub of cellular activities. Traffic 2006;7:499 – 507.

27. Zygourakis K. Quantification and regulation of cell migration. In: Mary Ann Liebert, Inc. Tissue engineering. 1996; volume 2, number 1.

28. Webb DJ, Donais K, Whitmore LA, et al. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. Nat Cell Biol 2004;6:154 – 61.

29. Honore S, Kamath K, Braguer D, et al. Synergistic suppression of microtubule dynamics by discodermolide and paclitaxel in non-small cell lung carcinoma cells. Cancer Res 2004;64:4957 – 64.

30. Kaverina I, Krylyshkina O, Small JV. Microtubule targeting of substrate contacts promotes their relaxation and dissociation. J Cell Biol 1999;146:1033 – 44.

31. Mimori-Kiyosue Y, Tsukita S. ''Search-and-capture'' of microtubules through plus-end-binding proteins (+TIPs). J Biochem 2003; 134:321 – 6.

32. Kaverina I, Krylyshkina O, Small JV. Regulation of substrate adhesion dynamics during cell motility. Int J Biochem Cell Biol 2002;34:746 – 61.

33. Krylyshkina O, Anderson KI, Kaverina I, et al. Nanometer targeting of microtubules to focal adhesions. J Cell Biol 2003;161:853 – 9.

34. Etienne-Manneville S. Actin and microtubules in cell motility: which one is in control? Traffic 2004;5:470 – 7.

35. Ezratty EJ, Partridge MA, Gundersen GG. Microtubule-induced focal adhesion disassembly is mediated by dynamin and focal adhesion kinase. Nat Cell Biol 2005;7:581 – 90.

36. Jordan MA, Wilson L. Microtubules as a target for anticancer drugs. Nat Rev Cancer 2004;4:253 – 65.

37. Vaughan KT. Surfing, regulating and capturing: are all microtubule-tip-tracking proteins created equal? Trends Cell Biol 2004;14:491 – 6.

38. Vaughan PS, Miura P, Henderson M, Byrne B, Vaughan KT. A role for regulated binding of p150(Glued) to microtubule plus ends in organelle transport. J Cell Biol 2002;158:305 – 19.

39. Peth A, Boettcher JP, Dubiel W. Ubiquitin-dependent proteolysis of the microtubule end-binding protein 1, EB1, is controlled by the COP9 signalosome: possible consequences for microtubule filament stability. J Mol Biol 2007;368:550 – 63.

40. Wen Y, Eng CH, Schmoranzer J, et al. EB1 and APC bind to mDia to stabilize microtubules downstream of Rho and promote cell migration. Nat Cell Biol 2004;6:820 – 30.

41. Dickinson RB, Caro L, Purich DL. Force generation by cytoskeletal filament end-tracking proteins. Biophys J 2004;87:2838 – 54.

Downloaded from http://aacrjournals.org/mct/article-pdf/7/7/2080/1880134/2080.pdf by guest on 19 July 2024