Microtubule Depolymerization Rapidly Collapses Capillary Tube Networks in Vitro and Angiogenic Vessels in Vivo through the Small GTPase Rho*†

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Maintenance of endothelial cell tube integrity is dependent on an intact cytoskeleton. We present data indicating that rapid collapse of endothelial tubular networks in vitro occurs in a dose-dependent manner after administration of microtubule-depolymerizing reagents but not after actin depolymerization. Pretreatment of endothelial cell networks with C3 exoenzyme or recombinant adenoviruses expressing dominant negative RhoA resulted in complete blockade of tube collapse, indicating a role for RhoA in these events. Microtubule depolymerization also resulted in activation of RhoA, whereas increased expression of constitutively active RhoA induced cell rounding and apoptosis of endothelial cells. Furthermore, following treatment with the chemotherapeutic agent vinblastine, rapid capillary tube network collapse occurred followed by endothelial cell apoptosis. Vinblastine, but not control agents, induced cleavage of procaspase-3, procaspase-9, and procaspase-8, along with the known caspase targets p21-activated kinase-2 and gelsolin, indicating that tube collapse caused a defined apoptotic response. Using a model of vascular endothelial growth factor-stimulated angiogenesis in vitro, vinblastine treatment also resulted in collapse and apoptosis of angiogenic blood vessels. Apoptotic endothelial cells stained strongly for cleaved caspase-3, and terminal dUTP nick-end labeling staining revealed fragmented nuclei in vinblastine-treated but not control angiogenic areas. Together, these findings indicate that microtubule-depolymerizing agents directly induce endothelial network collapse in vitro and in vivo leading to endothelial cell apoptosis in a manner dependent on the small GTPase, RhoA. In addition, these findings reveal a novel function for microtubule disrupting chemotherapeutic agents, namely their ability to rapidly collapse newly formed angiogenic vessels, which may contribute to their effectiveness in limiting angiogenesis and tumor growth.

The development of an angiogenic blood supply from the existing vasculature is critical for solid tumor progression (1, 2). Blood vessel formation both in vivo and in vitro is intricately regulated by a number of factors including extracellular matrix, growth factors, membrane-bound proteinases, and integrins (3, 4). Signal generation by these molecules is critically integrated with intracellular cues that lead to cytoskeletal rearrangements that orchestrate the various steps in angiogenesis including endothelial cell (EC) proliferation, branching, sprouting, and lumen formation (4). Both the actin and microtubule cytoskeletons play a key role in the formation and maintenance of EC shape changes as well as cell proliferation. Therefore, the cytoskeleton is a critical component regulating the complex series of signaling events that dictate EC shape changes (e.g. morphogenesis) occurring during angiogenesis.

We have a particular interest in identifying the molecular mechanisms through which ECs not only assemble into tubes but also maintain those structures. We have identified previously that Cdc42 and Rac1 GTPases are required for ECs to initiate lumen formation and undergo morphogenesis when placed in three dimensions (5). An interesting new question is to determine how members of the Rho GTPase family function in maintaining the shape of EC tubular structures. Endothelial cells undergoing morphogenesis form lumens and branched structures, assembling into tubes, when placed in either threedimensional collagen or fibrin matrices (5–10). Here, established EC networks are treated with microtubule- and actin-disrupting agents to determine which cytoskeletal components are necessary to maintain tubular structures.

Microtubule perturbation is an effective chemotherapeutic strategy in cancer therapy. Generally, microtubule-depolymerizing agents are thought to interfere with the cell cycle, which limits tumor and EC proliferation. Recent studies (11) have indicated a direct relationship between the state of microtubules and the process of apoptosis through anti-apoptotic proteins such as survivin. Furthermore, past studies indicate the ability of the pro-apoptotic protein, p53, to bind microtubules and be released from them following microtubule depolymerization (12). Vinblastine is a vinca alkaloid that is utilized as an effective chemotherapeutic agent (13). It acts as a reversible microtubule-collapsing agent, and in combination with other therapies, such as vascular endothelial growth factor (VEGF) receptor antagonism, has proven to be an effective tumor and angiogenesis regression agent in animal models (14–16). Although vinblastine therapy has been shown to prevent tumor and endothelial cell proliferation (13, 14, 17), which may par-
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Immunoperoxidase Staining and TUNEL Assays—Tissues were prepared for von Willebrand factor (vWF) expression, cleaved caspase-3, and green fluorescent protein (GFP). The adenosinergic system (20) is designed so that adenosinergic infected fibroblasts co-express GFP along with secretion of VEGF165. Formalin-fixed paraffin sections were cut 5 μm thick and deparaffinized in xylene. Sections were rehydrated through a series of graded ethanol washes before being placed in water. Antigens were unmasked using fresh 0.1% trypsin in 10 mM CaCl2 for 10 min at room temperature. Slides were rinsed and incubated in 3% H2O2 for 15 min in the dark. After a wash in phosphate-buffered saline, sections were blocked with RPMI 1640 + 10% FCS for 30 min at room temperature. Polyclonal antisera raised to caspase-3 (BD Pharmingen), vWF (DAKO Laboratories) (21), and polyclonal anti-enhanced GFP antibody (15) purified using protein A-Sepharose were incubated overnight at 4 °C in humidified chambers. Antibody detection was accomplished using the Rabbit IgG Vectastain ABC kit (Vector Laboratories) according to the product insert. TUNEL staining was performed according to manufacturer's instructions (Roche Applied Science). Peroxidase was detected using ABC chromogen (DAKO) for 2–10 min. Reactions were stopped with water, and sections were counterstained using a 1:100 dilution of 0.5% toluidine blue and 1% sodium borate in water.

Caspase-3 Inhibition and Detection—ZVAD-fmk (caspase inhibitor I) and Z-DEVD-fmk (caspase-3 inhibitor II) (Calbiochem) were suspended in M2 to 10 μM. Ecs were suspended in collagen matrices and allowed to undergo morphogenesis. At 36 h of culture, 50 μM dose of ZVAD was added. At 48 h of culture, an additional 50 μM dose of ZVAD was added. Extracts were collected and analyzed for caspase-3 expression and activation by Western blot analysis. Polyclonal antisera to cleaved caspase-3 fragments (Cell Signaling Technologies) and monoclonal antibodies recognizing pro-caspase-3 (BD Biosciences) were used at 1:1000 dilutions overnight in 4% milk. DEVD hydrolysis assays (Chemicon) were conducted by seeding confluent monolayers (60-mm dishes) overnight for 16 h. Cells were rinsed once in 5 ml of M199 and vinblastine, or vehicle control was added at 10 μM dose in M199 with reduced serum II supplement (1:250) for 3 h. Cells were harvested (250 μl) of assay buffer, incubated on ice for 10 min, and spun for 5 min at 4 °C. Assays were carried out at 37 °C according to manufacturer's instructions and read at A405.

RESULTS

Microtubule Disruption Rapidly Induces Collapse of Capillary Tube Structures in Three-dimensional Collagen Matrices—Here we investigate the molecular mechanisms through which the structure of EC tubular networks are maintained. Initial experiments revealed that an intact microtubular cytoskeleton is necessary for endothelial cells undergoing morphogenesis in three dimensions to maintain their structure (Fig. 1). During the process of morphogenesis, endothelial cells suspended individually in three-dimensional collagen or fibrin matrices assemble into tubes to form lumens and branched structures that closely resemble capillary tubular networks (5–10). To determine whether an intact microtubule or actin cytoskeleton is required to maintain these tubular structures, ECs were allowed to assemble into networks before treatment with various agents. The addition of the microtubule-disrupting agent vinblastine induced rapid collapse (occurring within 1 h), whereas the disruption of actin microfilaments with cytochalasin B had minor effects. Pretreatment with C3 exoenzyme, an inhibitor of the small GTPase Rho, interfered with the ability of vinblastine to collapse luminal structures, indicating Rho is required for this process. Similar to vinblastine, colchicine and nocodazole induced complete collapse of EC luminal structures that was also blocked by pretreatment with C3 exoenzyme (not shown). The microtubule-stabilizing agent taxol likewise induced collapse of EC luminal structures at 48 h, although not as complete as the collapse seen with microtubule-depolymerizing agents (not shown). No effects were observed with buffer controls.

ECs forming tubular networks in three-dimensional collagen gels at 48 h were treated with varying doses of vinblastine, colchicine, and cytochalasin B (Fig. 2). Quantitation of collapse...
is shown where the addition of cytochalasin B up to 10 μM results in collapse of only 50% of luminal structures. In contrast, doses of vinblastine are 100 times more potent, and the addition of vinblastine collapsed 90% of luminal structures. Although the effects of colchicine are similar, the collapse response is not as complete compared with vinblastine.

In other experiments, we determined the reversibility of the cytoskeletal disrupting agents. Drugs were removed after 1 h of collapse, and cultures were washed every 30 min for 3 h before new culture media were added. Interestingly, although the effects of cytochalasin B and nocodazole were reversible, as branched and luminal structures began to reform, cultures treated with vinblastine or colchicine were unable to recover over the next 24 h (data not shown).

Vinblastine-induced Collapse of EC Structures Specifically Involves the Small GTPase RhoA—We further investigated the involvement of specific members of the Rho GTPase family using recombinant adenoviruses to deliver dominant negative forms of RhoA, Rac1, and Cdc42 GTPases to ECs. After 16 h, ECs were placed in three-dimensional collagen matrices and allowed to form lumens for 24 h before addition of cytoskeletal disrupting agents. Nocodazole (NOCOD) and colchicine (COLCH) (10 μM) were added 1 h before fixation. Cultures were stained, and lumen formation was quantitated. B, photographs illustrating the requirement for RhoA in microtubule depolymerization-induced EC luminal collapse. Arrows indicate luminal structures. Arrowheads indicate collapsed areas. Similar results were seen with the addition of vinblastine (10 μM) (data not shown). Bar = 30 μm.

**FIG. 3.** The small GTPase RhoA is required for EC luminal collapse following microtubule disruption. A, recombinant adenoviruses (5, 20) were used to deliver green fluorescent protein (GFP) or dominant negative forms of RhoA, Rac1, and Cdc42 GTPases to ECs. After 16 h, ECs were placed in three-dimensional collagen matrices and allowed to form lumens for 24 h before the addition of cytoskeletal disrupting agents. Nocodazole (NOCOD) and colchicine (COLCH) (10 μM) were added 1 h before fixation. Cultures were fixed in 2% paraformaldehyde in phosphate-buffered saline, and lumen formation was quantitated. B, photographs illustrating the requirement for RhoA in microtubule depolymerization-induced EC luminal collapse. Arrows indicate luminal structures. Arrowheads indicate collapsed areas. Similar results were seen with the addition of vinblastine (10 μM) (data not shown). Bar = 30 μm.
ment with C3 exoenzyme and indicate Rho activation, but not Rac or Cdc42, is required for EC luminal structures to collapse following microtubule disruption with nocodazole or colchicine. Photographs of these effects are shown in Fig. 3B. Adenovirally infected ECs expressing either GFP or N19RhoA were allowed to undergo morphogenesis before the addition of nocodazole. Whereas nocodazole induced complete collapse in GFP-expressing cells, the collapse was blocked in cells expressing the dominant negative form of RhoA (N19RhoA) (Fig. 3B). An additional experiment tested whether other Rho isoforms participated in the microtubule-dependent tube collapse response. ECs expressing dominant negative RhoC were able to

FIG. 4. Microtubule disruption initiates pro-caspase and gelsolin cleavage following EC lumen collapse. A, ECs were placed in three-dimensional collagen matrices and allowed to undergo morphogenesis for 48 h before the addition of cytoskeletal disrupting agents. At 48 h, vinblastine (Vbl), cytochalasin B (CCB), colchicine (Col), and nocodazole (Noc) were added at the doses indicated for an additional 4, 8, and 24 h before extracts were made. Western blot analyses were conducted to determine pro-caspase-3 levels and gelsolin cleavage. Arrowhead indicates gelsolin cleavage product. B, pretreatment of EC networks with ZVAD, a caspase inhibitor, does not affect vinblastine-induced luminal collapse. Vinblastine was added after 48 h of EC network formation. Cultures were fixed at the time points indicated, and luminal collapse was quantitated. Data shown are average values (n = 3) ± S.D. Western blot analyses of extracts were probed with antibodies to pro-caspase-3 and cleaved caspase-3. Note that treatment with ZVAD prevented complete conversion of pro-caspase-3 to lower molecular weight form. C, vinblastine induced EC luminal collapse and initiated caspase activation. ECs were placed in three-dimensional collagen matrices and allowed to undergo morphogenesis for 48 h before the addition of Vbl (10 μM) and buffer control for an additional 0.5, 2, 8, and 19 h before extracts were made. Western blot analyses were conducted to determine pro-caspase-8, pro-caspase-9, and cleaved caspase-3 levels. Arrowhead indicates caspase-3 and caspase-9 cleavage products. D, DEVD hydrolysis assays were carried out with EC extracts following treatment with vinblastine or control (3 h). Values represent fold increase in absorbance readings (405 nm) compared with control.
block vinblastine-induced luminal collapse, whereas expression of RhoB did not (data not shown). These data indicate that activated RhoA (and possibly RhoC) is required for microtubule depolymerization-induced collapse of EC tubes.

**Vinblastine-induced Collapse of EC Structures Is Followed by Apoptosis**—Time course experiments were performed to characterize the collapse response and determine whether structural collapse initiated EC apoptosis. EC cultures were allowed to develop for 48 h in three-dimensional collagen matrices before the addition of vinblastine, colchicine, nocodazole, or cytochalasin B. Capillary tube structures were allowed to collapse for 4, 8, or 24 h. Western blot analyses of endothelial extracts probed for pro-caspase-3 and gelsolin revealed that both proteins were cleaved following microtubule depolymerization (Fig. 4A). Pro-caspase-3 levels are diminished at 8 h in the presence of vinblastine. At 24 h, pro-caspase-3 levels are decreased in cultures treated with 10 μM vinblastine, 1 μM vinblastine, 10 μM nocodazole, or 10 μM colchicine. Gelsolin is known to be cleaved during apoptosis (22, 23). A visible fragment of gelsolin (indicated by arrowheads) is seen in the presence of vinblastine as early as 4 h. Also evident at 24 h is a decrease in the intact 85-kDa form of gelsolin with vinblastine, colchicine, and nocodazole treatment. To investigate the temporal series of events leading to luminal collapse and apoptosis of EC luminal networks following microtubule depolymerization.
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Microtubule-disrupting agents induced almost complete collapse of EC tubular structures, whereas CCB, an actin-disrupting agent showed minimal effects on EC tube structures. Comparative molecular analysis of gelsolin, procaspase-3, and cleaved caspase-3 following treatment with vinblastine versus cytochalasin B are shown in Fig. 5, A and B, respectively. Cultures were fixed and extracts were made 8 h after collapse was initiated and probed for gelsolin, procaspase-3, and cleaved caspase-3 at the doses indicated. After 8 h of vinblastine treatment (Fig. 5A), 10 \(\mu\)M doses resulted in gelsolin cleavage, with a decrease in the full-length form of gelsolin (85 kDa) that coincides with the appearance of a 50-kDa fragment of gelsolin not seen in cytochalasin B experiments (Fig. 5B). Similarly, vinblastine elicited cleavage of procaspase-3, where levels were reduced compared with control, even at very low doses of vinblastine. In agreement with this, cleaved caspase-3 was also detected at low doses of vinblastine. The caspase-3 cleavage, indicative of apoptosis, correlated well with luminal collapse (see Fig. 2). In contrast, only the highest dose of cytochalasin B elicited collapse of luminal structures (Fig. 2) and caspase-3 cleavage (Fig. 5B). Taken together, these data confirm that microtubule depolymerization precedes EC luminal collapse and initiates apoptosis.

Vinblastine-induced Initiation of Apoptosis and Collapse of EC Structures Involves Activation of Rho—To demonstrate whether vinblastine treatment initiated Rho activation, EC monolayers were extracted at 0, 1, 3, 20, and 30 min after treatment with vinblastine (Fig. 6A). Lysates were incubated with GST-rhotekin-Sepharose and assayed for GTP-bound Rho. Data from two separate experiments showed a clear increase in Rho-GTP levels after 1 min of vinblastine treatment that was sustained and increased further at 30 min, indicating vinblastine treatment resulted in activation of Rho. These data agree with previous reports where microtubule collapse with nocodazole resulted in Rho activation in fibroblasts (25, 26).

To determine whether RhoA activation may initiate apoptosis, mutant forms of RhoA were expressed in ECs using recombinant adenoviruses. ECs expressing N19RhoA, V14RhoA, and GFP control were placed in three-dimensional collagen matrices (arrowheads) that is absent in the GFP control. Angiogenic vessels that developed at the perimeter of collagen matrices (small arrows) were photographed prior to treatment (Start) with vinblastine (details under “Materials and Methods”). After 7 h, the same field was photographed (7 h of treatment). Bar = 1 mm. C, the addition of vehicle control had no effect on angiogenic rings formed at the perimeter of collagen matrices. In addition to collapse of angiogenic rings, after 7 h of vinblastine treatment, cleared zones were also observed (large arrows) on the surface of the CAM itself. Bar = 1 mm.

curred rapidly, within 1.5 h. Caspase-3 cleavage, indicative of apoptosis, lagged considerably and did not become evident until between 3 and 6 h after vinblastine addition. Pretreatment of networks with ZVAD-fmk, a general caspase inhibitor prior to the addition of vinblastine, exhibited no ability to block vinblastine-induced luminal collapse. This time course indicated that caspase activation occurred subsequent to collapse of luminal structures and was only detectable hours after luminal collapse. Additional experiments revealed cleavage of procaspase-8 and pro-caspase-9 using extracts that were taken 0.5, 2, 8, and 19 h after the addition of vinblastine or control buffer (Fig. 4C). These data indicate that pro-caspase-8 cleavage occurred, as did pro-caspase-9 cleavage, where a smaller fragment of caspase-9 (arrowhead) is detected along with cleaved caspase-3 (Fig. 4C). Also, PAR-2 cleavage was observed. PAR-2 has been reported previously to be a target of activated caspases (24). In addition, using a DEVD hydrolysis assay, a 4-fold increase was observed following microtubule depolymerization by vinblastine (Fig. 4D). These data were confirmed by Western blot analysis of cleaved caspase-3 (data not shown). Together, these data suggest that microtubule depolymerization rapidly collapsed EC luminal networks, leading to subsequent pro-caspase activation and apoptosis.

Figure 7: Vinblastine-induced collapse of VEGF-induced angiogenic vessels. A, recombinant adenoviruses were used to transfer the VEGF165 isoform or green fluorescent protein (GFP) control to human dermal fibroblasts for 24 h. Fibroblasts were suspended in collagen matrices that were equilibrated and placed onto the surface of CAM (indicated by *) and anchored with pre-wetted nitrocellulose membrane (NC). Angiogenesis was allowed to develop for 72 h after addition of collagen gels. Note the ring of angiogenic vessels at the perimeter of the VEGF165 collagen matrix (small arrows) that is absent in the GFP control. Bar = 1 mm. B, vinblastine treatment induced collapse of VEGF-stimulated angiogenic vessels. Angiogenic vessels that developed at the perimeter of collagen matrices (arrows) were photographed prior to treatment (Start) with vinblastin (details under “Materials and Methods”). After 7 h, the same field was photographed (7 h of treatment). Bar = 1 mm. C, the addition of vehicle control had no effect on angiogenic rings formed at the perimeter of collagen matrices. In addition to collapse of angiogenic rings, after 7 h of vinblastine treatment, cleared zones were also observed (large arrows) on the surface of the CAM itself. Bar = 1 mm.

Fig. 7. Vinblastine-induced collapse of VEGF-induced angiogenic vessels in the chick chorioallantoic membrane (CAM) assay. A, recombinant adenoviruses were used to transfer the VEGF165 isoform or green fluorescent protein (GFP) control to human dermal fibroblasts for 24 h. Fibroblasts were suspended in collagen matrices that were equilibrated and placed onto the surface of CAM (indicated by *) and anchored with pre-wetted nitrocellulose membrane (NC). Angiogenesis was allowed to develop for 72 h after addition of collagen gels. Note the ring of angiogenic vessels at the perimeter of the VEGF165 collagen matrix (small arrows) that is absent in the GFP control. Bar = 1 mm. B, vinblastine treatment induced collapse of VEGF-stimulated angiogenic vessels. Angiogenic vessels that developed at the perimeter of collagen matrices (arrows) were photographed prior to treatment (Start) with vinblastine (details under “Materials and Methods”). After 7 h, the same field was photographed (7 h of treatment). Bar = 1 mm. C, the addition of vehicle control had no effect on angiogenic rings formed at the perimeter of collagen matrices. In addition to collapse of angiogenic rings, after 7 h of vinblastine treatment, cleared zones were also observed (large arrows) on the surface of the CAM itself. Bar = 1 mm.
To investigate the ability of known anti-apoptotic molecules to affect the collapse event, recombinant adenoviruses were used to express Bcl-2 (Fig. 6C). Under control conditions, an adenovirus expressing GFP was used, and EC collapse occurred nearly completely within 0.5 h. ECs expressing Bcl-2 collapsed in a similar manner, indicating that expression of the anti-apoptotic protein did not block collapse. Notably, expression of N19RhoA blocked the collapse for up to 24 h after the addition of vinblastine. These data reveal that RhoA activation occurred proximal to the collapse response and was required for tube collapse and subsequent EC apoptosis. The sequence of events that occurs following microtubule disruption of three-dimensional EC networks is illustrated in Fig. 6D.

**Vinblastine Induces Rapid Collapse of Angiogenic Vessels in Vivo and Endothelial Cell Apoptosis**—To determine whether our observations in vitro were consistent with effects of vin-
Vinblastine observed in vivo (14–17), we developed a model for induction of angiogenesis using the CAM assay. In this model, angiogenesis was initiated through adenoviral delivery of VEGF<sub>165</sub> by human dermal fibroblasts. Fibroblasts expressing VEGF<sub>165</sub> were placed in three-dimensional collagen gels, which were anchored to the CAM using sterile nitrocellulose. As shown in Fig. 7A, an angiogenic response (indicated by arrows) is seen around the periphery of collagen gels (marked with asterisk) containing fibroblasts expressing VEGF<sub>165</sub>. This response is not seen in control experiments where GFP is expressed in fibroblasts. The addition of vinblastine had a marked effect on the angiogenic vessels at the periphery of the collagen gels. Photographs illustrating this are shown in Fig. 7B. Before the addition of vinblastine (Start), marked angiogenic rings are seen (marked by arrows) that collapse 7 h after vinblastine treatment (see arrowheads). No obvious changes are seen with the vehicle control (Fig. 7C, left panel). In addition to collapse and disappearance of the angiogenic ring stimulated by VEGF<sub>165</sub> (marked by arrowheads), we also observed cleared zones (marked by arrows) after vinblastine treatment but not in control groups (Fig. 7C, right panel). Interestingly, many vessels in the area of treatment appeared unaffected, perhaps indicating that stable vessels (i.e., non-angiogenic) do not collapse in response to vinblastine. Another known effect of microtubule disruption in the microcirculation is the induction of arteriolar vasoconstriction that limits blood flow into the area of treatment (27). This transient decrease in perfusion may contribute to the angiogenic vessel collapse as well.

Paraffin sections of collagen gels and surrounding tissue from the choriouillantoic membrane assay treated with control buffer or vinblastine were stained with hematoxylin and eosin to analyze the VEGF-induced angiogenic response and the effects of vinblastine treatment. In the control group (Fig. 8A), intact vascular structures containing red blood cells were observed (marked by arrows). In the vinblastine-treated group (Fig. 8B), collapsed areas (marked by arrowheads) were observed that did not contain continuous channels and lacked red blood cells. To confirm that these were endothelial structures, sections were stained with polyclonal antisera recognizing vWF (21). In the control group (Fig. 8C), the staining pattern appears consistent with capillary-like structures that appear intact and contain red blood cells (marked by arrows). Vinblastine treatment, however, dramatically alters the morphology of these structures, and vWF staining is localized to collapsed areas (Fig. 8D), where it appears nuclear fragmentation is occurring (marked by arrowheads). Adjacent to collagen matrices, no open capillary-like structures were observed positive for vWF staining, indicating that endothelial structures lining vascular channels were collapsed. Control polyclonal antisera raised to GFP stained only fibroblasts (marked by arrowheads) placed in the collagen gels that co-express GFP and VEGF (Fig. 8E). No signal was observed without primary antibody (Fig. 8F). The percentage of open lumens remaining following vinblastine and control treatments are shown in Fig. 8G. The addition of vinblastine resulted in a 3-fold reduction in the number of open lumens adjacent to the collagen matrix. Staining for cleaved caspase-3 is illustrated in Fig. 9 (A–D). Minimal staining was observed in control groups (Fig. 9, A and C), whereas cleaved caspase-3 localized directly to collapsed areas following vinblastine treatment (Fig. 9, B and D; see arrowheads). To confirm these findings, TUNEL staining of sections was employed to further demonstrate that vinblastine treatment induced EC apoptosis in vivo. A positive signal indicates labeling of DNA strand breaks, which is known to occur during...
apoptosis. In the control group, a modest amount of staining was observed, consistent with EC proliferation that occurs during angiogenesis. In controls, nuclei appeared morphologically normal with smooth edges (see arrows). However, with vinblastine treatment, the collapsed areas that stained positively for vWF also were strongly positive by TUNEL staining, indicating these areas contain apoptotic endothelial cells (marked by arrowheads). The appearance of fragmented nuclei is consistent with the occurrence of apoptosis in these areas.

**DISCUSSION**

Here we find that microtubule-depolymerizing agents, but not actin disruption, rapidly collapsed EC tubular networks in vitro and induced EC apoptosis. Furthermore, RhoA is required for microtubule depolymerization-induced collapse, and microtubule disruption activated RhoA. Finally, similar effects were observed in vivo, where vinblastine-induced microtubule depolymerization caused collapse and apoptosis of angiogenic vessels.

The Microtubular Cytoskeleton Is Necessary for Maintenance of Three-dimensional Capillary Structures in Vitro and in Vivo—We have investigated the requirement for an intact cytoskeleton in maintaining EC tube integrity. Microtubule disruption induced a rapid dose-dependent collapse of EC lumens, whereas actin disruption did not. Thus, microtubules appear to be predominantly involved in maintaining the overall shape and structure of EC tubes. Disruption of actin slightly altered the shape of individual ECs within the tube structures but overall does not dramatically affect the structure of tubular networks. Interestingly, formation of EC lumens in three-dimensional collagen and fibrin matrices requires the Cdc42 and Rac GTPases (5) as does morphogenesis in Matrigel (28). We have demonstrated previously (7, 10) that several other actin cytoskeletal regulatory proteins including gelsolin, VASP, and profilin are up-regulated during the morphogenic events during tube formation. It follows then that the cytoskeleton and regulatory proteins would also be required to maintain these structures once they have formed. Previous studies (6) have shown that disruption of actin and microtubules using cytochalasin B and microtubule-depolymerizing agents completely blocks formation of EC lumens, indicating that both the actin and microtubule cytoskeletons are important for lumen formation in our assay system. Here we find that microtubules play a critical role in maintaining the structure of EC tubular networks as well.

Microtubule Depolymerization-induced Collapse of Networks of EC Tubes in Vitro via the Small GTPase RhoA—It has been reported previously that microtubule collapse is associated with activation of the small GTPase Rho (25, 29–32), stress fiber formation (33), as well as rounding and apoptosis in cultured cells (26). Also, Rho has been reported to be involved in neurite retraction (34). Furthermore, RhoA has been linked to cell rounding that occurs during mitosis (35), an observation that is consistent with our data. From this, we conclude that activation of the small GTPase Rho occurs with microtubule depolymerization and is necessary for structural collapse of endothelial luminal structures, because blockade of Rho resulted in blockade of EC luminal collapse. We find then that RhoA is involved in the maintenance of three-dimensional capillary structures.

Rho Activation Leads to Apoptosis of ECs Placed in Three-dimensional Collagen Matrices—Both the Rho and Rac small GTPases have been reported to regulate apoptosis (36). Although several reports (37, 38) have indicated that blockade of Rho resulted in apoptosis of endothelial cells, our data are not consistent with these observations. Expression of dominant negative RhoA or treatment with the Rho inhibitor C3 exoenzyme blocked collapse of luminal structures in vitro. Expression of constitutively active RhoA, however, resulted in apoptosis of ECs placed in three-dimensional collagen gels as indicated by caspase-3 cleavage. Other reports have linked the Rho effector, Rho-associated kinase (39, 40), to the caspase-induced blebbing effects seen during apoptosis. These data are consistent with ours and suggest a significant role for activated Rho in regulating EC apoptosis.

Vinblastine Treatment of Angiogenic Vessels in Vivo Resulted in Collapse of Luminal Structures and EC Apoptosis—An intact microtubular cytoskeleton is required for maintenance of EC capillary-like structures, and treatment with the microtubule-disrupting agents resulted in collapse of the structures in a Rho-dependent manner. Others (41) have shown that docetaxel, a derivative of taxol, impairs the microtubule-organizing center and also impairs angiogenesis in vivo. Microtubule-depolymerizing agents at low doses induced tube formation on Matrigel substrates and in the chick CAM assay (42) as well as neovascularization in rat gliomas (43). Microtubule depolymerization may release a cascade of factors that initiate apoptosis and inactivate anti-apoptotic molecules such as p53 (12, 44), survivin (11, 45), or IAP and Bax that phosphorylate Bcl-2 (46). Bcl-2 is normally anti-apoptotic (pro-survival), but when phosphorylated, as occurs with microtubule targeting agents, Bcl-2 becomes inactive (46). This possibility remains to be tested further in three-dimensional EC morphogenesis assays.

These data represent a detailed analysis of the effects of cytoskeletal depolymerizing agents on EC tubes in three-dimensional matrices. Our findings indicate that regulation of the small GTPase Rho and the microtubule cytoskeleton cooperatively maintain the shape of three-dimensional networks of endothelial cells. Additional biochemical studies are necessary to determine the molecular pathway that is responsible for Rho-dependent EC apoptosis following microtubule disruption, but our data suggest that microtubule perturbation directly targets angiogenic vessels in a manner that requires Rho.

Our data also strongly indicate a new functional role for microtubule-disrupting chemotherapeutic agents such as vinblastine. Previous data (15) indicate an interesting ability for vinblastine to serve as an anti-angiogenic agent, particularly in conjunction with anti-VEGF antibodies. The known ability of vinblastine to decrease EC proliferation directed angiogenesis has long been considered its primary mechanism of action (14). Our work here suggests a second possibility, namely the ability of vinblastine to induce rapid collapse of newly formed capillary tubes and angiogenic vessels through disruption of the microtubule cytoskeleton that is required to maintain the shape of tubular networks. Furthermore, we show that RhoA plays a role in these events. These data also present a new strategy in anti-angiogenic therapy, which is to disrupt the three-dimensional architecture of dynamic and unstable EC capillaries in an area of angiogenesis.

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