HEMOSTASIS, THROMBOSIS, AND VASCULAR BIOLOGY

Antiangiogenesis Is Produced by Nontoxic Doses of Vinblastine

By Angelo Vacca, Monica Iurlaro, Domenico Ribatti, Monica Minischetti, Beatrice Nico, Roberto Ria, Antonio Pellegrino, and Franco Dammacco

The effects of vinblastine (VBL) on endothelial cell functions involved in angiogenesis, namely proliferation, chemotaxis, spreading on fibronectin (FN), secretion of matrix-metalloproteinase-2 (MMP-2) and MMP-9, and morphogenesis on Matrigel were tested in vitro, whereas its effects on angiogenesis were studied in vivo by using the chick embryo chorioallantoic membrane (CAM) model. In vitro, at noncytotoxic doses (0.1, 0.25, 0.5, 0.75, and 1 pmol/L), VBL impacted all these functions, except secretion of MMPs, in a dose-dependent fashion. By contrast, proliferation of other primary cells such as fibroblasts and lymphoid tumor cells was not impacted. In vivo, VBL at 0.5, 0.75, and 1 pmol/L again displayed a dose-dependent antiangiogenic activity. Lack of cytotoxicity in vitro and in vivo was shown both morphologically, and also because the antiangiogenic effects were rapidly abolished when VBL was removed. Apoptosis was not induced. At the ultrastructural level, impairment of cell functions in vitro was associated with thin disturbance of the cytoskeleton, in the form of slight depolymerization and accumulation of microfilaments, which was equally reversible. Results suggest that VBL has an antiangiogenic component at very low, nontoxic doses, and that antiangiogenesis by VBL could be used to treat a wide spectrum of angiogenesis-dependent diseases, including certain chronic inflammatory diseases, Kaposi's sarcoma, and cancer.

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Submitted February 12, 1999; accepted August 10, 1999.


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0006-4971/99/9412-0026$3.00/0
cell number was estimated on day 4 by the colorimetric assay of Kueng et al14; cells were fixed for 15 minutes at room temperature with 2.5% glutaraldehyde, stained with 0.1% crystal violet in 20% methanol for 20 minutes, solubilized with 10% acetate, and read at 595 nm in a Microplate Reader 3550 (Bio-Rad Lab, Richmond, CA). The cell number was derived from a calibration curve set up with a known number of cells, and calculated as mean ± 1 standard deviation (SD) per medium.

In the second series, HUVEC or EA.hy926 cells were exposed to control media and VBL doses every 24 hours once, twice, and three times, and counted as above.

Chemotaxis assay. This was performed in Boyden chambers as previously described.15 HUVEC or EA.hy926 cells pretreated for 24 hours with each VBL dose in complete medium were harvested in trypsin/acetate (0.05%/0.02% in PBS), collected by centrifugation, resuspended in DMEM supplemented with 0.1% bovine serum albumin (BSA), and seeded in triplicate for each dose and control in the upper compartment of the chamber (1.2 × 10^6 cells/400 µL), whereas the lower compartment was filled with 200 µL of the NIH 3T3 CM as chemoattractant (positive control), or with DMEM supplemented with 0.1% BSA in the negative control (to evaluate random migration). The compartments were separated by a polycarbonate filter (12 µm pore size; Nucleapore, Costar Co, Cambridge, MA) coated with 0.005% gelatin to allow cell adhesion. After incubation for 6 hours in a humidified 5% CO 2 atmosphere at 37°C, cells on the upper side of the filter were removed, whereas those that had migrated to the lower side were fixed in absolute ethanol, stained with toluidine blue, counted in 5 to 8 160 immersion fields, and calculated as mean ± 1 SD per filter and per medium.

Adhesion assay on fibronectin (FN). After 96-well plates were coated with a FN solution (20 µg/mL) at 4°C overnight, HUVEC were plated (5 × 10^5/well) in triplicate in starvation medium alone (positive control) or containing each VBL dose for 90 minutes at 37°C in 5% CO 2 humidified atmosphere, as described.15 Cells were fixed with glutaraldehyde 2.5% in PBS at 30 and 90 minutes and their number was calculated as described for the proliferation assay.

From 30 to 45 minutes the assay assesses cell attachment that takes place via the α5β3 integrin expressed by both HUVEC and EA.hy926 cells.16 From 45 to 90 minutes the same assay assesses cell spreading mediated by microtubules and microfilaments.2

Morphogenesis assay on Matrigel. Unpolymerized Matrigel (17 mg/mL; Collaborative Biomedical Products, Two Oaks, Park Bedford, MA) was placed in the wells (300 µL/well) of a 24-well microtiter plate (1.28 cm 2/well) and allowed to polymerize for 1 hour at 37°C. HUVEC or EA.hy926 cells were plated (2 × 10^5 cells/well) in 1 mL of SFM containing 50% NIH 3T3 CM (positive control), to which VBL doses were added in experimental wells. After 6 hours of incubation17 in a 5% CO 2 humidified atmosphere at 37°C, cell growth and three-dimensional organization were observed through a reverted, phase-contrast photomicroscope. Cells plated in SFM alone served as the negative control.

MMP-2 and MMP-9 sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) zymography and activity. T25 flasks of EA.hy926 cells at 90% confluence were rinsed twice with SFM and incubated for 24 hours in this medium either alone (positive control) or containing each VBL dose. The CM were then collected and the total protein content was measured by absorbance with the Bradford method (Bio-Rad), using BSA as a standard protein.

To visualize the gelatinolytic activity of the secreted MMP-2 and MMP-9, an SDS-PAGE zymography was performed. Briefly, aliquots of 5 µg CM proteins were applied to 7.5% SDS-PAGE gels polymerized with type A gelatin from porcine skin (Sigma) at a final concentration of 0.6 mg/mL (0.06% wt/vol). After electrophoresis in a Protean II dual lab system (Bio-Rad), the gels were washed in 2.5% Triton 1 × 1 hour to remove SDS, incubated for 18 hours at 37°C, and stained in 0.1% Coomassie brilliant blue. The gelatinolytic regions were observed as white bands against a blue background. MMP activity was measured by scoring the intensity of bands by computerized image analysis (Apple Computer Inc, Cupertino, CA).

Evaluation of apoptosis of HUVEC. The fluorescent propidium iodide (PI) stain weakly the subdiploid DNA produced during apoptosis, whereas it stains strongly the diploid DNA of nonapoptotic cells. The percentage of apoptotic HUVEC was thus detected by measurement of weak PI staining, as described elsewhere.18 HUVEC were plated (2 × 10^5/well) in 78.5 cm 2 Petri dishes (Falcon 3003; Becton Dickinson) in complete medium containing 10% FCS. After 24 hours, the medium was replaced and replaced on days 0 and 2 by the same medium (negative control) or by this medium containing VBL 0.25 pmol/L or 1 pmol/L, or dexamethasone (Merck Co, Inc, West Point, PA) 1 nmol/L, an apoptogenic drug.19 On day 4, cells harvested in trypsin/acetate (0.05%/0.02% in PBS) were treated for 3 hours with 70% ethanol at 4°C, then incubated overnight with 100 µL PI in the presence of RNase, and tested in a flow cytometry (FACScan, Becton Dickinson) for the magnitude of the subdiploid DNA fluorescent peak (M1 region) in comparison with that of diploid DNA peak (M2 region).

Morphologic study. HUVEC or EA.hy926 cells were plated (1 × 10^6/well) in 96-well plates in DMEM supplemented with 10% FCS, allowed to adhere until confluence was reached, and exposed to each VBL dose for 6, 8, and 10 days. A daily morphologic analysis was performed directly with a reverted, phase-contrast photomicroscope, and after the fixation and staining as described in the proliferation assay.

Chorioallantoic membrane (CAM) assay. Fertilized White Leghorn chicken eggs (10/group) were incubated at 37°C at constant humidity. On incubation day 3, a square window was opened in the shell and 2 to 3 mL of albumen was removed to allow detachment of the developing CAM. The window was sealed with a glass and the eggs were returned to the incubator. On day 8, 1 mm 2 gelatin sponges (Gelfoam; Upjohn Co, Kalamazoo, MI) loaded with 3 µL of PBS alone as the negative control, or containing 3 µg (1 mg/mL) of the angiogenic recombinant basic fibroblast growth factor (FGF-2; Pharmacia, Milan, Italy) alone as the positive control20 or together with each VBL dose, were implanted on top of the CAM. The sponge traps the sample and allows slow release of the product. CAM were examined daily until day 12, when the angiogenic response peaks.20 On day 12, blood vessels entering the sponge within the focal plane of the CAM were recognized macroscopically (at 50×), counted by two observers in a double-blind fashion21 under a Zeiss SR stereomicroscope (Zeiss; Oberkochen, Germany), and photographed in ovo with the MC63 Camera system (Zeiss). To better highlight vessels, the CAM were injected into a large allantoic vein with India ink solution, fixed in Serra’s fluid, dehydrated in graded ethanols, and rendered transparent in methylbenzoate.22 On day 12, after macroscopic counting, the embryos and their membranes were fixed in ovo in Bouin’s fluid. The sponges and the underlying and immediately adjacent CAM portions were removed, embedded in paraffin, sectioned at 8 µm along a plane parallel to the CAM surface, and stained with a 0.5% aqueous solution of toluidine blue (Merck). Angiogenesis was measured by a slightly modified planimetric point count method:4 to 6 250× fields covering almost the whole of every third section within 30 serial slides of each sponge per sample were analyzed within a superimposed 144 intersection point square reticulum of 0.125 mm 2. Only transversely sectioned microvessels, ie, capillaries and small venules with or without a 3 to 10 µm lumen occupying the intersection points, were counted and calculated as the mean ± 1 SD per section, per CAM, and groups of CAM.

Electron microscopy. T25 flasks of HUVEC at 90% confluence were maintained for 24 hours in DMEM supplemented with 10% FCS and 1% glutamine alone or containing each VBL dose. The cells were then washed by PBS and fixed in 3% glutaraldehyde in 0.1 mol/L PBS for 3 hours, washed in the same buffer for 12 hours, and post-fixed in 1% osmium tetroxide.23 Afterwards, the cells were scraped with a rubber bar, dehydrated in graded ethanol, and embedded in Epon 812.
Ultrathin sections were cut with a diamond knife on a LKB ultratome, stained with uranyl acetate followed by lead citrate, and examined in a Zeiss 9A electron microscope.

RESULTS

Inhibition of the angiogenic phenotype of cultured human microvascular endothelial cells by VBL. The angiogenic phenotype is an ensemble of cell functions, namely proliferation, chemotaxis, adhesion to, and spreading on extracellular matrix constituents, such as FN, morphogenesis and MMP secretion, which are expressed by cells when cultured in optimal growth medium alone or supplemented with angiogenic growth factors.24,25

The first series of experiments focused on the effects of VBL on the proliferation of HUVEC and EA.hy926 cells. These cells were exposed on days 0 and 2 to complete medium alone (positive control) or supplemented with each VBL dose, or to starvation SFM (negative control), and their proliferation rate was measured on day 4 by a colorimetric method. Figure 1A shows that VBL was ineffective on HUVEC at 0.1 pmol/L, but significantly inhibited their proliferation at 0.25 pmol/L (78%, as mean, of the positive control; \( P < .001 \) Wilcoxon rank test). This inhibition was progressively enhanced with increasing doses (92% at 1 pmol/L; \( P < .05 \) for the within-sample comparisons by Wilcoxon-Wilcox test). Figure 1B shows that when cells were exposed to control media and VBL every 24 hours for 1 to 3 times, it again gave more evident inhibition of cell proliferation at 0.25 pmol/L, in agreement with the number and overall duration of exposures: compared with their positive control, the three ways of exposure resulted in 20%, 50% and 85% mean inhibition respectively (\( P < .01 \), Wilcoxon-Wilcox test). Higher doses inhibited more strongly. Similar results were obtained with EA.hy926 cells (data not shown).

Induction of apoptosis was ruled out because by using FACS analysis of HUVEC weakly stained with fluorescent PI for subdiploid DNA, a marker of apoptosis,18 we found that (Fig 2)
the percentages of weakly fluorescent cells (M1 region) were marginal after exposure to VBL 0.25 pmol/L (2%, Fig 2A) and 1 pmol/L (2.1%, Fig 2B), similar to what was observed in unexposed cells (2.2%, Fig 2C), used as the negative control. By contrast, high percentages of apoptotic HUVEC (66.8%) were produced by dexamethasone 1 mmol/L, an apoptogenic drug19 (Fig 2D), which was used as the positive control. Speculatively, the percentages of nonapoptotic HUVEC, ie, cells strongly fluorescent due to diploid DNA (M2 region), were high in cell preparations exposed to VBL 0.25 pmol/L (98%) and 1 pmol/L (97.9%), as in unexposed cells (97.8%), whereas they were much lower (33.2%) in preparations exposed to dexamethasone; apoptotic bodies, nuclear fragmentation, and homogenization, cellular shrinking, or membrane blebbing were not detected, and the inhibitory effect was fully and rapidly reversible.

The inhibitory effect of low-dose VBL was restricted to endothelial cells, because neither NIH 3T3 fibroblasts nor Burkitt’s lymphoma (Namalwa), B-cell lymphoblastic leukemia (LIK), and T-cell lymphoblastic leukemia (CEM) cell lines displayed reduced proliferation when exposed to 0.25 pmol/L or 1 pmol/L (Table 1).

The inhibitory effect of VBL on HUVEC and EA.hy926 cell chemotaxis was assessed in Boyden chambers. Figure 3 shows that when HUVEC were pretreated with each VBL dose and left to migrate towards the NIH 3T3 CM or 0.1% BSA solution (chemoattractant and negative control respectively), the number of migrated cells compared to the positive control was −38% as mean (P < .01, Wilcoxon rank test) and −54% (P < .001) at 0.1 pmol/L and 0.25 pmol/L, respectively, and lowered progres-

![Fig 3. Effect of VBL on chemotaxis of HUVEC. Cells (1.2 x 10⁵) exposed for 24 hours to each VBL dose were seeded in the upper compartment of Boyden chambers, whereas the CM of NIH 3T3 cells was placed as the chemoattractant in the lower compartment. Nonexposed cells were used in the positive and negative controls. The latter were without the chemoattractant. Cells that had migrated to the lower surface of the filter separating the compartments after 6 hours were counted after coding the samples. Bars represent the mean ± 1 SD of the number of migrated cells in 5 to 8 400× fields of the filter per sample in 1 representative experiment out of 5. Significance by the Wilcoxon rank test.](http://www.bloodjournal.org)

![Fig 4. Effect of VBL on the adhesion of HUVEC on the FN substrate. The cells were incubated for 90 minutes in the specific growth medium without FCS (positive control) and in this medium containing each VBL dose. The attachment to and the spreading on FN were assessed at 30 minutes and 90 minutes, respectively. Bars represent the mean ± 1 SD in 1 representative experiment out of 6. Significance by the Wilcoxon rank test.](http://www.bloodjournal.org)

Table 1. Proliferation of HUVEC and Other Primary Cells Exposed to Low-Dose Vinblastine

<table>
<thead>
<tr>
<th>Vinblastine Dose</th>
<th>HUVEC</th>
<th>NIH 3T3</th>
<th>Namalwa</th>
<th>LIK</th>
<th>CEM</th>
</tr>
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<tbody>
<tr>
<td>0.25 pmol/L</td>
<td>8.4 ± 3.2</td>
<td>68.2 ± 4.3</td>
<td>48.7 ± 4.6</td>
<td>37.4 ± 3.7</td>
<td>54.5 ± 5.1</td>
</tr>
<tr>
<td>1 pmol/L</td>
<td>3.5 ± 1.1</td>
<td>58.5 ± 5.2</td>
<td>41.5 ± 5.8</td>
<td>44.9 ± 4.8</td>
<td>51.6 ± 4.2</td>
</tr>
<tr>
<td>(33.2 ± 5.6)</td>
<td>(51.2 ± 4.7)</td>
<td>(42.3 ± 4.7)</td>
<td>(47.1 ± 6.7)</td>
<td>(59.8 ± 5.8)</td>
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</tr>
<tr>
<td>(4.1 ± 2.3)</td>
<td>(7.3 ± 2.8)</td>
<td>(11.2 ± 3.4)</td>
<td>(6.4 ± 1.8)</td>
<td>(8.3 ± 3.5)</td>
<td></td>
</tr>
</tbody>
</table>

Results are expressed as the mean ± 1 SD in 1 representative experiment of 4 performed. Positive control values are in parentheses. Negative control values are in brackets.

*The cells (2 x 10⁵ per 0.32-cm² well) were exposed to VBL on days 0 and 2 and counted on day 4, as described in the proliferation assay (Fig 1A).
sively as the VBL dose increased ($P < .01$, Wilcoxon-Wilcoxon test). Chemotaxis of EA.hy926 cells was declined to $-40\%$ when 0.1 pmol/L was used (data not shown).

In other experiments, the ability of HUVEC to attach to and spread on FN after treatment with each VBL dose was studied (Fig 4). Attachment evaluated at 30 minutes of treatment was not impacted. By contrast, cell spreading evaluated at 90 minutes was significantly impacted at 0.5 pmol/L (3490 ± 160 spread treated cells vs 3800 ± 70 spread control cells, $P < .05$, Wilcoxon rank test), 0.75 pmol/L (3170 ± 210, $P < .01$), and 1 pmol/L (2830 ± 80, $P < .01$, Wilcoxon-Wilcoxon test).

The effect of VBL on vessel morphogenesis of HUVEC and EA.hy926 cells was investigated. Data on HUVEC are presented. Cells seeded on Matrigel in the presence of NIH 3T3 CM spread throughout the Matrigel surface and aligned to form branching, anastomosing tubes with multicentric junctions that formed a closely-knit meshwork of capillary-like structures (Fig 5A). Thin sections perpendicular to the Matrigel surface showed that these capillaries were formed of single or multiple layers of cells, and were both solid or canalized with a narrow lumen (not shown). In contrast, when CM was admixed with VBL at increasing doses, a progressive loss of this picture was observed: 0.5 pmol/L and even more so 1 pmol/L caused most cells to remain spherical and isolated, and few aggregated as small clumps or generated irregular tubes, mostly without lumen (Fig 5B and C). The picture at 1 pmol/L resembled that obtained with the negative control (Fig 5D).

Lastly, SDS-PAGE gelatin zymography of CM of EA.hy926 cells exposed and not exposed for 24 hours to each VBL dose did not show reduced MMP-2 and MMP-9 secretion by exposed cells. The secreted MMP-2 and MMP-9 gave rise to gelatinolytic bands with an apparent Mr of 62 kD and 88 kD respectively (Fig 6A), indicating that both enzymes were present in the CM in their cleaved, activated form. The amount of MMP, as evaluated by computerized image analysis of the band intensity (Fig 6B), did not decrease in the CM of VBL-exposed cells, even at 1 pmol/L. A decrease was observed at 2 pmol/L, a cytotoxic dose (see below).

**VBL in the chick embryo CAM.** Ten CAM per series were examined macroscopically on incubation day 12. Eight out of 10 (80%) CAM implanted with sponges loaded with the angiogenic factor FGF-2 (positive control) displayed a vasoproliferative response, and in all 10 CAM per series treated with FGF-2 plus VBL 0.5 pmol/L, 0.75 pmol/L, or 1 pmol/L, the response was inhibited; 9 of 10 (90%) CAM treated with PBS (negative control) displayed no response. Vessels entering the sponges were macroscopically recognized and counted after their highlighting by India ink injection into a major allantoic vein. CAM implanted with sponges loaded with the FGF-2

\[ \text{Fig 5. HUVEC morphogenesis on Matrigel. Cells (2 x 10^5) were plated per 1.25 cm}^2 \text{ well precoated with Matrigel and grown for 6 hours (A) in the specific medium alone (positive control) or containing (B) VBL 0.5 pmol/L, (C) 1 pmol/L, or (D) in the medium without FCS (negative control). Sub (A), cells arranged in branching, reciprocally anastomosing tubes forming a closely knit capillary-like plexus; sub (B) and (C), progressive alterations of the plexus parallel with increasing VBL dose. Bar = 30 \mu m from (A) to (D).} \]
showed numerous allantoic vessels converging like spokes towards the sponge, which was completely filled with the ink (Table 2 and Fig 7A). When the sponge was loaded with PBS, physiologic angiogenesis was observed as fewer allantoic vessels arranged partly around the sponge and partly converging towards it (Table 2). By contrast, very few vessels were detectable with 0.5 pmol/L and even fewer with 0.75 pmol/L and 1 pmol/L VBL (Table 2 and Fig 7B).

Histologic examination and planimetric vessel counting were also performed (Table 3). FGF-2–loaded sponges displayed a dense collagenous matrix and numerous blood vessels among the sponge trabeculae and at the boundary between the sponge and the CAM mesenchyme (Table 3 and Fig 7C). Vessels pierced the sponge at some points. By contrast, no vessels could be detected inside the PBS–loaded sponges, and fewer were found at the boundary than in the positive control. Sponges loaded with 0.5 pmol/L, 0.75 pmol/L, and 1 pmol/L VBL gave no vessels. The boundaries gave counts overlapping those of the PBS at 0.5 pmol/L, but lower at 0.75 pmol/L, and no vessels at 1 pmol/L (Table 3 and Fig 7D).

Cytotoxicity of VBL on endothelial cells and other primary cells in vitro and in the CAM. Cells were exposed at day 0 and every 2 days to each VBL dose in specific growth medium, and observed daily for morphologic alterations. At 1 pmol/L, VBL induced morphologic changes in endothelial cells and other primary cells.
Fig 7.
produced signs of cytotoxicity on HUVEC, namely vacuolization, loss of elongated shape, and cytoplasmic swelling in about 60% of cells on day 6. On day 8, most cells displayed these changes, and cell detachment began. At 2 pmol/L, most cells underwent morphologic alterations on day 4 (Fig 8A and B) and cell detachment rapidly occurred afterwards. At 4 pmol/L, these findings were observed on day 2. Similar behavior was displayed by EA.hy926 cells (Fig 8C and D). By contrast, NIH 3T3 cells gave cytoplasmic vacuolization and swelling at 30 pmol/L on day 4 and at 50 pmol/L on day 2. On Namalwa, LIK, and CEM cells cytoplasmic vacuolization and toxic granules were evident with 40 pmol/L on day 4 (Fig 8E and F) and 65 pmol/L on day 2.

In the CAM, 1 pmol/L was cytotoxic for endothelial cells and other mononuclear stromal cells at day 16 (antiangiogenesis experiments ended at day 12), when they displayed nuclear pyknosis and cytoplasmic degeneration and homogenization. VBL at 2 pmol/L and 4 pmol/L was cytotoxic on days 12 and 10, respectively.

**Ultrastructural findings.** HUVEC cultured in specific medium appeared as elongated cells, containing well-developed organelles, lipid droplets, and a network of microtubules and microfilaments extending across the cytoplasm and thickened near the subcortical plasma membrane (Fig 9A). Cells exposed to 0.1 pmol/L VBL for 24 hours displayed very limited lesions of cytoskeleton microfilaments in the form of small perinuclear areas of condensation and depolymerization (Fig 9B and C). These areas were more evident and numerous after exposure at 1 pmol/L, the cells appeared as rounded cells irregularly shaped with numerous bundles of thickened microfilaments (Fig 10A and B). These changes regressed after 12 hours and 24 hours when 0.1 pmol/L and 1 pmol/L, respectively, were removed, and the cells returned to the normal elongated shape (Fig 10C).

**DISCUSSION**

Here we show that VBL inhibits certain functions of human microvascular endothelial cells (HUVEC and EA.hy926 cells), namely proliferation, chemotaxis, spreading on FN, and morphogenesis in vitro (though not MMP-2 and MMP-9 secretion), and angiogenesis of the chick embryo CAM in vivo. These effects were directly dose-dependent and obtained at very low doses, namely from 0.1 to 1 pmol/L in vitro, and from 0.5 to 1 pmol/L in vivo. By contrast, 0.25 pmol/L and 1 pmol/L did not impact proliferation of mouse fibroblasts (NIH 3T3 cells) and human lymphoid tumor cells (Namalwa, LIK, and CEM cells).

It has been shown that the VBL uptake into microvascular endothelial cells is already operative at very low doses (5 to 10 nmol/L) in the extracellular milieu. The doses we used caused no nonspecific cytotoxicity: 1 pmol/L caused cytoplasmic vacuolization, a loss of elongated cell shape, cytoplasmic swelling, and detachment from FN 2 days after the experiments ended, and 1 pmol/L per CAM gave vascular degeneration and cytoplasmic fragmentation of endothelial cells 4 days afterwards. By contrast, doses of 2 pmol/L or greater were rapidly cytotoxic both in vitro and in vivo. Additional proof of the absence of cytotoxicity in our models was provided by the complete reversal of both in vitro and in vivo inhibition when VBL was removed.

VBL is specifically toxic for cytoskeleton microfilaments and microtubules and hence for the mitotic spindle, which results in metaphase blockage and necrosis of tumor cells. These effects have already been shown on human microvascular endothelial cells: HUVEC exposed in vitro to VBL at doses $4 \times 10^5$-fold greater than ours (0.4 µmol/L) displayed irreversible signs of cytotoxicity (spherical shape, cytoplasmic vacuolar degeneration, nuclear pyknosis, and disruption of the tight cell-to-cell contacts) within 20 minutes to 2 hours, and 50% survival after only 4 hours, parallel with irreversible accumulation of F-actin microfilaments, other cytoskeleton structures. Similar results have been obtained in vitro on porcine aortic endothelial cells exposed to VBL at doses $1 \times 10^6$-fold greater (1 µmol/L). In vivo, in a Lewis rat and different mouse models, VBL at doses equivalent to those applied in vitro, ie, 7.5 to 10 mg/kg (at or slightly above 50% of the maximum tolerated dose) also provoked diffuse necrosis of vascular endothelial cells, resulting in intratumor vessel collapse, punctuate hemorrhages, reduced overall tumor blood flow and tumor patchy necrosis, in the absence of necrotic damage of endothelial cells and blood flow disturbance in normal tissues.

Angiogenesis in tumors is sustained by rapidly proliferating endothelial cells, whose evident sensitivity to the antiproliferative effect of VBL explains the endothelial cell necrosis restricted to tumors in the animal models. We have attained inhibition of functions of human microvascular endothelial cells essential for angiogenesis without cytotoxicity or cell necrosis. Proliferation and chemotaxis are important steps for neovessel sprouting. Morphogenesis develops by migration, spreading on FN, and the mutual alignment of the endothelial cells to form tubular branching structures that are anastomosed in a capillary-plexus network. Inhibition of these functions agrees well with that of the in vivo angiogenesis that has been obtained by us in the CAM model, insofar as the same functions are recapitulated by angiogenesis. Overall data suggest that VBL has an antiangiogenic component when applied at very low, nontoxic doses. The cell functions studied are tightly linked to the integrity of the cytoskeleton, and hence they are typically impaired by VBL as a consequence of its mechanism of action. Accordingly, we have shown at the ultrastructural level that even 0.1 pmol/L VBL...
produced a thin disturbance of the endothelial cell cytoskeleton in the form of minimal focal depolymerization and accumulation of microfilaments. This was equally reversible, like the impairment of cell functions. VBL at 100 nmol/L to 10 µmol/L has been shown to inhibit other endothelial cell functions associated with cytoskeleton functionality, namely active internalization of the E-selectin ELAM-1 by HUVEC and secretion of endothelin-1 by porcine aortic cells.

The highest antiangiogenic dose applied in vivo was 1 pmol/L per CAM on incubation day 8. Because on this day the CAM-embryo weight was 10 grams, the dose evaluated as µg/kg was 0.24 µg/kg, and the equivalent dose in a 70 kg adult subject was 16 µg, which is lower than the daily dose in tumor management (1 mg). VBL could be considered as an antiangiogenic agent along with suramin hexasodium, linomide, the recombinant human platelet factor 4, the fumagillin derivative...
AGM-1470,\textsuperscript{40} and Taxol,\textsuperscript{41} which also inhibit the proliferation and chemotaxis of endothelial cells.

The antiangiogenic activity of low-dose VBL deserves further investigation, alone or together with other antiangiogenic agents\textsuperscript{42} for the treatment of tumors characterized by vivid angiogenesis, and of other angiogenesis-dependent diseases, such as Kaposi’s sarcoma,\textsuperscript{43} rheumatoid arthritis,\textsuperscript{44} and psoriasis.\textsuperscript{15}
Fig 10. Ultrathin sections of HUVEC cells exposed to (A, B) 1 pmol/L VBL and (C) 24 hours after removing the VBL-containing medium. (A, B) Round-shaped endothelial cell displaying numerous bundles of thickened microfilaments distributed in the cytoplasm (arrow). (C) Endothelial cell showing normal distribution of cytoskeleton organelles. Bar (A), (C) = 16.6 μm; (B) = 0.5 μm.
ACKNOWLEDGMENT

The authors thank Prof F. Bussolino (IRCCS, University of Turin Medical School, Turin, Italy), Prof F. Silvestris, and Dr P. Cafforio (DIMO, University of Bari Medical School, Bari, Italy) for helpful advice in performing some experiments.

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