

Antiangiogenic Properties of *Viscum Album* Extracts Are Associated with Endothelial Cytotoxicity

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Abstract. *Back ground:* *Viscum album* (VA) extracts are widely used in cancer therapy and are known to be cytotoxic to tumors and endothelial cells. Angiogenesis plays an important role in the growth, sustenance and metastasis of tumors. Inhibition of angiogenesis is now being explored as a new therapeutic avenue for cancer. *Materials and Methods:* The cytotoxicity of VA extracts was analyzed by Annexin V labeling and propidium iodide uptake in EA-hy926 endothelial cells. The antiangiogenic effect was studied *in vitro* by treating the EA-hy926 cells in matrigel and subsequent analysis of vascular formation. Computer-assisted image analysis of vascular formation was analyzed to quantify the *in vitro* data. *In vivo* studies were performed by implanting matrigel ± VA extracts in Balb/C mice that had been subjected to IP treatment with VA extracts. *Results:* The combination of systemic and intra-matrigel treatment with the VA *Qu Spez* extract caused significant inhibition of angiogenesis. The VA *P* extract treatment showed insignificant change in vessel formation. *Conclusion:* These results may provide novel guidelines towards improved strategies using VA extracts based on the inhibition of angiogenesis of tumors.

Viscum album (VA) preparations consisting of mistletoe lectins (MLs) have been proposed as adjuvant immunomodulatory

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therapy in cancer patients (1). The biologically active components of VA extracts include the ML and viscotoxins, and several other substances including amino acids, polysaccharides and lipids (2). ML I, II and III belong to the ribosome-inactivating protein (RIP) family of type II, which includes highly toxic ricin and abrin. RIP of type II are composed of an *N*-glycosidase (A chain) and a galactoside-recognizing lectin (B-chain) connected by a disulfide bridge (3). Treatment with VA extract, and or with purified ML has been shown to be associated with tumor regression in several *in vivo* experimental models of tumoral implantation (4, 5). Extensive investigations have explored the molecular mechanisms underlying the beneficial effect of VA and ML preparations. Several mutually non-exclusive effects have been proposed to explain the control of tumor progression. The mechanisms underlying such antitumoral activity of VA and ML implicate either a direct cytotoxicity of tumor cells or immunomodulation (1, 6-8).

Angiogenesis is the process by which blood vessels develop from pre-existing vessels. Malignant neoplasms need adequate blood supply and nutrients to sustain their growth. Tumor angiogenesis enables a tumor mass to expand and spread hematogenously, and plays an essential role in tumor progression and metastasis (9). The mechanisms involved in tumor angiogenesis consist of a wide range of phenomena including the enhanced division of endothelial cells (EC) within the tumor and up-regulation of cell adhesion molecules. Tumor cells produce various angiogenic factors that stimulate the EC in a venule to degrade the vascular basement membrane and to migrate into the surrounding tissues towards the tumor mass and to promote the proliferation of EC in a capillary sprout. Targeting angiogenesis is thus being explored as a cancer therapy to check tumor growth and metastasis (10, 11). Novel strategies, based on several antiangiogenesis approaches, have emerged and are undergoing extensive study

in advanced clinical trials (12, 13). The preliminary results of these clinical trials have suggested that single-agent antiangiogenic therapy is poorly active in advanced tumor, while combination therapy, although controversial is being explored for efficient inhibition of angiogenesis (14).

Some of the recent observations on the biological effects of VA and ML preparations have suggested that VA extracts may exert an antiangiogenic effect (15). Amongst others, we have recently shown that VA preparations induce the apoptosis of immortalized EC in a dose- and time-dependent manner (16). A recent study has shown that VA extracts inhibit tumor and EC growth by delaying cell cycle progression and by causing apoptotic cell death. The mitochondrial pathway and/or death receptor pathway involvement of these extracts on the EC raises interesting questions regarding the functional impact *i.e.* angiogenesis (17). Together these observations have encouraged us to investigate, concurrently, the induction of apoptosis of EC along with the antiangiogenic effects of VA extracts. In addition, in the present study, the relationship between the concentration of the viscotoxins and lectins in the standardized extracts and their ability to inhibit angiogenesis was examined.

Materials and Methods

Cell culture. The endothelial cell line EA-hy926 was cultured in 75 cm² tissue culture flasks (Costar, Cambridge, MA, USA) in RPMI medium (Gibco BRL Life Technologies, Grand Island, NY, USA) containing Earle's salts, L-glutamine and 25 mM HEPES and supplemented with 20% fetal calf serum (FCS; Dutscher, Brumath, France), 100 U/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone (Gibco BRL, Cergy Pontoise, France) and HAT (Hypoxanthine Aminopterin Thymidine medium; Sigma Aldrich, Lyon, France). At confluence, the primary cultured cells were harvested after 3 min trypsination using 33% trypsin (Biological Industries, Kibbutz Beit Haemek, Israel) in PBS and sub-cultured.

Viscum album extracts. VA preparations (VA Qu Spez and VA P) were a kind gift from Weleda AG (Arllesheim, Switzerland). VA consists of aqueous extracts of mistletoe plants growing in oak tree. The preparations (1 ml ampoules of 5 mg for VA Qu Spez and 1 ml ampoules of 10 mg for VA P) contain a standardized concentration of ML and viscotoxins. VA preparations are formulated in endotoxin-free, sodium chloride (NaCl 0.9%) isotonic solution as 5 mg/ml vials. During the manufacturing process, VA preparations are prepared by standardizing the levels of mistletoe lectins and viscotoxins.

While, VA P is less cytotoxic due to its lower lectin concentration, VA Qu Spez with higher lectin content is more cytotoxic. In view of the differential cytotoxic properties of VA P and VA Qu Spez related to their differing concentrations of viscotoxins and lectins, the antiangiogenic effect of the extracts may be related to the presence of other components in the extracts.

Assay for apoptosis. The EA-hy926 cells were incubated for 24 h with 12.5 µg/ml or 50 µg/ml of VA extracts in low serum

conditions. Using Annexin V-FITC (Bender Medsystems, Lonza, Saint Beauzire, France) (2.5 µg/ml), labeling was carried out by staining the cells for 30 min on ice. The cells were gently trypsinized, washed with 1X PBS-Azide with 2 mM CaCl₂ and resuspended in PBS-Azide before adding propidium iodide (PI) (50 µg/ml) to each sample. Using flow cytometry, the samples were analyzed for green fluorescence (annexin V labeling) and for red fluorescence (PI uptake). Cells were considered apoptotic if they were annexin V-positive and PI negative.

In vitro angiogenesis assay. Unpolymerised matrigel (10 mg/ml; BD Biosciences, Le Pont de Claix, France) was placed in the wells (400 µl/well) of a 24-well micro titer plate and allowed to polymerize for 1 h at 37°C. The matrigels were either untreated or mixed with different concentrations of VA P or VA Qu Spez. EA-hy926 cells were plated (50×10³ cells/well) in 400 µl of M199 medium containing 1% FCS and basic fibroblast growth factor (bFGF) onto the matrigel. After incubation in a 5% CO₂ humidified atmosphere, the cell growth and bi-dimensional organization were observed after 24 h through a inverted phase-contrast microscope (Olympus, Paris, France).

Image analysis methods. Phase-contrast images of the EC cultures were taken under the inverted phase-contrast light microscope and saved as TIFF files as such files can be used for further processing. The images were then processed and analyzed using computer-assisted image analysis. The main processing steps applied to the images are shown in Figure 1. Briefly, after the application of an edge detection filter to the original image, the image was thresholded by selecting its moving mean grey value as the threshold value. In the present imaging conditions, this allowed quite a good identification of the cell profiles present in the field. To this binary image, a geometric filter was applied to the profiles selected resulting in the identification of the network formed by the tubules and the interconnecting cell clusters in the image. By applying binary thinning procedures, the skeleton of this image was derived. For each phase-contrast image, the total angiogenesis area (Figure 4A) was defined as the total area occupied by the completely or incompletely formed vessels, while the alveolar space (Figure 4B) was defined as the average distance between the completely and incompletely formed vessels in the final skeleton image. The areas of the angiogenesis network and the alveolar space were analyzed statistically using StatView software (Adept Scientific, Bethesda, MD, USA).

Mouse matrigel angiogenic assay. *In vivo* angiogenesis was assayed in female Balb/C mice (6-8 weeks old, Janvier, Le Genest Saint Isle, France) by the growth of blood vessels from subcutaneous tissue into a matrigel (Matrigel Growth Factor Reduced; BD Biosciences) plug. The matrigel in liquid form at 4°C was mixed with 50 µg/ml of the VA extracts or used alone and 0.5 ml was injected into the abdominal subcutaneous tissue of the mice along the peritoneal midline. The mice were also injected with VA preparations intraperitoneally (20 µg/day) while control mice were not injected. The mice were sacrificed after 7 days and the matrigel plugs were excised and processed for histological analysis. The samples were fixed in formalin at 4°C and embedded in paraffin. The matrigel treatment/systemic treatment combinations were: 0/0, 0/P, 0/Q, P/P and Q/Q as shown in Table I.

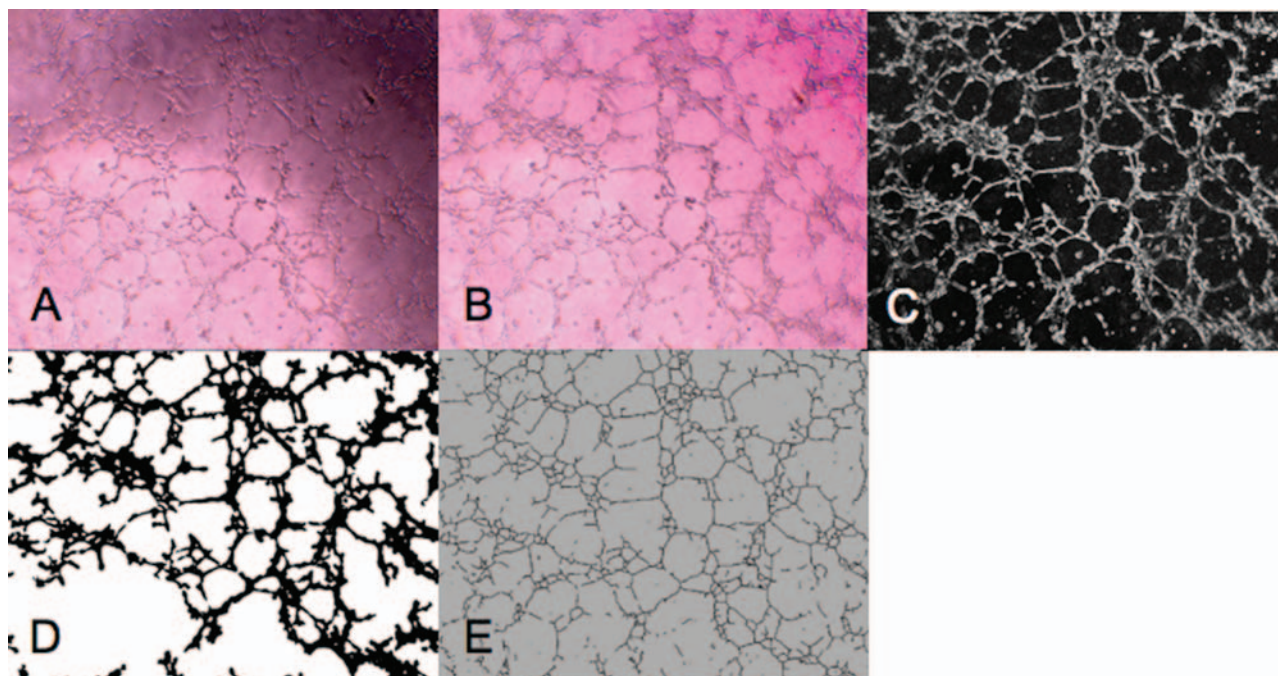


Figure 1. Sequence of computer assisted analysis of *in vitro* angiogenesis. The phase contrast image (A) was subjected to shadow correction (B). This image was then thresholded (C) with the application of an edge detection filter. A geometric filter was applied and the cell network was identified (D) and the skeleton (E) was generated to analyze the alveolar space.

Table I. *In vivo* matrigel treatments.

Designation of the group	Number of mice in the group	Matrigel treatment before implantation	Systemic treatment after implantation
0/0	6	No	No
0/P	6	VA P (50 µg)	No
0/Q	6	VA QuSpez (50 µg)	No
P/P	6	VA P (50 µg)	VA P (20 µg/day)
Q/Q	6	VA QuSpez (50 µg)	VA QuSpez (20 µg/day)

Semi-quantitative analysis of vascular density within the matrigel plugs. From each matrigel plug, three 5 µm-thick sample sections, at different levels within the tissue, were obtained and stained with hematoxylin and eosin for microscopic observation. All the sections were then semi-quantitatively scored for vascular density in a blinded fashion, by two different observers (JPDVH, FP). The matrigel sections were analyzed at high power and scored from 0 to 3 according to the number of vascular channels within the field. For every matrigel, the average score of the three sample sections was calculated.

Results

Effect of the VA extracts on the apoptosis of EA-hy926 cells.

The apoptosis and *in vitro* studies were performed simultaneously using similar concentrations of VA extracts of the same batch and the same population of cells that was used in both studies. As shown in Figure 2, there was an

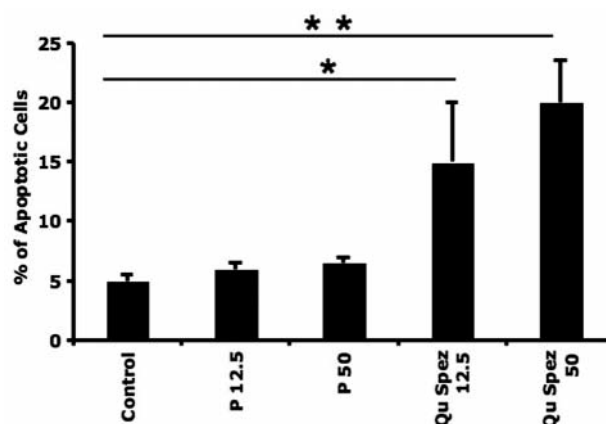


Figure 2. Effect of the VA extracts in the percentage of apoptotic cells. EA-hy926 cells were treated with VA P or VA Qu Spez (12.5 µg/ml or 50 µg/ml each) for 24 h. Cells that were positive for annexin V and negative for propidium iodide were considered apoptotic. Cells grown under normal culture conditions without VA extracts were the controls.

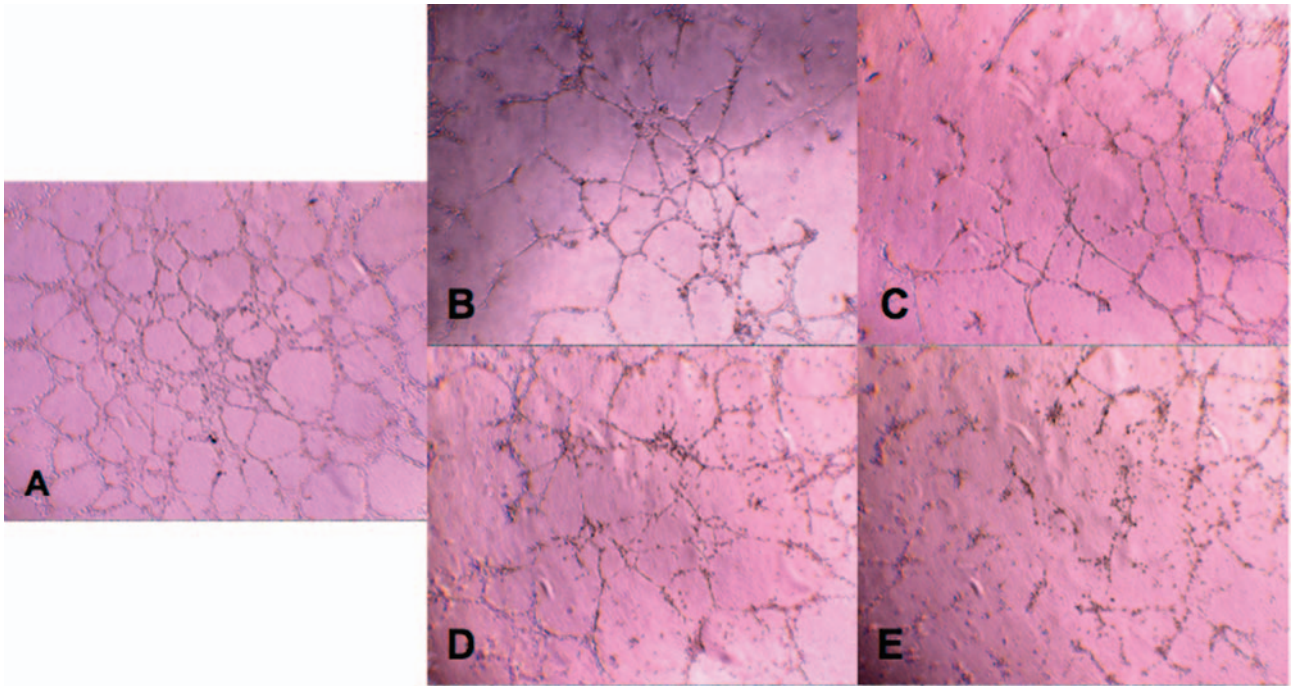


Figure 3. Effect of VA extracts on the vessel growth in vitro. EA-hy926 cells were layered on the matrigel with or without VA extracts. Control (A), VA P treatments (B; 12.5 µg/ml and C; 50 µg/ml); VA Qu Spez treatments (D; 12.5 µg/ml and E; 50 µg/ml).

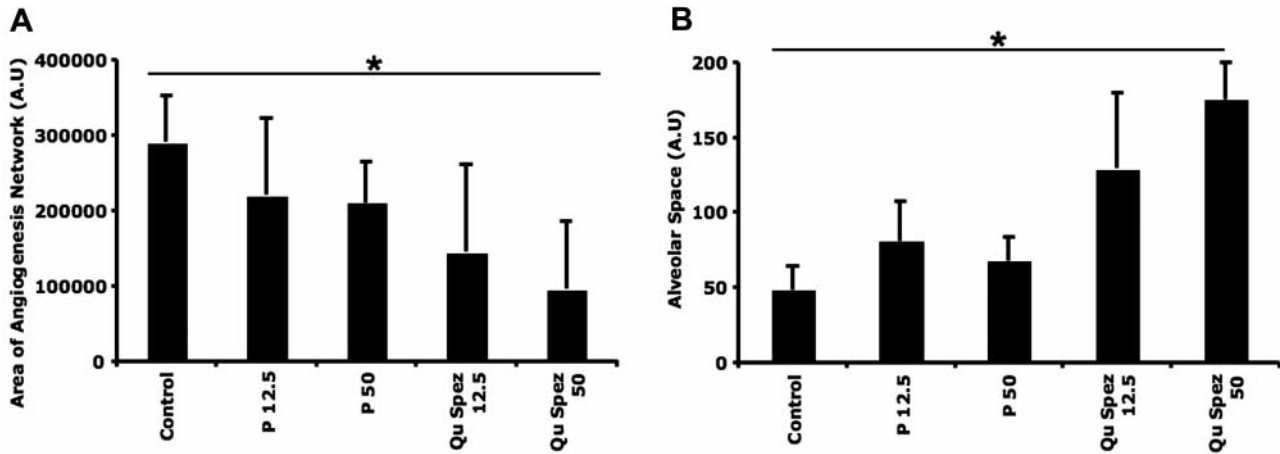


Figure 4. Effect of VA extracts on the area of angiogenesis network and alveolar space. The average angiogenesis area (A) and the alveolar space (B) were analyzed using the StatView Software.

increase in the percentage of apoptotic cells in both the VA Qu Spez treatments. However, the increase in the percentage of apoptotic cells in the VA P treatments was not significant.

Effect of VA extracts on capillary tube formation. As illustrated in Figure 3, A, the EA-hy926 cells after 24 h culture on matrigel had become organized into an

anastomotic capillary network. While co-incubation of the cells with VA P was associated with an insignificant reduction in the network (Figure 3, B and C), the treatment of the cells with VA Qu Spez was associated with a substantial reduction in the capillary network, in a dose-dependant manner (Figure 3, D and E). VA Qu Spez at 50 µg/ml induced a nearly complete disruption of capillary

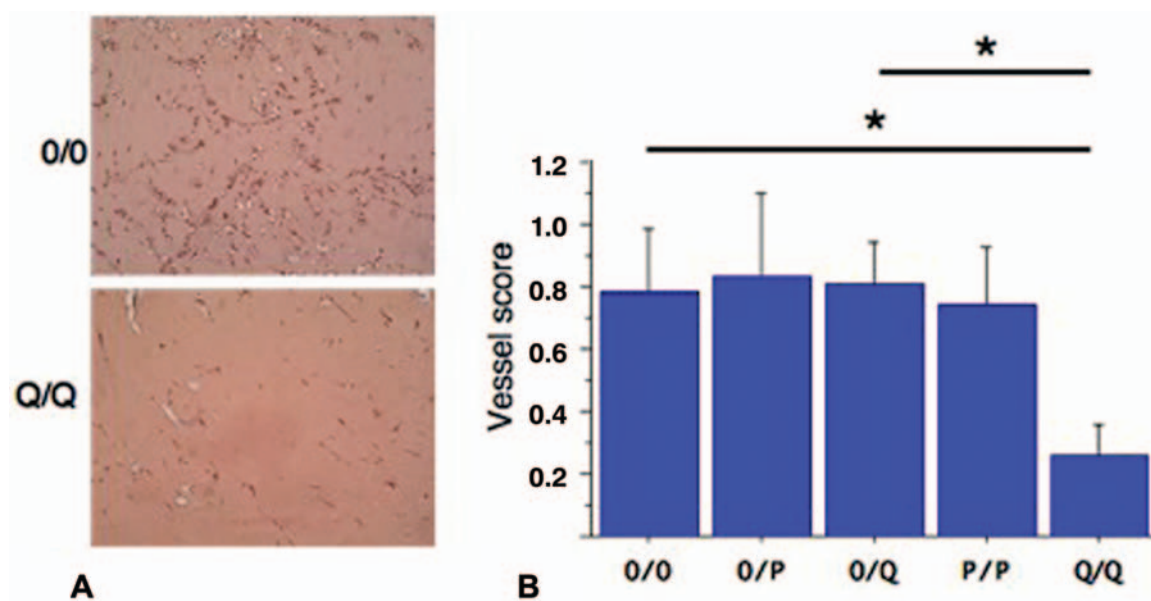


Figure 5. Effect of VA extracts on angiogenesis *in vivo*. Balb/C mice were implanted with matrigel plugs that were either untreated or mixed with 50 $\mu\text{g/ml}$ of VA extracts. Panel A: vascular formation 7 days after implantation with matrigel plugs that were either untreated (O/O) or intra-matrigel pre-treated with VA Qu Spez followed by systemic VA Qu Spez (Q/Q). Panel B: vessel score of intra-matrigel pre-treatment/systemic treatment (O: Control, no VA treatment; P: VA P and Q: VA Qu Spez).

tube formation. Antiangiogenesis *in vitro* was then analyzed by computer-assisted image analysis. VA Qu Spez at 50 $\mu\text{g/ml}$ caused an almost 66% decrease in the area of angiogenesis network (Figure 4, A). Correspondingly, there was an increase in the alveolar space with VA Qu Spez at the higher concentration (Figure 4, B).

Effect of VA extracts on the angiogenesis in vivo. There was a dramatic decline in the vascular density in the matrigel treated with VA Qu Spez at the time of the implantation and followed by intraperitoneal treatment (Q/Q) as compared to the control (O/O) untreated mice (Figure 5A). Semi-quantitative analysis of the vessel score showed an almost 66% reduction of the vascular density in the Q/Q group compared to the control (Figure 5B). Systemic treatment alone was not effective in the reduction of angiogenesis. VA P as either systemic treatment (O/P) or local plus systemic treatment (P/P) was not associated with a reduction in *in vivo* angiogenesis.

Discussion

In a previous study, we showed that the VA preparation, VA Qu FrF, induced EC apoptosis in human umbilical vein endothelial cells (HUVECs) and in the immortalized human venous endothelial cell line IVEC (16). Interestingly, among four VA preparations, VA Qu FrF, VA Qu Spez, VA M Spez and VA P, VA P was not cytotoxic towards IVEC. HUVEC

apoptosis by VA extracts was clearly demonstrated by the cleavage of PARP (poly(A)DP-ribose polymerase) suggesting the involvement of caspases (18).

The previous study regarding the anti-angiogenesis effect of ML concerned the use of extracts from *Viscum album L. coloratum* (Korean mistletoe) (15). Attempting to correlate the molecular observations with the functional significance, in the present study angiogenesis *in vitro* and *in vivo* was mimicked using promoters of angiogenesis rather than fat emulsions as used in the previous study involving CAM (chorio-allantoic membrane) and Korean mistletoe lectin (VCA) (15).

However, European mistletoe and Korean mistletoe extracts differ in their composition of MLs and function. Our preliminary studies on the effect of VA extracts on the modulation of a specific vascular epithelial growth factor (VEGF) receptor (flt-1/VEGF receptor type 1) in HUVECs showed no significant changes in its gene expression (data not shown). This supported the previous observations of the involvement of apoptotic pathways rather than the inhibition of angiogenesis receptors.

In the present study, we hypothesized that the functional relevance of the EC cytotoxicity of VA extracts could be the inhibition of angiogenesis. The use of two different preparations (VA P, VA Qu Spez) with different lectin and viscotoxin concentrations helped us in assessing the differential antiangiogenic effects since their cytotoxic properties depend on the composition of the preparations.

The PI and annexin V labeling showed that the percentage of apoptotic cells was higher with the VA Qu Spez treatments compared to the control and VA P treatments. However, the effects of the VA extracts on the angiogenic process were different in the *in vitro* and *in vivo* studies.

The analysis of the *in vitro* matrigel images showed that VA Qu Spez had a dose-dependent effect on angiogenesis while VA P did not have much impact. This was apparent from the diminution in the angiogenesis area and expansion in the alveolar space with the VA Qu Spez treatment. Moreover, the VA Qu Spez concentration (50 µg/ml) at which it was antiangiogenic was shown to be highly cytotoxic towards the EA-hy926 cells. The results obtained with VA P showing a tendency towards anti-angiogenesis *in vitro* raise the possibility of a non-cytotoxic, non-lectin compound being present in VA P. Although the apoptosis and *in vitro* data suggested that VA extracts have direct cytotoxic effect on the EC, the functional relevance *in vivo* was not elucidated.

To validate the results *in vivo*, the impact of the VA extracts on the vessel development in sub-cutaneously implanted matrigel plugs was analyzed. The marked reduction (about 66%) in the vessel score in the mice that were subjected to systemic treatment of VA Qu Spez along with intra-matrigel treatment (Q/Q) showed that the presence of VA Qu Spez at the site is necessary for the reduction of angiogenic growth. This gives a strong argument for the need to inject VA extracts at the site of a tumor itself.

The antiangiogenic properties of VA extracts are due to the apoptosis of EC that is dependent on their lectin content. VA extracts can thus be regarded as cytostatic in addition to being cytotoxic.

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