NUTRITION AND CANCER, *55*(1), 86–93

Mastic Oil from *Pistacia lentiscus* var. *chia* Inhibits Growth and Survival of Human K562 Leukemia Cells and Attenuates Angiogenesis

Heleni Loutrari, Sophia Magkouta, Anastasia Pyriochou, Vasiliki Koika, Fragiskos N. Kolisis, Andreas Papapetropoulos, and Charis Roussos

Abstract: *Mastic oil from* Pistacia lentiscus *var.* chia, *a natural plant extract traditionally used as a food additive, has been extensively studied for its antimicrobial activity attributed to the combination of its bioactive components. One of them, perillyl alcohol (POH), displays tumor chemopreventive, chemotherapeutic, and antiangiogenic properties. We investigated whether mastic oil would also suppress tumor cell growth and angiogenesis. We observed that mastic oil concentration and time dependently exerted an antiproliferative and proapoptotic effect on K562 human leukemia cells and inhibited the release of vascular endothelial growth factor (VEGF) from K562 and B16 mouse melanoma cells. Moreover, mastic oil caused a concentration-dependent inhibition of endothelial cell (EC) proliferation without affecting cell survival and a significant decrease of microvessel formation both in vitro and in vivo. Investigation of underlying mechanism(s) demonstrated that mastic oil reduced 1) in K562 cells the activation of extracellular signal–regulated kinases 1/2 (Erk1/2) known to control leukemia cell proliferation, survival, and VEGF secretion and 2) in EC the activation of RhoA, an essential regulator of neovessel organization. Overall, our results underscore that mastic oil, through its multiple effects on malignant cells and ECs, may be a useful natural dietary supplement for cancer prevention.*

Introduction

Substantial evidence from epidemiological and laboratory studies supports that regular consumption of dietary phytochemicals is strongly associated with reduced risk of cancer (1–3). The essential oil and gum from the plant *Pistacia lentiscus (L.)* var. *chia* have been widely used as food and beverage flavoring additives and traditional medicines in the Mediterranean region since ancient times without any reported toxicity in humans. Numerous recent studies have demonstrated that they possess antimicrobial properties against a broad range of bacterial and fungal pathogens (4–7) and antioxidant activity as well (8). Lately, a hexane extract of mastic gum was demonstrated to induce apoptosis in HCT116 human colon cancer cells (9). Analysis of the chemical composition of mastic oil revealed that is a complex mixture of volatile compounds, mainly terpenes (5,10), with established beneficial biological properties (11). The monoterpene perillyl alcohol (POH), which corresponds to 0.84% of mastic oil, is of great clinical interest due to its established chemopreventive and chemotherapeutic potential demonstrated in a variety of rodent tumor models (12,13), and it has currently advanced to phase I–II clinical trials (14,15). Recently, we have shown that POH, besides its antitumor effect, acts as an angiogenesis inhibitor (16).

Aberrant angiogenesis plays an important role in the progression not only of solid tumors but of hematological malignancies as well (17,18). In fact, increased angiogenesis in bone marrow is one of the characteristics of various types of leukemia (19). Vascular endothelial growth factors (VEGFs) essentially coordinate the angiogenic process by activating several signal pathways upon binding to the tyrosine kinase VEGF receptors (VEGFRs), largely restricted to the vascular endothelium (20,21). It has been recently shown that certain leukemia cells not only produce VEGF but also express functional VEGFRs resulting in an autocrine and paracrine loop for tumor growth and propagation (22,23). Therefore, the use of VEGF and VEGFR inhibitors for the treatment of leukemias seems particularly attractive because it may target not only the vascular environment but also the malignant cells per se.

It is currently widely accepted that health benefits associated with phytochemicals present in complex mixtures of nutrients are attributed to their additive and/or synergistic interactions rather than to the effect of single constituents (24).

H. Loutrari, S. Magkouta, and C. Roussos are affiliated with "G.P. Livanos and M. Simou Laboratories," Evangelismos Hospital, Department of Critical Care and Pulmonary Services, Medical School, University of Athens, 10675 Athens, Greece. A. Pyriochou, V. Koika, and A. Papapetropoulos are affiliated with the Laboratory of Molecular Pharmacology, Department of Pharmacy, University of Patras, 26504 Patras, Greece. F. N. Kolisis is affiliated with the Biotechnology Laboratory, School of Chemical Engineering, National Technical University of Athens, 15700 Athens, Greece.

This has also been indicated for mastic oil in a recent investigation correlating the antimicrobial activity with individual components or the mastic oil itself (25). The results from this study support that the observed activity of mastic oil was due to the combination of several compounds, including some of the trace elements, working synergistically. Based on these observations, we sought out to determine whether mastic oil would exhibit tumor and angiogenesis preventive properties. POH was also examined in parallel experiments as a reference compound. We demonstrated that mastic oil was able to inhibit cell proliferation and survival of human leukemia K562 cells and decrease VEGF release from K562 and mouse melanoma B16 cells. In addition, mastic oil attenuated endothelial cell (EC) proliferation and neovessel formation both in vitro on Matrigel and in vivo on the chicken embryo chorioallantoic membrane (CAM). Finally, mastic oil reduced the activation levels of extracellular signal–regulated kinases 1/2 (Erk1/2) in K562 cells and of RhoA small GTPase in ECs, thus modulating important signaling pathways in target cells.

Materials and Methods

Materials

Mastic oil was kindly donated by the Chios Mastic Growers Association. POH was from Fluka Chemie AG (Buchs, Switzerland). Microvascular ECs (MVECs) were obtained from Vec Technologies (Rensselear, NY). K562 and B16 cell lines were originally obtained from the American Type Culture Collection (Manassas, VA). Tissue culture plastic ware was from Corning-Costar (Corning, NY). Cell culture media and supplements were obtained from Life Technologies (Paisley, UK). A VEGF enzyme-linked immunoabsorbent assay (ELISA) Duo set kit was provided by R&D Systems (Minneapolis, MN). The CyQUANT and EnzChek Caspase-3 Assay kits were purchased from Molecular Probes (Eugene, OR). Antibodies against Erk1/2 and RhoA were from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology, respectively. GST-Rhotekin RBD agarose was purchased from Upstate (Lake Placid, NY). All other materials were purchased from Sigma Chemical Co. (St. Louis, MO).

Cell Culture

K562 and B16 cells were maintained in RPMI-1640 and MVECs in Dulbecco's Modified Eagle's medium. Media were supplemented with 10% FCS, L-glutamine, and antibiotics (10 U/ml penicillin and 100 mg/ml streptomycin). Human umbilical vein ECs (HUVECs) were isolated as previously described (16) and cultured in M199 supplemented with 20% FCS, 200 μg/ml EC growth supplement, 5 U/ml heparin sodium, L-glutamine, and antibiotics (as previously described). Cells were incubated in a humidified 37°C incubator containing 5% CO_2.

Cell Proliferation and Apoptosis Assays

For cell proliferation assays, endothelial (MVEC or HUVEC) or K562 cells were plated at 4×10^3 or 10^4 cells per well, respectively, in a 96-well plate. Twenty-four hours later, proliferating cells were treated with mastic oil (0.01–0.02% vol/vol), POH (0.5 mM), or dimethyl sulfoxide (DMSO) vehicle (0.1%); after 24–72 h, cell numbers were measured as described previously (16) using the CyQUANT Cell Proliferation assay kit. Briefly, cells were washed with phosphate-buffered saline, and then 200 μl of CyQUANT GR dye in cell lysis buffer was added to each well. This dye exhibits strong fluorescence enhancement when bound to nucleic acids. After 5 min of incubation at room temperature under light protection, the sample fluorescence was measured. A reference standard curve with ECs or K562 cells in the linear detection range (50–50,000 cells) was created for converting sample fluorescence to cell number.

Cell apoptosis was monitored by measuring caspase-3 activity as previously described (16). Briefly, 1×10^6 K562 cells per well in 24-well plates or confluent ECs in 12-well plates were incubated with fresh media containing mastic oil (0.01–0.1% vol/vol), POH (1 mM), DMSO vehicle (0.1%), or 10 μM cyclohexamide. After 24–72 h, the cells were collected and processed according to EnzCheck Caspase-3 Assay kit #1 instructions. Results were normalized as relative fluorescence units per milligram of protein.

ELISA for VEGF Detection

K562 (1×10^6) or B16 cells grown to confluence in 24-well plates were treated with fresh media containing mastic oil (0.01–0.1% vol/vol), POH (1 mM), or DMSO vehicle (0.1%) for 24–48 h. Conditioned media were then collected for ELISA analysis, whereas cells were solubilized and total protein was measured. VEGF ELISA was performed according to the manufacturer's specifications. Results were normalized as picograms per milligram of protein.

Angiogenesis Assays

The Matrigel tube formation assay was performed as described in Ref. 26. Briefly, HUVECs (10^5 cells per milliliter) were added onto Matrigel-coated wells in 96-well plates in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle (0.1% DMSO) and incubated for 6 h. The cells were then fixed, and the length of tube-like structures was measured in the total area of the wells using Scion Image software (Scion Corp., Frederick, MD) as previously described (27).

The in vivo chicken embryo CAM angiogenesis model was used as previously described (27). In brief, Leghorn fertilized eggs were incubated for 4 days at 37°C, when a window was opened on the egg's shell, exposing the CAM. The window was covered with tape, and the eggs were returned to the incubator. Different amounts of mastic oil (1×10^{-4} to 1×10^{-6}) or vehicle (0.1% DMSO) were applied onto an area of 1 cm^2 (restricted by a plastic ring) of the CAM on Day 9 of embryo development. Forty-eight hours later, CAM was fixed in situ and

excised from the eggs, and pictures were taken through a stereoscope equipped with a digital camera. The total length of the vessels was measured using the Scion Image software.

Western Blotting

K562 cells grown in 24-well plates (1×10^6) were treated with fresh media containing mastic oil (0.01–0.04% vol/vol) or vehicle (0.1% DMSO) for 5–30 min and then subjected to cell lysis and Western blotting. Briefly, cells were collected and solubilized with lysis buffer (50 mM Tris-HCl, pH 7.6; 150 mM NaCl; 50 mM NaF; 1 mM Na_3VO_4; 0.5% sodium deoxycholate; 1 mM ethylenediaminetetraacetic acid (EDTA); 0.1 mM ethylene glycol-bis(2-amionoethylether)-N,N,N′,N′-tetraacetic acid (EGTA); 1% Triton-X; 1% sodium dodecyl sulfate (SDS); 1 mM phenylmethanesulfonyl fluoride (PMSF); 10 μg/ml aprotinin; 5 μg/ml leupeptin; and 10 ng/ml pepstatin). Lysates containing equal amounts of protein were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) on 12% gels, transferred to nitrocellulose membranes, and probed with primary antibodies that specifically recognize the phosphorylated or total form of Erk1/2. Immunoreactive bands were visualized by enhanced chemiluminescence detection.

Determination of Active RhoA Levels

Serum-starved confluent HUVECs in 10-cm dishes were stimulated by adding 10% FCS containing mastic oil (0.01–0.04% vol/vol), POH (0.5 mM), or vehicle (0.1% DMSO) for 2 min. RhoA activation was then determined by a pull-down assay using GST-Rhotekin RBD agarose beads essentially as described in Ref. 28. In brief, cells were solubilized with lysis buffer (25 mM HEPES, pH 7.5; 150 mM NaCl; 1% vol/vol Igepal CA-630; 10 mM $MgCl_2$; 1 mM EDTA; 10 % glycerol; 10 μg/ml leupeptin; 10 μg/ml aprotinin; 1 mM PMSF; and 1 mM sodium orthovanadate), and cell lysates containing equal amounts of protein were incubated with 30 μg GST-RBD agarose beads for 45 min at 4°C. Active RhoA bound to the beads was then eluted and analyzed in parallel with aliquots containing 20 μg protein of total cell lysates by 12% SDS-PAGE and Western blotting using anti-RhoA antibody.

Data Analysis and Statistics

Data are presented as mean ± SE of the indicated number of observations. Statistical comparisons between control and other groups were performed using the Mann-Whitney nonparametric test. Differences among means were considered significant when $P < 0.05$ from control.

Results

Effects of Mastic Oil on Tumor Cells

Mastic oil inhibits K562 cell growth and survival: To estimate the effect of the examined compounds on cell proliferation, cultures of dividing cells were treated for various time periods, and thereafter the cell number was measured. Mastic oil induced a concentration-dependent decrease in cell number compared with control treatment, indicating an inhibition in the proliferation of K562 cells. Following a 48-h incubation with 0.01 and 0.02% vol/vol mastic oil, the cell number was reduced to 91.9% and 68.5% of control samples, respectively (Fig 1A).

Moreover, treatment of cells with mastic oil (0.01–0.1% vol/vol) caused a concentration-dependent stimulation of caspase-3 activity in K562 cells after 72-h of incubation (Fig. 1B). It is interesting to note that the apoptotic effect of mastic oil and POH followed a similar time-response pattern while cyclohexamide (used as a positive control) caused an earlier induction of caspase-3 activity at 24–48 h of incubation (not shown).

Mastic oil down-regulates VEGF release from tumor cells: VEGF is produced by most tumor cells and appears to be one of the main mediators relevant to the proangiogenic tumor phenotype. Treatment of K562 cells with mastic oil (0.01–0.1% vol/vol) for 24–48 h caused a concentration- and time-dependent reduction of the secreted VEGF (Fig. 2A). A similar inhibition of VEGF protein production was also observed in a B16 mouse melanoma cell line in response to increasing concentration of mastic oil (Fig. 2B).

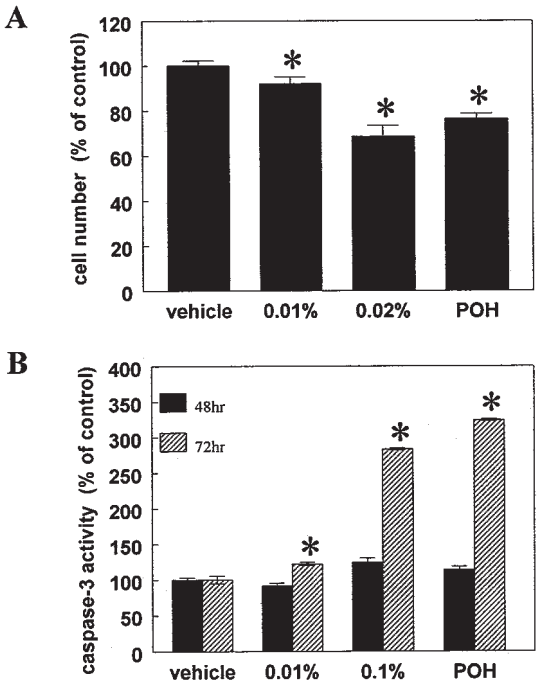

Figure 1. Mastic oil inhibits K562 (A) cell proliferation and (B) survival. (A) In a 96-well plate, 10^4 cells per well were plated and 24 h later were treated with mastic oil (0.01–0.02% vol/vol), perillyl alcohol (POH, 0.5 mM), or vehicle. Cell numbers were measured after 48 h. (B) Cells (1×10^6) grown in 24-well plates were treated with mastic oil (0.01–0.1% vol/vol), POH (1 mM), or vehicle and analyzed after 48–72 h for caspase-3 activity. Results are expressed as mean ± SE; $n = 9–12$; *$P < 0.05$ from vehicle.

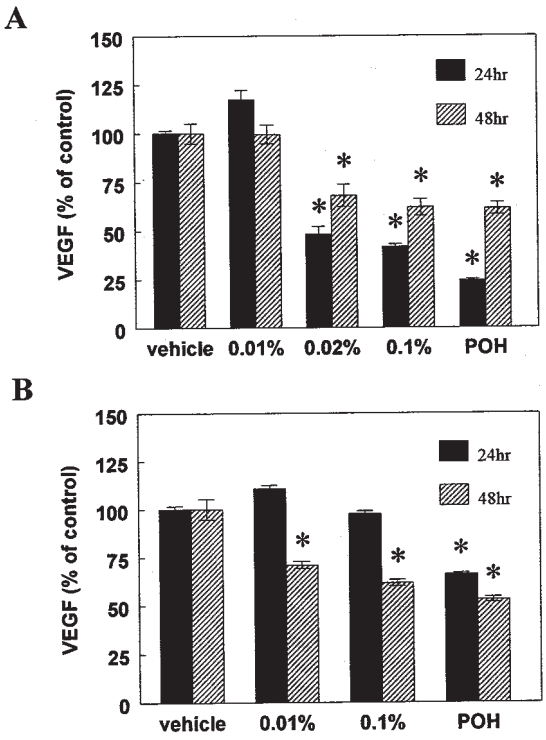

Figure 2. Mastic oil reduces vascular endothelial growth factor (VEGF) secretion from tumor cells. (A) K562 cells (1×10^6) or (B) confluent cultures of B16 cells grown in 24-well plates were treated with mastic oil (0.01–0.1% vol/vol), perillyl alcohol (POH, 1 mM), or vehicle. At the indicated times, culture supernatants were collected and analyzed by enzyme-linked immunoabsorbent assay for the presence of VEGF. Results are expressed as mean ± SE; $n = 9$; $^*P < 0.05$ from vehicle.

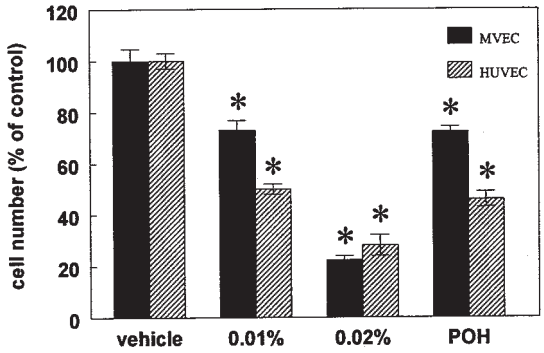

Figure 3. Mastic oil inhibits endothelial cell (EC) proliferation. Microvascular or human umbilical vein ECs were plated in a 96-well plate at 4,000 cells per well. The next day, cell cultures were treated with mastic oil (0.01–0.02% vol/vol), perillyl alcohol (POH, 0.5 mM), or vehicle for 48 h, and cell numbers were measured. Results are expressed as mean ± SE; $n = 12$; $^*P < 0.05$ from vehicle.

Effect of Mastic Oil on ECs

Mastic oil inhibits EC proliferation: EC proliferation is a key component of normal and tumor-associated angiogenesis. As shown in Fig. 3, mastic oil efficiently reduced the number of growing MVECs and HUVECs in a concentration-dependent manner (0.01–0.02% vol/vol), indicating inhibition of cell proliferation. EC treatment with mastic oil (0.01–0.04% vol/vol) did not induce any significant change in caspase-3 activity, suggesting that mastic oil does not affect EC survival. Representative values (% of vehicle) from HUVEC incubated with 0.04% vol/vol mastic oil for 24, 48, and 72 h were 104.4 ± 3.7, 101.0 ± 8.5, and 96.4±2.5, respectively.

Mastic oil attenuates capillary-like organization of EC on Matrigel: The effect of mastic oil on the morphological differentiation of ECs was studied in the Matrigel assay. As shown in Fig. 4A, vehicle-treated HUVECs grown on Matrigel developed tube-like networks. However, mastic oil inhibited the organization of the cells in structures mimicking vascular networks. Visual observations were confirmed by morphometric analysis (Fig. 4B) showing that mastic oil (0.01–0.04% vol/vol) induced a concentration-dependent reduction of the total length of the tube-like structures.

Mastic oil inhibits neovascularization in the CAM: The effect of mastic oil on the in vivo formation of new vessels was evaluated using the CAM system of angiogenesis. As demonstrated by morphometric analysis, mastic oil induced in the examined concentrations a small but statistically significant reduction in the number of CAM vessels under basal conditions (Fig. 5).

Molecular Targets for Mastic Oil in Leukemia and Endothelial Cells

To elucidate the signaling mechanism(s) through which mastic oil exerted its effects on leukemia cells, we examined by Western blotting the levels of phosphorylated Erk1/2 relative to total Erk1/2 following treatment of K562 cells with different concentrations of mastic oil (0.01–0.1% vol/vol) and for various incubation times (5–30 min). Mastic oil caused a concentration- (Fig. 6A) and time- (Fig. 6B) dependent inhibition of Erk1/2 phosphorylation without affecting total levels of Erk1/2. This inhibition was reversible and reached a maximum within the first 10 min of incubation. It should be noted that K562 cells express higher levels of Erk1; a minor band for Erk2 could only be detected with the phosphospecific antibody.

To explore the signaling pathways that are affected by mastic oil in ECs, we induced serum-starved HUVECs with complete medium containing mastic oil (0.01–0.04% vol/vol) and then determined the levels of activated RhoA relative to total levels of RhoA. As shown in Fig. 7, mastic oil concentration dependently inhibited RhoA activation without changing the total levels of RhoA. POH exhibited a similar effect (not shown).

Discussion

The search for new effective cancer chemopreventive and chemotherapeutic agents, especially for those that have a nat-

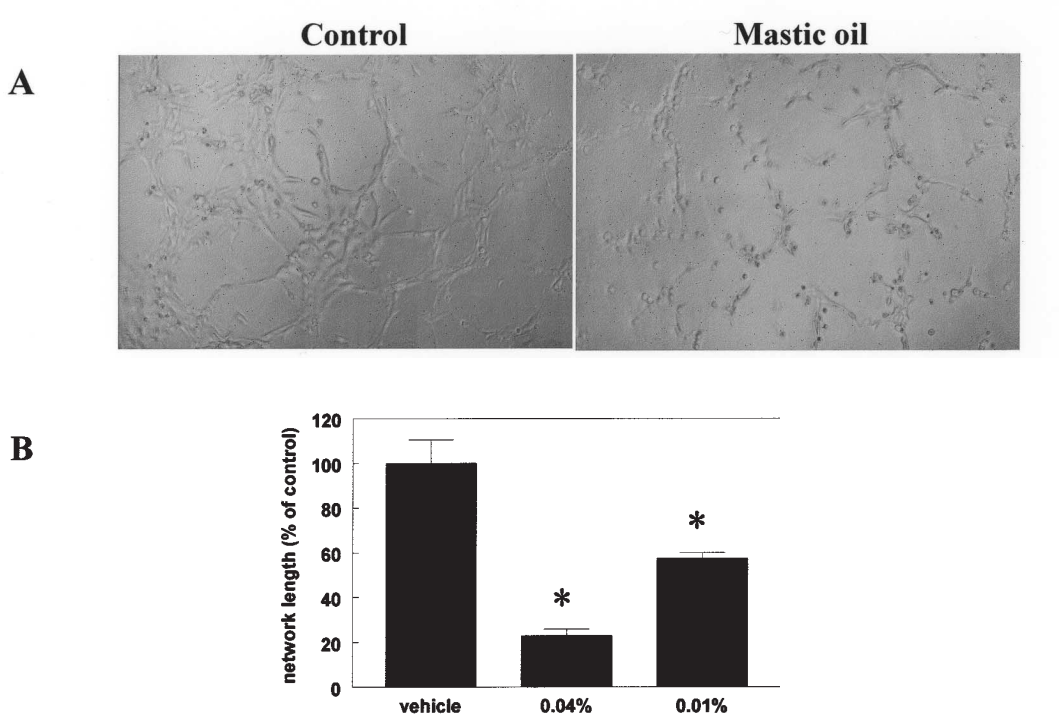

Figure 4. Mastic oil attenuates endothelial cell (EC) differentiation on Matrigel. Human umbilical vein ECs (10^5 cells per milliliter) were added into Matrigel-coated wells in 96-well plates in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle and incubated for 6 h. (A) Representative photomicrographs showing the formation of tube-like structures on Matrigel after vehicle or mastic oil (0.01% vol/vol) treatment. (B) The length of the tube network was measured in the total well area. Results are expressed as mean ± SE; $n = 9$; $^*P < 0.05$ from vehicle.

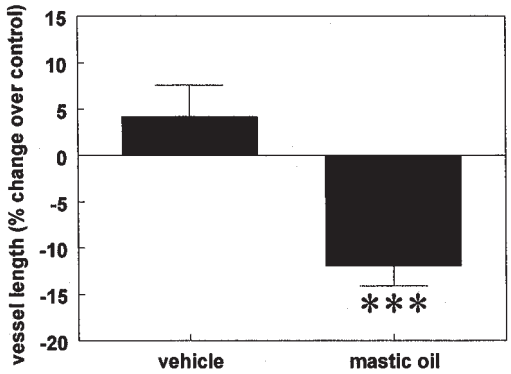

Figure 5. Mastic oil inhibits angiogenesis on the chorioallantoic membrane (CAM). Mastic oil (1×10^{-4}) or vehicle was applied onto 1 cm^2 of the CAM on Day 9 and incubated for 48 h at 37°C. CAM was fixed and excised from the eggs, and the total length of the vessel network was measured using image analysis software. Results are expressed as mean ± SE; $n \geq 24$; $^{***}P < 0.001$ from vehicle.

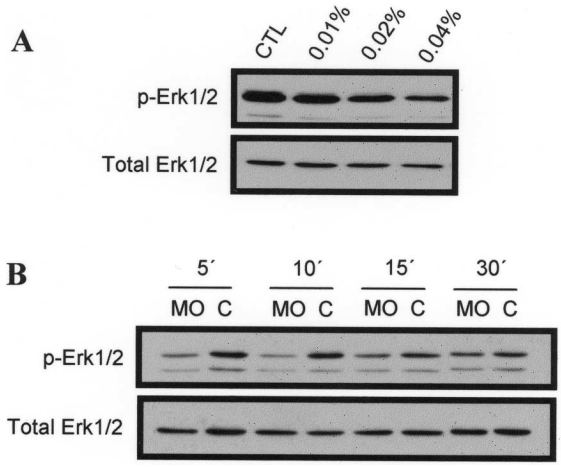

Figure 6. Mastic oil inhibits extracellular signal–regulated kinase 1/2 (Erk1/2) phosphorylation in K562 cells. Cells were grown in a 24-well plate at 1×10^6 per well. (A) A concentration-dependent effect was assessed after cell treatment with mastic oil (0.01–0.04% vol/vol) or vehicle (CTL) for 10 min and analysis of lysates by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting using antibodies specific for the phosphorylated (p-Erk1/2) or total Erk1/2. The p-Erk1/2/total Erk1/2 ratios for mastic oil–treated cells were 85.2 ± 2.2%, 72.3 ± 10.3%, and 60.4 ± 4.6% of the corresponding ratios of vehicle-treated cells (control) after incubation with 0.01, 0.02, and 0.04% mastic oil, respectively. (B) Time course effect was determined after treatment with 0.04% vol/vol mastic oil (MO) or vehicle (C) for the indicated time (min) and analysis as in (A). The p-Erk1/2/total Erk1/2 ratios for mastic oil–treated cells were 43.6 ± 1.9%, 24.1 ± 4.6%, 73.05 ± 4.1%, and 127.2 ± 29.7% of the corresponding ratios of vehicle-treated cells (control) after incubation for 5, 10, 15, and 30 min, respectively. Blots are representative of three independent experiments.

ural origin and relatively low toxicity, has recently increased and become an important issue in public health–associated research policy (29). In the present study, we demonstrated for the first time the pleiotropic effects of mastic oil, a natural mixture of phytochemicals, on tumor cell growth and angiogenesis, and we investigated its potential molecular targets in tumor and ECs. POH, one of mastic oil's bioactive components, was also included in our study because it pos-

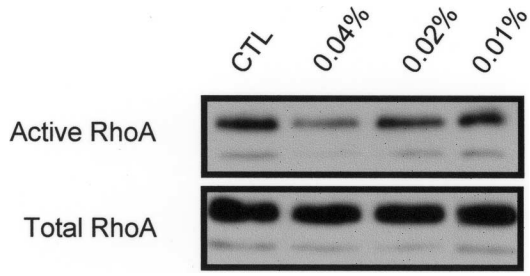

Figure 7. Mastic oil inhibits RhoA activation in endothelial cells (ECs). Starved human umbilical vein ECs were serum stimulated in the presence of mastic oil (0.01–0.04% vol/vol) or vehicle (CTL) for 2 min. Cell lysates were incubated with GST-RBD beads, and eluted active RhoA samples were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and Western blotting, in parallel with aliquots of total cell lysates, using anti-RhoA antibody. The active RhoA/total RhoA ratios for mastic oil–treated cells were 92.6 ± 6.0%, 80.4 ± 8.3%, and 38.3 ± 3.5% of the corresponding ratios of vehicle-treated cells (control) after incubation with 0.01, 0.02, and 0.04% mastic oil, respectively. Blots are representative of three independent experiments.

sesses well-documented tumor chemopreventive, chemotherapeutic, and antiangiogenic properties (12,13,16).

The antitumor activity of mastic oil was assessed on the human K562 cell line established from a patient with chronic myelogenous leukemia. In analogy to solid neoplasms, accumulating evidence underscores the requirement of angiogenesis for the progression of several types of leukemias (17–19). It is known that leukemias originate from hematopoietic stem cells at different stages of their maturation and that hematopoietic cells and ECs are mutually influenced in their growth (19). Leukemia cells release endothelial growth factors (22,23,30), and activated ECs release cytokines that stimulate leukemia cell growth (31). VEGF is almost invariably expressed by all established leukemia cell lines as well as freshly isolated primary human leukemia cells. Furthermore, it has been recently shown that, in addition to ECs, certain leukemia cells also express functional VEGFRs, resulting in an autocrine and paracrine mechanism for leukemia cell growth and migration (22,23). K562 cells have been shown to produce functional VEGFRs that promote matrix metalloproteinase activation and cell migration (23). Therefore, VEGF, by stimulating both endothelial and leukemia cells, seems to fulfill a fundamental role in promoting leukemia cell proliferation, survival, migration, and angiogenesis. In the present study, we have shown that mastic oil concentration and time dependently decreased the number of dividing K562 cells, indicating under the examined experimental conditions an inhibition of cell proliferation, and induced the apoptotic death of K562 cells in the stationary phase following 72 h of treatment. A proapoptotic effect of a hexane extract of mastic gum has been also reported on HCT116 human colon cancer cells (9). Moreover, treatment of K562 cells with mastic oil reduced the levels of secreted VEGF. Mastic oil had a similar effect on B16 mouse melanoma cells, indicating a broad activity of mastic oil on VEGF released from malignant cells. To exclude that inhibition of cell growth or induction of apoptotic cell death contributed to the observed reduction in VEGF, we determined VEGF release following a 24- to 48-h treatment of nondividing cells, a timeframe during which apoptosis is not observed. Moreover, results of VEGF release were normalized for protein content. Therefore, the decrease in VEGF levels after mastic oil treatment was due to a direct effect on VEGF production and/or secretion.

Proliferation, survival, and differentiation of ECs are key events of angiogenesis, and compounds that target these processes may be of clinical relevance as antiangiogenic drugs (32). In this study, the effects of mastic oil on ECs were assessed in three in vitro assays, namely, cell proliferation, apoptosis, and tube-like formation assay using MVECs and/or HUVECs. Mastic oil exerted a concentration-dependent inhibitory activity in both cell proliferation and Matrigel-induced differentiation without affecting cell survival. Furthermore, to investigate the ability of mastic oil to interfere with the angiogenic process in vivo, we used the well-established CAM model of neovascularization and observed that mastic oil caused a small but significant reduction in the number of new vessels. These data suggest that mastic oil is an effective inhibitor of EC proliferation and differentiation in vitro and angiogenesis in vivo.

We next investigated the potential mechanisms through which mastic oil may exert its major effects on tumor cells and ECs. In leukemia cells, we focused on the mitogen-activated protein signal transduction pathway leading to the activation of Erk1/2. There is direct evidence that the Raf/Mek/Erk pathway primarily mediates mitogenic and antiapoptotic signals in leukemia cells (33,34). It has also been demonstrated that VEGF induces myeloma cell proliferation by activating this signaling cascade (35), and more recently it has been shown that down-modulation of Erk1/2 activation inhibits VEGF secretion by human myeloma cells (36). In this study, we found that mastic oil concentration and time dependently reduced the levels of Erk1/2 phosphorylation, suggesting that it might inhibit leukemia cell proliferation and survival and VEGF secretion by attenuating activation of this signaling pathway. A similar effect on Erk1/2 activation was reported previously for POH in K562 cells (16), in line with earlier evidence suggesting that POH blocks Ras small GTPase, an upstream activator of the RAF/Mek/Erk signaling cascade (37).

In ECs, we investigated the effect of mastic oil on the levels of RhoA activation. RhoA is an important member of the Rho GTPase family, known to interact with the cytoskeleton and regulate cytokinesis in different cell types including EC (38,39). Recent evidence suggests that RhoA GTPases are involved in the process of EC morphogenesis during neovascularization in vitro and in vivo (40–42). In the present work, we showed that mastic oil markedly reduced the levels of the active form of RhoA (GTP-RhoA) in a concentration-dependent manner without altering the expression of total levels of RhoA. This result suggests that mastic oil might inhibit the EC assembly into new blood vessels by disrupting the RhoA signaling cascade. POH was found to exert similar effects with mastic oil in ECs. Interestingly, POH has been shown to reduce RhoA levels in tumor cells (43).

As mentioned previously, POH was used in the present study as a reference compound. Comparison of the concentrations at which mastic oil and POH are effective (as determined herein and in Ref. 16) as well as consideration of the POH content of mastic oil (approximately 1%) suggest that mastic oil is more potent than POH in inhibiting tumor growth and angiogenesis. We speculate that this can be attributed to the additive and/or synergistic action of its bioactive constituents, as has been already shown for the chemopreventive effect of other mixtures of phytochemicals (24) and for the antimicrobial activity of mastic oil (25).

In conclusion, our results suggest that mastic oil is a tumor and angiogenesis inhibitor targeting components of critical signaling cascades in both leukemia and ECs. If present findings are extended to other malignancies and to in vivo tumor models, they can form the basis for establishing mastic oil as a naturally occurring chemopreventive and antiangiogenic agent.

Acknowledgements and Notes

This work was supported in part by a grant from the Greek General Secretariat of Research and Technology and The Chios Gum Mastic Growers Association and funds from the Thorax Foundation. Address correspondence to H. Loutrari, G.P. Livanos and M. Simou Laboratories, Evangelismos Hospital, Department of Critical Care and Pulmonary Services, Medical School, University of Athens, 3 Ploutarchou Street, Athens, Greece 10675. Phone: +30–210–7235521. FAX: +30–210–7239127. E-mail: elloutrar@med.uoa.gr.

Submitted 25 January 2006; accepted in final form 3 April 2006.

References

1. Milner JA: Strategies for cancer prevention: the role of diet. *Br J Nutr* **87,** S265–S272, 2002.
2. Sabichi AL, Demierre MF, Hawk ET, Lerman CE, and Lippman SM: Frontiers in cancer prevention research. *Cancer Res* **63,** 5649–5655, 2003.
3. Brenner DE and Gescher AJ: Cancer chemoprevention: lessons learned and future directions. *Br J Cancer* **93,** 735–739, 2005.
4. Huwez FU, Thirlwell D, Cockayne A, and Ala'Aldeen DA: Mastic gum kills Helicobacter pylori. *N Engl J Med* **339,** 1946, 1998.
5. Magiatis P, Melliou E, Skaltsounis AL, Chinou IB, and Mitaku S: Chemical composition and antimicrobial activity of the essential oils of Pistacia lentiscus var. chia. *Planta Med* **65,** 749–752, 1999.
6. Marone P, Bono L, Leone E, Bona S, Carretto E, et al.: Bactericidal activity of Pistacia lentiscus mastic gum against Helicobacter pylori. *J Chemother* **13,** 611–614, 2001.
7. Daifas DP, Smith JP, Blanchfield B, Sanders G, Austin JW, et al.: Effects of mastic resin and its essential oil on the growth of proteolytic Clostridium botulinum. *Int J Food Microbiol* **94,** 313–322, 2004.
8. Andrikopoulos NK, Kaliora AC, Assimopoulou AN, and Papapeorgiou VP: Biological activity of some naturally occurring resins, gums and pigments against in vitro LDL oxidation. *Phytother Res* **17,** 501–507, 2003.
9. Balan KV, Demetzos C, Prince J, Dimas K, Cladaras M, et al.: Induction of apoptosis in human colon cancer HCT116 cells treated with an extract of the plant product, Chios mastic gum. *In Vivo* **19,** 93–102, 2005.
10. Papageorgiou V, Sagredos A, and Moser R: GLC-MS computer analysis of the essential oil of mastic gum. *Chim Chronica New Ser* **10,** 119–124, 1981.
11. Wagner KH and Elmadfa I: Biological relevance of terpenoids. Overview focusing on mono-, di- and tetraterpenes. *Ann Nutr Metab* **47,** 95–106, 2003.
12. Belanger JT: Perillyl alcohol: applications in oncology. *Altern Med Rev* **3,** 448–457, 1998.
13. Crowell PL: Prevention and therapy of cancer by dietary monoterpenes. *J Nutr* **129,** S775–S778, 1999.
14. Hudes GR, Szarka CE, Adams A, Ranganathan S, McCauley RA, et al.: Phase I pharmacokinetic trial of perillyl alcohol (NSC 641066) in patients with refractory solid malignancies. *Clin Cancer Res* **6,** 3071–3080, 2000.
15. Stearns V, Coop A, Singh B, Gallagher A, Yamauchi H, et al.: A pilot surrogate end point biomarker trial of perillyl alcohol in breast neoplasia. *Clin Cancer Res* **10,** 7583–7591, 2004.
16. Loutrari H, Hatziapostolou M, Skouridou V, Papadimitriou E, Roussos C, et al.: Perillyl alcohol is an angiogenesis inhibitor. *J Pharmacol Exp Ther* **311,** 568–575, 2004.
17. Bergers G and Benjamin LE: Tumorigenesis and the angiogenic switch. *Nat Rev Cancer* **3,** 401–410, 2003.
18. Moehler TM, Ho AD, Goldschmidt H, and Barlogie B: Angiogenesis in hematologic malignancies. *Crit Rev Oncol Hematol* **45,** 227–244, 2003.
19. Molica S, Vacca A, Levato D, Merchionne F, and Ribatti D: Angiogenesis in acute and chronic lymphocytic leukemia. *Leuk Res* **28,** 321–324, 2004.
20. Yancopoulos GD, Davis S, Gale NW, Rudge JS, Wiegand SJ, et al.: Vascular-specific growth factors and blood vessel formation. *Nature* **407,** 242–248, 2000.
21. Ferrara N, Gerber HP, and LeCouter J: The biology of VEGF and its receptors. *Nat Med* **9,** 669–676, 2003.
22. Bellamy WT, Richter L, Frutiger Y, and Grogan TM: Expression of vascular endothelial growth factor and its receptors in hematopoietic malignancies. *Cancer Res* **59,** 728–733, 1999.
23. Dias S, Hattori K, Zhu Z, Heissig B, Choy M, et al.: Autocrine stimulation of VEGFR-2 activates human leukemia cell growth and migration. *J Clin Invest* **106,** 511–521, 2000.
24. Liu RH: Potential synergy of phytochemicals in cancer prevention: mechanism of action. *J Nutr* **134,** S3479–S3485, 2004.
25. Koutsoudaki C, Krsek M, and Rodger A: Chemical composition and antibacterial activity of the essential oil and the gum of Pistacia lentiscus Var. chia. *J Agric Food Chem* **53,** 7681–7685, 2005.
26. Pipili-Synetos E, Papadimitriou E, and Maragoudakis ME: Evidence that platelets promote tube formation by EC on matrigel. *Br J Pharmacol* **125,** 1252–1257, 1998.
27. Papadimitriou E, Polykratis A, Courty J, Koolwijk P, Heroult M, et al.: HARP induces angiogenesis in vivo and in vitro: implication of N or C terminal peptides. *Biochem Biophys Res Commun* **282,** 306–313, 2001.
28. Ren XD, Kiosses WB, and Schwartz MA: Regulation of the small GTP-binding protein Rho by cell adhesion and the cytoskeleton. *Embo J* **18,** 578–585, 1999.
29. Greenwald P: Clinical trials in cancer prevention: current results and perspectives for the future. *J Nutr* **134,** S3507–S3512, 2004.
30. Giuliani N, Colla S, Lazzaretti M, Sala R, Roti G, et al.: Proangiogenic properties of human myeloma cells: production of angiopoietin-1 and its potential relationship to myeloma-induced angiogenesis. *Blood* **102,** 638–645, 2003.
31. Rafii S, Mohle R, Shapiro F, Frey BM, and Moore MA: Regulation of hematopoiesis by microvascular endothelium. *Leuk Lymphoma* **27,** 375–386, 1997.
32. Saaristo A, Karpanen T, and Alitalo K: Mechanisms of angiogenesis and their use in the inhibition of tumor growth and metastasis. *Oncogene* **19,** 6122–6129, 2000.
33. Kang CD, Yoo SD, Hwang BW, Kim KW, Kim DW, et al.: The inhibition of ERK/MAPK not the activation of JNK/SAPK is primarily re-

quired to induce apoptosis in chronic myelogenous leukemia K562 cells. *Leuk Res* **24,** 527–534, 2000.
34. Platanias LC: Map kinase signaling pathways and hematologic malignancies. *Blood* **101,** 4667–4679, 2003.
35. Podar K, Tai YT, Davies FE, Lentzsch S, Sattler M, et al.: Vascular endothelial growth factor triggers signaling cascades mediating multiple myeloma cell growth and migration. *Blood* **98,** 428–435, 2001.
36. Giuliani N, Lunghi P, Morandi F, Colla S, Bonomini S, et al.: Downmodulation of ERK protein kinase activity inhibits VEGF secretion by human myeloma cells and myeloma-induced angiogenesis. *Leukemia* **18,** 628–635, 2004.
37. Hohl RJ and Lewis K: Differential effects of monoterpenes and lovastatin on RAS processing. *J Biol Chem* **270,** 17508–17512, 1995.
38. Bishop AL and Hall A: Rho GTPases and their effector proteins. *Biochem J* **348,** 241–255, 2000.
39. Fryer BH and Field J: Rho, Rac, Pak and angiogenesis: old roles and newly identified responsibilities in endothelial cells. *Cancer Lett* **229,** 13–23, 2005.
40. Davis GE, Bayless KJ, and Mavila A: Molecular basis of endothelial cell morphogenesis in three-dimensional extracellular matrices. *Anat Rec* **268,** 252–275, 2002.
41. Cascone I, Giraudo E, Caccavari F, Napione L, Bertotti E, et al.: Temporal and spatial modulation of Rho GTPases during in vitro formation of capillary vascular network. Adherens junctions and myosin light chain as targets of Rac1 and RhoA. *J Biol Chem* **278,** 50702–50713, 2003.
42. Hoang MV, Whelan MC, and Senger DR: Rho activity critically and selectively regulates endothelial cell organization during angiogenesis. *Proc Natl Acad Sci USA* **101,** 1874–1879, 2004.
43. Holstein SA and Hohl RJ: Monoterpene regulation of Ras and Ras-related protein expression. *J Lipid Res* **44,** 1209–1215, 2003.