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ORIGINAL ARTICLE

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Elemene displays anti-cancer ability on laryngeal cancer cells in vitro and in vivo

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Abstract *Purpose*: The goal of this study is to investigate the inhibitory effects and mechanism of elemene on the growth of laryngeal cancer cells in vitro and in vivo. *Methods*: Laryngeal cancer cells (HEp-2 cells) were grown in elemene, cisplatin, or a combination of the drugs. The cytotoxic, or apoptotic, effects of elemene on the cells were evaluated by a 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide assay, flow cytometry, and a caspase-3 activity assay. A Western blot was used to semi-quantify the protein expression of eukaryotic initiation factors (eIF4E and eIF4G), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF); RT-PCR analysis semi-quantified the mRNA transcript expression of bFGF and VEGF. The HEp-2 cells were transplanted subcutaneously to BALB/c nude mice to produce solid tumors. Elemene and cisplatin were administered to the mice either as individual drugs or in combination. The tumors were excised and immunostained to determine the effect each drug had on tumor size, eIF levels, angiogenic factors, and microvessel density (MVD). *Results*: Elemene inhibited the growth of HEp-2 cells in vitro in a dose- and time-dependent manner with an IC_{50} of 346.5 μM (24 h incubation). Increased apoptosis was observed in elemene-administered cells. Elemene is suspected to enhance caspase-3 activity, and thus inhibit protein expression of eIFs (4E, 4G), bFGF, and VEGF. In vivo, the growth of HEp-2 cell-transplanted tumors in nude mice was inhibited by intraperitoneal injection of elemene. Compared with control groups, elemene significantly inhibited the protein expression of eIFs (4E and 4G), bFGF, and VEGF and decreased the MVD. *Conclusions*: Elemene inhibits the growth of HEp-2 cells in vitro and in vivo. These data provide useful information for further clinical study on the treatment of LSCC by elemene.

Keywords Elemene · Laryngeal squamous cell carcinoma · Apoptosis · EIF · Angiogenesis

Abbreviations *A*: Absorbances · *BFGF*: Basic fibroblast growth factor · *DAB*: 3,3′-diaminobenzidine tetrahydrochloride · *EIF*: Eukaryotic initiation factor · *GI*: Growth inhibition · *IR*: Inhibition rate · *LSCC*: Laryngeal squamous cell carcinoma · *MVD*: Microvessel density · *PI*: Propidium iodide · *PNA*: P-nitroanalide · *VEGF*: Vascular endothelial growth factor

Introduction

Laryngeal squamous cell carcinoma (LSCC) is one of the most common cancers in the world [1]. Unfortunately, high rates of local recurrence at the primary site have kept cure rates low. Surgery is currently the only treatment modality that consistently prolongs survival; other treatment attempts such as radiotherapy, chemotherapy [2], and immunotherapy [3] have not been effective in the majority of patients.

New approaches with chemotherapeutic agents have recently been tested in the treatment of LSCC. Gemcitabine can suppress ribonucleoside diphosphate reductase and inhibit tumor growth in head and neck cancer [4]. Paclitaxel and Docetaxel have been effective in inhibiting the growth of LSCC cell lines grown in vitro and in vivo by inhibiting angiogenesis; these drugs

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have been used in the second-line management of carcinoma of the head and neck [5]. Data supporting the use of these agents in clinical practice, however, remains weak.

A new treatment trend is to combine standard chemotherapeutic agents with traditional medicines like elemene. Elemene is a terpene compound isolated from Rhizoma Zedoariae, a Chinese medicinal herb [6]. There is only limited information in the literature on elemene essential oil compounds; β-elemene is reportedly the most abundant constituent, and other components, such as α-elemene and γ-elemene, are present only in trace amounts [7]. The anti-cancer effects of elemene have long been recognized by traditional physicians, but they have only recently been established scientifically. Reports indicate elemene can efficiently inhibit the growth of HL-60 cells and Hela cells in vitro, and the tumors of W-256 and Lewis in vivo [8, 9]. Therefore, elemene has generated a great deal of interest as a possible therapeutic modality in LSCC.

A possible target of such therapeutic interventions is stimulation of the apoptotic pathways, especially via caspase-3. Caspase-3 is an effector caspase that is activated by apical caspases (caspase-2, -8, -9, and -10) and is required for apoptosis-associated DNA fragmentation and membrane blebbing [10]. Its importance has been demonstrated in the caspase-3 deficient breast cancer cell line MCF-7, which is relatively insensitive to many chemotherapeutic agents. Reconstitution of caspase-3 restores the apoptotic pathways and sensitizes the cells to chemotherapeutic agents [11]. This is likely because caspase-3 is a key caspase in the apoptotic signaling cascade and is important in both death-receptor and mitochondria-mediated apoptosis [12]. Caspase-3 performs a central role as both an integrator and an amplifier of the apoptotic pathways.

Another function of caspase-3 is cleavage of eukaryotic initiation factors (eIFs) [13]. The eIF4E is a 24 kDa phosphoprotein which specially binds to the mRNA cap at the first step of mRNA recruitment, and unwinds the secondary structure at the 5′ untranslated region (5′UTR) of the mRNA [14]. The eIF4G is a 154 kDa polypeptide which increases the binding of eIF4E to the cap of mRNA [15]. "Weak mRNAs", such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF), which are both important for tumor angiogenesis, contain long GC-rich 5′UTRs and possess the potential for forming stable secondary structures and/or upstream AUGs. With low eIF4E levels, these weak mRNAs are translated poorly. As eIF4E levels increase, the secondary structure of the mRNA becomes less stable; so weak mRNAs are translated more efficiently. This pushes the cells to grow more rapidly, and they become neoplastic [16]. By cleaving the eIFs, caspase-3 helps to keep translation of bFGF and VEGF low, thus reducing cell growth. Though caspase-3 is a likely target, the direct effect of elemene on the growth of LSCC cells has not been previously reported. Experimental support is crucial for clinical studies on the treatment of LSCC by elemene.

The current study aims to investigate the anti-cancer effect and mechanism of elemene on human LSCC (HEp-2 cells) in vitro and in vivo. Elemene's success was evaluated in comparison with a known chemotherapeutic agent, cisplatin, and an elemene-cisplatin combination was tested for a synergistic effect.

Materials and methods

Materials

Elemene and emulsion without elemene were purchased from Jingang Pharmaceutical Co. (Dalian, China). Cisplatin and other chemicals were obtained from Sigma Co. (Shanghai, China). Human LSCC cell line HEp-2 cells, WI-38 cells (human normal cells as normal control), and Hela cells (human cervical cancer cells as positive control) were provided by the Cell Bank of the Chinese Institute of Biochemistry and Cell Biology. Six-week-old athymic female nude mice (BALB/c) were obtained from the Shanghai Cancer Institute. The mice were kept in laminar air-flow benches. This animal study was approved by the Institutional Animal Care and Use Committee of China, and institutional guidelines for animal welfare and experimental conduct were followed.

Cell culture and cytotoxicity assay

All cell lines were maintained in RPMI1640 with 10% fetal bovine serum. The cells were maintained in a humidified atmosphere of air/CO_2 (19/1) and were subcultured every 2–3 days.

Approximately 5×10^4/ml cells were seeded in each well of a 96-well tissue culture plate and incubated in a CO_2 incubator for 12 h. A 10 μl stock solution of elemene and cisplatin was freshly prepared and added to wells of the plates at the desired concentrations: 100, 200, 300, 400, 500, and 600 μM elemene; 10 μM cisplatin; and 300 μM elemene + 10 μM cisplatin. After incubation with elemene and/or cisplatin for set periods of time (12, 24, and 48 h), survival of the cells in each plate well was determined by a 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide (Sigma) assay as previously reported [17]. Briefly, 10 μl of the 5 mg/ml stock solution of 3-[4,5-dimethylthiazol-2yl]-2,5-diphenyltetrazolium bromide was added to each well. After 4 h of incubation at 37°C, 100 μl of dimethyl sulfoxide was added to each well. After 15 min incubation at 37°C, the absorbances (A) at wavelengths of 570 and 630 nm were measured using a microplate reader (Bio-Rad, Model 550, CA, USA). The cell survival of each dose of elemene was calculated as the ratio of A in wells containing drug to that in control wells with no drug. IC_{50} values were calculated using a linear regression

from dose-dependent curves plotted from at least six points, and the percent growth inhibition (GI) was calculated according to the formula: $GI = (1\text{-}A_{drug}/A_{control})$.

Flow cytometric detection for Annexin-V

Apoptosis was measured using flow cytometry to quantify the levels of detectable phosphatidylserine on the outer membrane of apoptotic cells [18]. Briefly, HEp-2 cells were counted and plated at 5×10^4/ml in RPMI1640. Flasks were incubated with 300 μM elemene and/or 10 μM cisplatin. After 12–48 h, cells were harvested, suspended about 1×10^6/ml, and washed two times with ice-cold PBS. Cells were pelleted again and resuspended in 300 μl of dilute binding buffer from the Annexin-V FITC kit (Baosai Immunotech Co, Beijing, China). Then 5 μl dilute propidium iodide (PI) and 5 μl of dilute Annexin-V were added. The tubes were gently mixed and kept in the dark on ice for 10 min before analysis by flow cytometry (Coulter EPICs XL). To determine whether elemene was acting by inducing apoptosis, flow cytometric analysis was performed with Annexin-V FITC staining, and data were analyzed using the system software package. The PI-positive, Annexin-V FITC-negative cells were considered necrotic, and PI-negative, Annexin-V FITC-positive cells were considered early apoptotic, and PI-positive, Annexin-V FITC-positive cells were considered late apoptotic. Only the early apoptotic cells were considered to be of value in this study.

Colorimetric assays of caspase-3

The activity of caspase-3 was determined using a colorimetric analysis kit (BD ApoAlert™ Caspase-3 kit, Biosciences Clontech Co., USA), as previously described [19]. The assay was performed according to the manufacturer's protocol. Approximately 5×10^5/ml cells were collected, concentrated by centrifugation (250×*g*, 10 min, 4°C), and lysed in extraction buffer, 50 mM HEPES (pH 7.4), 0.1 mM EDTA, 0.1% CHAPS, and 1.0 mM dithiothreitol for 30 min with gentle agitation on ice. Cell extracts were cleared of debris by centrifugation (20,000×*g*, 5 min, 4°C). The test is based on the addition of a caspase-specific peptide conjugated to the color reporter molecule p-nitroanalide (pNA). Equal amounts of protein per treatment group were incubated with substrates DEVD-pNA or caspase inhibitor DEVD-fmk (as control) for 30 min at 37°C. The chromophore pNA was used to quantify the cleavage of the peptide by caspase-3 spectrophotometrically at 405 nm using a microplate reader (Bio-Rad, Model 550).

Western blot assay

The HEp-2 cells were grown in 10% fetal bovine serum-RPMI1640 at 5×10^4/ml. After incubation at 37°C to 80–90% confluence, we added 300 μM elemene or culture media (as a control) in each culture flask, and continued to incubate for another 12, 24, or 48 h. The tumor cells were washed twice with PBS and scraped into M-PERTM Mammalian Protein Extraction Reagent (Pierce, 78501) containing 1 mM phenylmethylsulfonyl fluoride, the details of which have been previously reported [20]. The same amounts of protein lysate from each specimen (50 μg protein) were analyzed on 6 and 15% SDS/PAGE. Primary incubation of the membranes was carried out using 1:400 dilutions of anti-p-eIF4E antibody (Santa Cruz sc-12885), anti-eIF4G antibody (Santa Cruz sc-9601), anti-bFGF antibody (Santa Cruz sc-79-G), anti-VEGF antibody (Santa Cruz sc-1878), and a 1:1,000 dilution of anti-actin antibody (Santa Cruz sc-1616) as an inner control protein. The membranes were visualized with an enhanced chemiluminescence system (Amersham Co, UK), according to the manufacturer's directions. The bands on the Western blot were scanned by VersaDoc Image Analysis System, and analyzed with the QualityOne Image Analysis software.

RT-PCR assay

Total RNA was isolated from elemene-administered HEp-2 cells by using a Superscript ™Preamplification System (GIBCOBRL 18089-011) for First Strand cDNA. The extraction of total RNA was carried out according to the manufacturer's instructions, and cDNA was generated from the total RNA as previously described [20]. The reversely transcribed product had a final volume of 20 μl, and the PCR reaction solution had a final volume of 50 μl. Amplification was performed using a DNA thermal cycler (Hybaid Omn-E) in three steps: Step 1, 1 cycle: 94°C 2 min, 55°C 1 min, 72C 2 min; Step 2, 35 cycles: 94°C 45 s, 50°C 40 s, 72°C 1 min; Step 3, 1 cycle: 72°C 10 min. Primer sequences were as follows: bFGF forward primer, 5′-GTG TGT GCT AAC CGT TAC CT-3′; bFGF reverse primer 3′-GCT CTT AGC AGA CAT TGG AAG-5′; VEGF forward primer, 5′-AAG GAG GAG GGC AGA ATC AT-3′; VEGF reverse primer, 3′-ATC CAA TTC CAA GAG GGA CC-5′; β-actin forward primer 5′-GTG GGG CGC CCC AGG CAC CA-3′; β-actin reverse primer 3′-CTC CTT AAT GTC ACG CAC GAT TTC-5′. Through the use of the VersaDoc Image Analysis System, the densitometric analysis compared the degree of band density of each PCR product using the QualityOne Analysis Software.

Transplantation of HEp-2 cells as solid tumors in nude mice and treatment of animals

Human cancer xenograft models were established using the methods reported previously [21]. When confluence reached 80%, cultured cells in monolayer were trypsinized and harvested by centrifugation. The HEp-2 cells

(5×10^6) were collected in 0.1 ml of HBSS, and these cell suspensions were injected subcutaneously into the right shoulder region of nude mice using a 27-gauge needle. The animals were monitored for changes in activity, physical condition, and body weight. Solid tumors were observed 30 days after injection of HEp-2 cells. When the largest diameters of the tumors reached 5 mm, the 42 nude mice were separated into seven groups: (1) normal saline (NaCl) negative control, (2) emulsion without elemene negative control, (3) 3 mg/kg cisplatin positive control, (4) 50 mg/kg elemene, (5) 100 mg/kg elemene, (6) 200 mg/kg elemene, and (7) 100 mg/kg elemene + 3 mg/kg cisplatin. The animals in these test groups received a total of eight intraperitoneal injections of drugs at 3-day intervals.

Tumor volume and weight

The volumes of the transplanted tumors were measured every 3 days by the same researcher over the entire experimental period. The largest and smallest diameters were measured using a vernier caliper, and the volumes of the tumors were estimated according to the formula: $V = 1/2\times a\times b^2$, as previously reported [22], where V is the tumor volume in mm^3, and a and b are the largest and smallest tumor diameters in millimeter, respectively. The mice were sacrificed by cervical dislocation on the 23rd day after the tumor-administration, and full necropsies were performed. Tumors were dissected and weighed individually before being fixed in 10% neutral buffered formalin and embedded in paraffin. The percent inhibition rate (IR) was calculated: IR = (1-Tumor weight $_{drug}$/Tumor weight $_{control}$). The mean tumor volumes and weights in the control and test groups were analyzed.

Immunohistochemical staining assay

Immunohistostaining was performed on 4 μm sections of formalin-fixed, paraffin-embedded tumor tissues. Immunohistostaining was conducted with 1:100 dilutions of anti-p-eIF4E antibody (Santa Cruz sc-12885), anti-eIF4G antibody (Santa Cruz sc-9601), anti-bFGF antibody (Santa Cruz sc-79-G), anti-VEGF antibody (Santa Cruz sc-1878), and anti-CD_{34} antibody (DAKO, M71651) at 37°C for 1 h. Then we applied a biotinylated secondary antibody and streptavidin-peroxidase complex and developed the tissues with 3,3′-diaminobenzidine tetrahydrochloride (DAB). The tissues were counter-stained with hematoxylin. Negative controls were processed in an identical manner as positive control samples, except they were incubated with rabbit serum rather than primary antibodies. A pathologist who was blinded to the drug administration evaluated the expression by light microscopy. The gradation of staining depended on the intensity of staining and the percentage of cells stained. Scores were ranked as no staining, weak staining, medium staining, and strong staining, and the values of 0, 100, 200, and 300 were, respectively, assigned to each intensity staining. The final scores were determined by multiplying the staining values by the percentage of positive stained cells, as previously described [23].

Microvessel density

Microvessel density (MVD) was recorded as the number of point counts of CD_{34}-positive vessels/field [24]. Briefly, ten fields/sections were randomly selected from non-necrotic areas of the tumors and examined with an Olympus microscope. The area with the highest number of vessels within the tumor was identified with 100 times magnification, and the individual vessels were counted in a 200 times field with the aid of a 36 square grid reticle. Single brown-staining endothelial cells or clusters of endothelial cells without a lumen were considered to represent a single countable microvessel. Vessel density counts were recorded independently in a blind manner by a pathologist.

Statistics

Statistical differences between mean values were calculated with student's t-tests. Differences were considered significant when $p < 0.05$.

Results

Inhibition of growth of HEp-2 cells in vitro

The addition of elemene to the cultures of HEp-2 cells showed dose- and time-dependent inhibition of cell growth. A morphological change was commonly observed in HEp-2 cells. Originally, cells appeared enlarged with prominent nuclei. Elemene caused individual cells to shrink and separate from neighboring cells. They appeared refringent, and finally detached from the monolayer by 24 h after elemene administration (Fig. 1a, b). The IC_{50} for elemene according to the dose-survival curve was calculated by a linear regression. The HEp-2 cells 12-, 24-, and 48-h IC_{50}'s were 461.3, 346.5, and 189.8 μM, respectively. The 24 h IC_{50} for WI-38 cells was 442.5 μM, and for Hela cells it was 345.3 μM. The GI (24 h) of 300 μM elemene was 42.2% and did not significantly differ from that of cisplatin 10 μM (41.2%; $p > 0.05$). The GI (24 h) of the drug combination (300 μM elemene + 10 μM cisplatin) was 64.5%, which was significantly higher than when either drug was administered individually ($p < 0.05$).

Annexin-V assay of apoptosis

Different fluorescent staining was able to distinguish necrotic from apoptotic cells (Fig. 1c). Flow cytometric

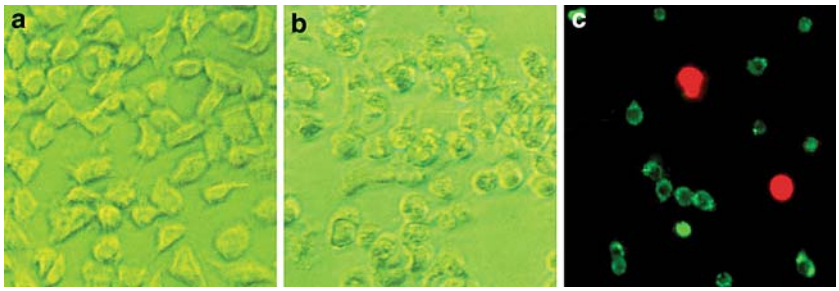


Fig. 1 The HEp-2 cells either incubated without **a** or with **b** elemene for 24 h at 300 μM in serum containing medium (original magnification 200 times). **c** fluorescence staining of HEp-2 cells showing Annexin-V FITC (*green*) membrane staining of apoptotic cells, and PI (*red*) nuclear counterstain of necrosis cells (original magnification 200 times)

analysis (Fig. 2a) revealed the constituent ratio of necrotic, early apoptotic, and late apoptotic cells (Fig. 2b). Elemene induced apoptosis in a time-dependent manner; the percentage of early apoptotic cells induced by elemene was significantly higher at 24 h than 12 h, and higher still at 48 h than 24 h ($p < 0.05$). There was no significant difference at any time interval between the number of apoptotic cells induced by elemene and cisplatin ($p > 0.05$). However, at every time interval, the combination of elemene and cisplatin induced significantly more apoptotic cells than either drug did individually ($p < 0.01$).

Elemene-induced caspase-3 activation in HEp-2 cells

In HEp-2 cells, elemene induced a significant elevation of caspase-3 activity to 2.91 arbitrary units above the baseline within 6 h after administration. The value of caspase-3 activity continued to increase to 5.34 after 12 h, then decreased to 3.46 after 24 h, and 0.09 after 48 h. These experiments demonstrated that elemene can stimulate the activation of caspase-3 in the early phases of apoptosis in HEp-2 cells.

Elemene-induced decrease of eIF4E, eIF4G, bFGF and VEGF proteins

Semi-quantitative Western blot analysis showed that the administration of 300 μM elemene to HEp-2 cells caused a marked decrease of eIF4E, eIF4G, bFGF, and VEGF protein at 12 h. Additional significant declines were seen at 24 and 48 h ($p < 0.05$) (Fig. 3a).

Elemene-induced decrease of bFGF and VEGF mRNA

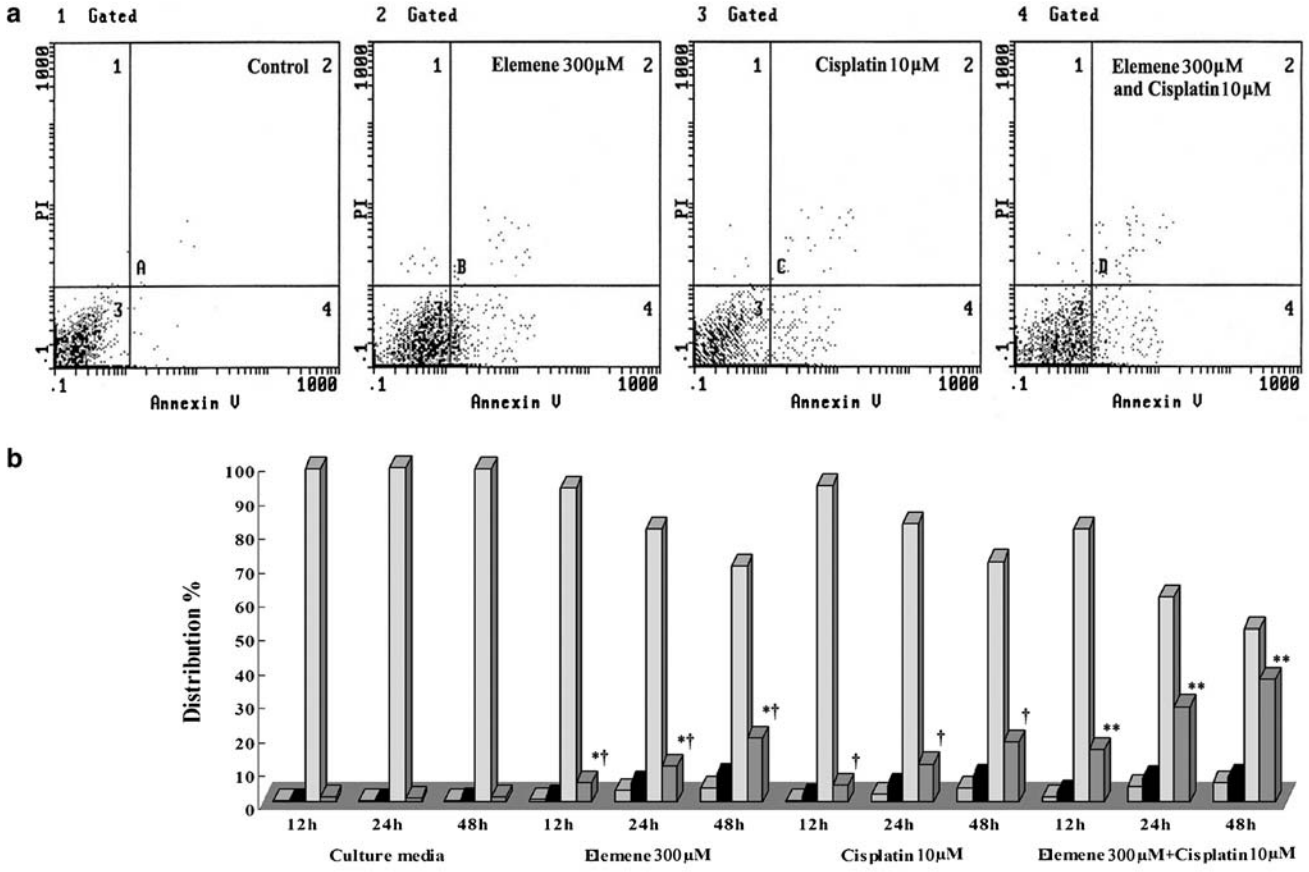
Semi-quantitative RT-PCR analysis showed a decrease of bFGF and VEGF mRNA band intensity within 12 h after the addition of the 300 μM elemene, and this decrease persisted when measured at 24 and 48 h (Fig. 3b); however, there were no significant differences in levels of bFGF and VEGF mRNA at any of the 300 μM elemene time intervals ($p > 0.05$).

Effects of elemene on the growth of HEp-2 cells transplanted tumors in vivo

The mice weight of different groups after elemene and/or cisplatin administration was a little decreased, but there were no significant differences ($p > 0.05$) (data not shown). The elemene-induced reductions of tumor volumes were progressive during the period of administration. As shown in Fig. 4a, tumor volumes were affected by intraperitoneal drug-administration. On the 23rd day following the initial injection, a significant suppression of tumor growth was observed. The tumor weights of all drug-administered groups were significantly lower than the control groups. The IRs of the seven groups are shown in Fig. 4b. The IR of 100 mg/kg elemene (41.7%) was not significantly different from that of 3 mg/kg cisplatin (44.6%; $p > 0.05$). However, the IR of the drug combination (100 mg/kg elemene + 3 mg/kg cisplatin) of 51.2% was significantly higher than either drug administrated separately ($p < 0.05$).

The expression of eIF4E, eIF4G, bFGF and VEFG in HEp-2 cells transplanted tumors in response to elemene administration

Paraffin-embedded samples were assessed for eIF4E, eIF4G, bFGF, and VEGF expression by immunohistochemistry. The majority of the immunoreactivity of the transplanted tumors was restricted to the cytoplasm of the tumor cells. The eIF4E, eIF4G, bFGF, and VEGF positive cells were clearly observed in the control groups (NaCl and emulsion without elemene). In contrast, there were significantly fewer positively staining cells in the 100 mg/kg elemene group and 3 mg/kg cisplatin group ($p < 0.01$), though these groups did not significantly differ from each other ($p > 0.05$). The scores of eIF4E, eIF4G, bFGF, and VEGF are shown in Fig. 5.



	Culture media			Elemene 300 μM			Cisplatin 10 μM			Elemene 300 μM + Cisplatin 10 μM		
	12h	24h	48h	12h	24h	48h	12h	24h	48h	12h	24h	48h
Necrosis cells	0.18	0.26	0.24	0.76	3.12	4.04	0.30	2.24	4.06	1.52	4.54	5.56
Advanced stage apoptosis cells	0.22	0.20	0.40	1.16	5.68	7.66	1.60	4.84	7.54	2.84	6.90	7.32
Normal tumor cells	98.12	98.50	98.10	92.70	80.60	69.40	93.20	81.90	70.80	80.40	60.50	51.00
Early stage apoptosis cells	1.48	1.04	1.26	5.38*†	10.60*†	18.90*†	4.90†	11.02†	17.60†	15.24**	28.06**	36.12**

*** p< 0.01 vs. negative control (with culture media); † p>0.05 elemene vs. cisplatin; ** p< 0.01 using combination vs. using separately**

Fig. 2 a Flow cytometry analysis of HEp-2 cells ($n = 5{,}000$) after elemene and/or cisplatin incubated 24 h (the figures of 12 and 48 h are not shown). 1 Gated: administration with culture media as negative control; 2 Gated: administration with elemene 300 μM; 3 Gated: administration with cisplatin 10 μM as positive control; 4 Gated: administration with elemene 300 μM + cisplatin 10 μM. The *first quadrant* represents necrotic cells; the *second quadrant* represents advanced stage apoptosis cells; the *third quadrant* represents normal tumor cells; the *fourth quadrant* represents early stage apoptosis cells. **b** The results of flow cytometry of HEp-2 cells after elemene administration 12, 24 and 48 h. The effects of apoptosis with elemene in HEp-2 cells were time-dependent and more available versus negative control (*$p < 0.01$); the effect between elemene and cisplatin was no significant difference (†$p > 0.05$); using drug combination inhibited HEp-2 cells more effectively than using separately (**$p < 0.01$)

Elemene-induced decrease in intratumoral MVD

To evaluate the effects of elemene on tumor-associated angiogenesis, tumor sections were immunostained with anti-CD_{34} antibodies to delineate the presence of endothelial cells. The tumors from mice administered with control NaCl and emulsion without elemene exhibited numerous endothelial cells throughout the tumor mass (Fig. 5). In contrast, tumors from mice administered with 100 mg/kg elemene and 3 mg/kg cisplatin exhibited a marked decrease of CD_{34} immunoreactivity. This was quantified by significantly lower MVD in the groups where the tumors were administrated 100 mg/kg elemene or 3 mg/kg cisplatin than in the control groups (Fig. 5). MVD was decreased by 59.1% in tumors treated with 100 mg/kg elemene, and was decreased by 54.5% in tumors treated with 3 mg/kg cisplatin.

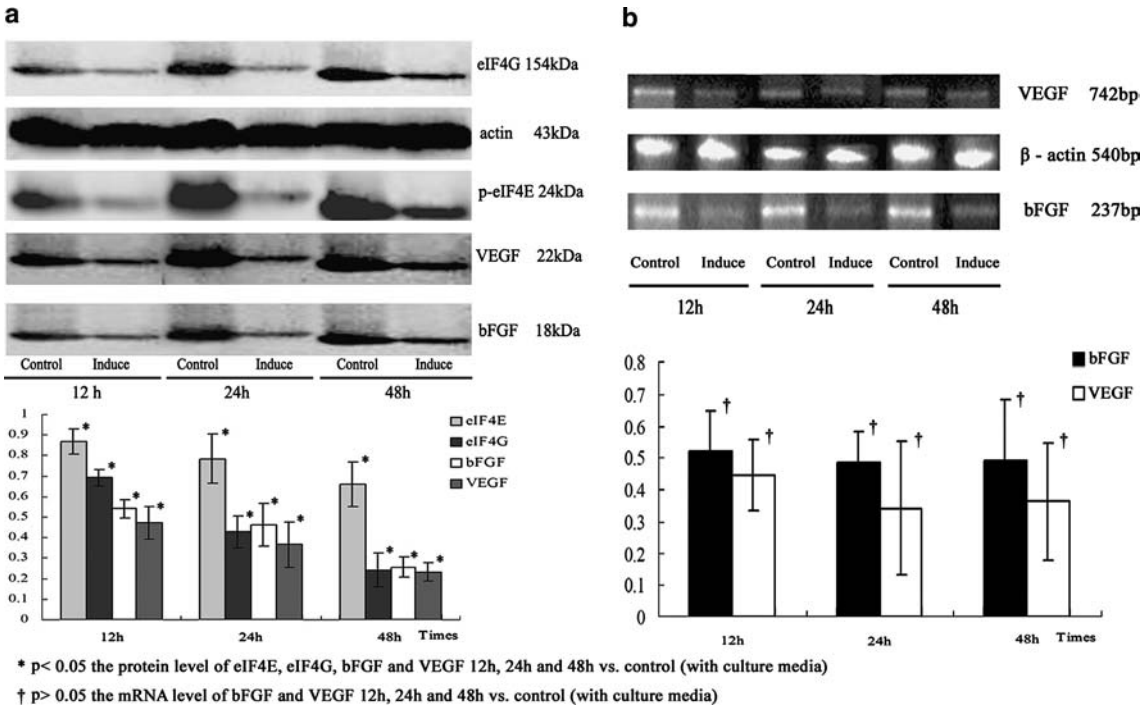


Fig. 3 **a** Effect of elemene on protein expression of eIFs (4E, 4G), angiogenic factors (bFGF, VEGF), and actin (as inner control) in HEp-2 cells. HEp-2 cells were cultured without (Control) or with elemene 300 μM for 12, 24, and 48 h (Induce). Cell lysates were subjected to Westernblot analysis using antibodies against eIF4E, eIF4G, bFGF, and VEGF, respectively. The protein level decreased and persisted for 12 to 48 h compared with control group, and the statistical significance existed between the three groups ($*p < 0.05$). **b** The effect of elemene (12, 24, and 48 h) on mRNA expression of angiogenic factors (bFGF, VEGF) in HEp-2 cells. HEp-2 cells cultured in complete medium were administered with culture media alone (Control) or elemene 300 μM for 12, 24 and 48 h (Induce). Total RNA was extracted and analyzed by RT-PCR. bFGF containing 237 bp, VEGF containing 742 bp and β-actin contains 540 bp (as control). The induction decreased and persisted for 12 to 48 h, but not found significant difference ($†p > 0.05$)

Discussion

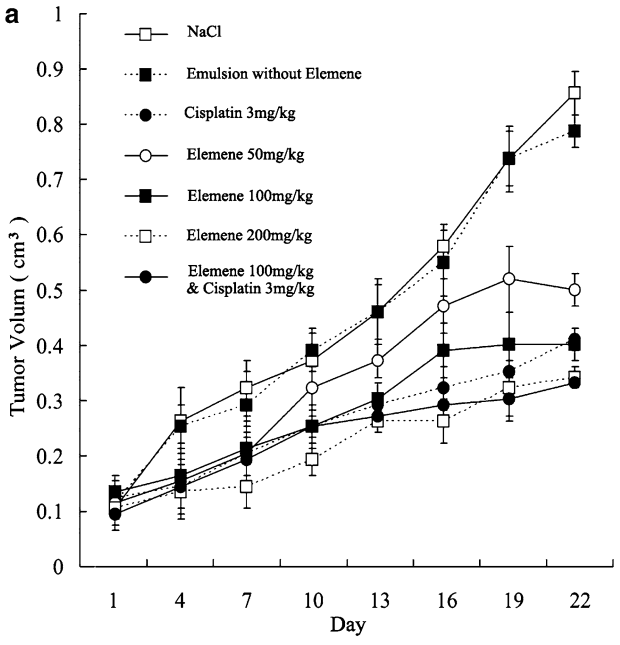
Current treatment of advanced LSCC includes surgery, radiation, or a combination of chemotherapy with radiation. With no significant improvements in recent years, LSCC remains difficult to treat, and patients are left with a high risk of recurrence [25]. Recent advances in understanding the biology of LSCC may offer new therapeutic approaches [26]. There is an increasing interest in anti-cancer treatment strategies that combine standard chemotherapy with novel agents that specifically target pathological signaling pathways of cancer cells [27].

New therapeutic approaches have attempted to use chemotherapeutic agents from Chinese medicinal herbs, such as Paclitaxel and Arsenic Trioxide, in the treatment of malignancies including non-small cell lung cancer and leukemia [28, 29]. Elemene is a terpene compound isolated from Rhizoma Zedoariae, a Chinese medicinal herb, and has been demonstrated to inhibit the growth of malignancies, including breast cancer and glioma cell lines [30, 31]. However, few studies have investigated the treatment of LSCC with elemene.

In this study, we observed the anti-proliferative activity of elemene on HEp-2 cells. Elemene was significantly more potent than controls, and inhibited tumor cell growth in a dose- and time-dependent manner. The 24 h elemene IC_{50} for HEp-2 cells was lower than that for WI-38 cells, which shows that elemene targets cancer cells. The 24 h GI of 300 μM elemene was not significantly different from that of 10 μM cisplatin, a widely used drug for the treatment of human malignancies, which suggests that elemene is comparably effective at fighting carcinoma as at least one current pharmaceutical option. The combination of 300 μM elemene and 10 μM cisplatin further inhibited the growth of HEp-2 cells. Therefore, not only is there a direct effect of elemene on the growth of HEp-2 cells, additional anti-proliferative activity is possible by combining elemene with cisplatin.

Parallel effects were seen in vivo on tumors from HEp-2 transplanted cells. In the nude mice model, elemene successfully inhibited the growth of tumors, as shown by the significant IR. Moreover, the IR for the drug combination group was higher than that of either elemene or cisplatin alone. The usefulness of much clinical chemotherapy against advanced solid tumors has been limited by host toxicity and tumor resistance [32]. Because elemene's anti-cancer effects are enhanced when used with another drug, combination therapy may help overcome this challenge.

a



b

Group	Tumor weight (g, $\bar{x} \pm s$)	IR (%)
NaCl	0.5560±0.0950	—
Emulsion without Elemene	0.5993±0.2102	—
Cisplatin 3mg/kg	0.3083±0.0684	44.6*†§
Elemene 50mg/kg	0.5272±0.1446	5.2 *
Elemene 100mg/kg	0.3242±0.0978	41.7*†§
Elemene 200mg/kg	0.2750±0.0742	50.5*
Elemene 100mg/kg & Cisplatin 3mg/kg	0.2715±0.0408	51.2*§

*** p< 0.05 IR of drug-administered group vs. control group**

† p> 0.05 elemene 100mg/kg group vs. cisplatin 3mg/kg group

§ p< 0.05 drug-administered combination vs. drug-administered individually

Fig. 4a Effect of elemene on HEp-2 cells transplanted tumors in nude mice. Tumor volumes were measured every 3 days as described in "Materials and methods." The tumor-bearing nude mice received peritoneal injections of elemene and/or cisplatin (elemene 50 mg/kg, 100 mg/kg, 200 mk/kg, cisplatin 3 mg/kg and elemene 100 mg/kg + cisplatin 3 mg/kg), and injection NaCl as well as emulsion without elemene for control. Points are mean ± SD of tumor volumes in seven groups ($n = 6$). **b** In the 23rd day, the IR of all the drug-administered groups showed significant inhibition of tumor growth compared with the control group (*$p < 0.05$). There was no significant difference between IR of elemene 100 mg/kg group and cisplatin 3 mg/kg group († $p > 0.05$). The group receiving elemene 100 mg/kg + cisplatin 3 mg/kg showed the highest inhibition. The IR of drug-administered combination was much higher when compared with the drug-administered individually (§ $p < 0.05$)

Many of elemene's effects relate to apoptosis, an important and well-controlled form of self-regulated cell death [33]. This process is of major importance for embryonic development and tissue homeostasis, and plays a significant role in neurodegeneration, autoimmune disease, carcinogenesis, cancer progression, and the killing of cancer cells by chemotherapeutic drugs [34]. In our study, induction of apoptosis by elemene in a time-dependent manner was confirmed by Annexin-V labeled flow cytometry analysis. The Annexin-V FITC binding assay was used to distinguish apoptotic cells from necrotic cells. The number of apoptotic HEp-2 cells significantly increased after the administration of both elemene and cisplatin, compared with elemene or cisplatin monotherapy and the controls.

To date, two major apoptotic pathways (the extrinsic apoptotic pathway and the intrinsic apoptotic pathway) have been identified [18, 35]. Despite the diversity of mechanisms inducing these two pathways, activation of either caspase-8 or caspase-9 causes activation of downstream effector caspases, including caspase-3, -6, and -7, ultimately leading to apoptosis. Caspase-3 is believed to play a pivotal role in apoptotic execution [12]. In our study, we found elemene stimulated the activation of caspase-3 and induced a significant increase in the value of caspase-3 activity in the early phase of active cell apoptosis in HEp-2 cells. In addition, a growing number of substances cleaved by caspase-3 have been identified, such as PARP [36], sterol-regulating element-binding protein [37], DNA fragmentation factor [38], DNA-dependent protein kinase [39], and eIFs [13]. Therefore, caspase-3 may have a potential apoptotic induction not only by forming the protease cascades, but also by cleaving the substrates in the cells.

Cell fate specification relies on many factors and levels of regulation. Translational regulation is also an important determinant of cell survival, proliferation, and maturation [40]. The critical step during translation initiation is when the m^7GpppN cap (where *m* is a methyl group and *N* is any nucleotide) interacts with a protein complex termed the eukaryotic initiation factor 4F (eIF4F), which consists of three subunits: eIF4E, eIF4G, and eIF4A [41]. The eIF4E and eIF4G have proven to be the main targets for translational control [42, 43]. In general, their inactivation is triggered by stress, and their activation is triggered by growth proliferation signals.

During apoptosis, protein synthesis is inhibited, and these translation factors are cleaved in a caspase-dependent manner. In some cases, cleavage is known to impair or otherwise alter the functions of these factors. This not only leads to the increased binding of 4E-BPs to eIF4E, and thus fewer eIF4F complexes [44], but eIF4G is also a target for caspase-3 mediated degradation during apoptosis [45]. These cleaved fragments of eIF4E and eIF4G cannot mediate cap-dependent translation. In this study, after the administration of 300 μM elemene to HEp-2 cells, the expression of eIF4E and eIF4G was significantly inhibited in a time-dependent manner. The inhibition of eIF4E and eIF4G expression was further observed by immunostaining tumors of HEp-2 transplanted cells. The observed reduction of eIF4E and eIF4G protein levels was likely due to cleavage by caspase-3, which may be one of the facilitating factors during elemene-induced apoptotic cell death.

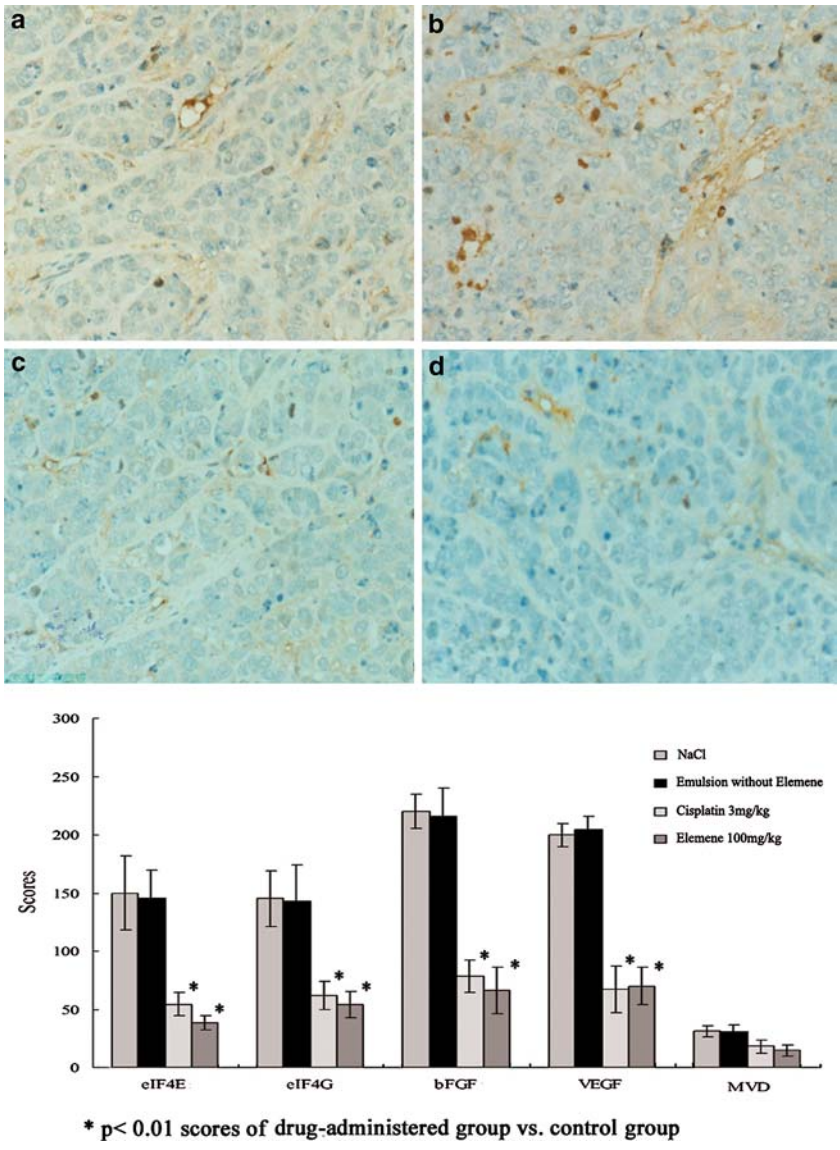


Fig. 5 Antiangiogenic effect of elemene in HEp-2 cells transplanted tumors in nude mice. High MVD in tumor without elemene administration as control (**a**: NaCl; **b**: emulsion without elemene), compared with HEp-2 cells solid tumor after cisplatin and elemene administration (**c**: cisplatin 3 mg/kg; **d**: elemene 100 mg/kg) demonstrated by staining with anti-CD_{34} (original magnification 200 times). The mean MVD for each administration group was determined by counting the number of CD_{34} positive vessels in each section in a blind manner. A 59.1% of decrease in MVD was observed after elemene 100 mg/kg administration, a 54.5% of decrease in MVD was observed after cisplatin 3 mg/kg administration. The morphological results of immunohistochemical analyses by antibodies against eIF4E, eIF4G, bFGF, and VEGF were not shown. The scores of eIF4E, eIF4G, bFGF, and VEGF were significantly decreased in the groups receiving elemene 100 mg/kg and cisplatin 3 mg/kg ($^{*}p < 0.01$)

Recruitment of the translational machinery to the 5′ end of mRNA by eIF4E and eIF4G is essential for the translation of most mRNAs including the angiogenic factors bFGF and VEGF [16]. The VEGF and bFGF are potent mitogens for endothelial cells and play an important role in angiogenesis [46], which is believed to be essential for growth and metastasis of solid malignancies. Studies have shown that high vessel density in tumor tissue is associated with poor prognosis in many solid tumors, including human small cell lung cancer [47], and there is a positive correlation between tumor bFGF and VEGF levels, blood vessel density, and disease progression in LSCC [48].

To test the possibility that elemene inhibits the growth of HEp-2 cells by suppressing expression of angiogenic factors, we studied the effect of elemene on the expression of bFGF and VEGF. In accordance with our hypothesis, we found that the expression of eIF4E, eIF4G, and angiogenic factors (bFGF and VEGF) was effectively retarded in vitro and in vivo, but there was no significant difference in mRNA level of bFGF and VEGF in vitro. This shows that inhibition at gene or mRNA level is not enough, and the mechanism of down-regulation is post-transcriptional, which is consistent with earlier research [20]. We then examined the relationship between the actual tissue levels of angiogenic factors and the MVD in solid tumors. This information is important for understanding the effect of angiogenic factors in vivo in LSCC. In our study, intratumoral MVD significantly decreased in response to elemene treatment. The same effect was shown previously in a colon cancer model [49].

It is likely that the anti-cancer effects of elemene are associated with the inhibition of bFGF and VEGF-mediated angiogenesis. Furthermore, it seems that angiogenic factors down-regulation is closely correlated with alterations in the expression of eIFs (4E and 4G). Our findings reveal a mechanism of tumor cell

survival, and highlight the ability of a specific integrin to regulate protein translation by influencing the activity of eIFs (4E and 4G). However, possible roles of eIFs (4E and 4G) in regulatory processes, and the mechanism of angiogenic factors deserve further investigation.

In conclusion, this study showed that elemene could inhibit the growth of HEp-2 cells in vitro and in vivo, thus demonstrating the anti-cancer and anti-angiogenic effects of elemene in human laryngeal cancer. The induction of apoptosis of these cells by elemene is a possible mechanism for this inhibition. It is likely that at least some of the anti-cancer effects of elemene and the sensitization of Hep-2 cells to elemene can be attributed to an up-regulation of caspase-3, which inhibits eIFs (4E and 4G) and down-regulates angiogenic factors (bFGF and VEGF). We believe that the activity exhibited by elemene could merit clinical investigation as a new agent in the treatment of LSCC. This study could provide support for a clinical trial of elemene in combination with cisplatin for the treatment of recurrent or advanced LSCC.

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