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Original Article

Hesperidin Inhibits Angiogenesis, Induces Apoptosis, and Suppresses Laryngeal Cancer Cell Metastasis

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Abstract

Introduction: Laryngeal carcinoma is the most common malignant head-and-neck tumor. Due to the low survival rate and the inadequate response to chemotherapy, effective therapy remains a challenge. **Objectives:** Therefore, the identification of new therapeutic options that preserve the larynx is needed. Hesperidin (Hsp) is a nontoxic plant flavanone that has proven effective against cancer. **Materials and Methods:** Hence, the current *in vivo* and *in vitro* study was conducted to determine whether Hsp might suppress metastasis of cancer larynx. **Results:** In an *in vivo* mouse metastasis model, Hsp suppressed metastasis of human Hep2 laryngeal cancer cells to the livers and lungs. *In vitro* assays, Hsp significantly inhibited angiopoietin 1 secretion (an angiogenic promotor) and increased annexin-V (an apoptotic indicator) in Hep2 cell culture at relatively low levels (10 μM). **Conclusions:** These studies suggest that Hsp deserves further investigation as a possible treatment option for laryngeal cancer.

Keywords: Angiogenesis, angiopoietin 1, Annexin V, apoptosis, hesperidin, laryngeal cancer

Introduction

In recent years, management of cancer larynx has received high attention, primarily due to low survival in the late stages and a new trend to change its therapy.[1] The aim of management should be laryngeal preservation and preservation of its function as well.[2] Hence, we need new conservative treatment modalities. The current study aimed at identifying natural compounds that might be used to inhibit the spread of laryngeal cancer cells. Hesperidin, a nontoxic flavanone present in citrus fruits, suppresses cell proliferation in many cancer types.[3]

Angiopoietin-1 (Ang1), the principal member of angiopoietins, is an angiogenic factor that promotes vessel remodeling and maturation. It binds to its endothelial tyrosine kinase receptor Tie2/Tek and triggers tyrosine phosphorylation. *In vivo*, Ang 1 promotes the interaction of the endothelial cells to the surrounding cells to promote the integrity and maintenance of the new blood vessels formed. *In vitro*, Ang1 is chemotactic and anti-apoptotic for endothelial cells and induces angiogenesis.[4] A few human tumors express Ang1 such as non-small cell lung carcinomas,[5] glioblastoma,[6] and some human tumor cell lines as cervical carcinoma cell HeLa.[7] However, in hepatocellular carcinoma[8] and thyroid cancer,[9] Ang1 was expressed at a similar level compared with normal tissues. It is not clear whether modulation of Ang1 expression would affect tumor growth and whether its inhibition will affect the spread of the tumor.

During apoptosis, it is translocated to the cell surface. Annexin V has a strong Ca^{++}-dependent affinity for phosphatidylserine, and so it can be used as a marker for detecting apoptosis.[10] Since few chemotherapeutic agents are effective in inhibiting metastatic laryngeal cancer, Hesperidin (Hsp) was tested for its ability to inhibit angiogenesis (by inhibiting Ang1 expression), disrupt the growth, induce apoptosis (detected by Annexin V) of laryngeal cancer cells, as well as to inhibit their spread in a mouse metastasis model.

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Materials and Methods

Cell lines and culture

The 85020207 Hep2 cell lines were supplied by Sigma. Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, USA) and 10% fetal bovine serum (FBS; Sigma-Aldrich Co.) were used. Split sub-confluent cultures (70%–80%) using 0.25% trypsin/ethylenediaminetetraacetic acid (Thermo Fisher Scientific) 5% CO_2; 37°C. Cells were allowed to reach 50%–60% confluence, washed in phosphate-buffered saline (PBS), and maintained in DMEM supplemented with 10% PBS for 24 h before treatment. Incubations were carried out in the presence of Hsp at 10, 20, or 40 mg/kg concentrations. Control represents cells treated with vehicle alone.

Viable cells were quantitated using sulforhodamine B (a cell protein dye binding) assay.[11] In brief, 1–1.5 × 10^4 cells in 100 μL 5% DMEM were incubated in 96-well plate in 5% CO^2 at 37°C overnight and then treated for 24 h with Hsp (30, 5, 9-dihydroxy-40-methoxy-7-orutinosyl flavanone) (Cat. H5254 Sigma-Aldrich Co., St. Louis, USA). Following this, it was dissolved in sterile-filtered dimethyl sulfoxide (DMSO; Cat. D2650; Sigma-Aldrich Co., St. Louis, USA). Solutions of Hsp were prepared weekly and stored at −20°C. Final DMSO concentration was 0.1% in Hsp and vehicle-treated cells. After that mRNA was extracted (Cincinnati, OH, USA). Total mRNA was reverse transcripted to cDNA and amplified (Invitrogen One-Step RT-PCR System, Cat. 12574-026). The primer sequences for Ang1 are as follows: 5′-CAACTGGAGC TGATGGACAC A-3′ (sense) and 5′-ACTGCCTCTG ACTGGTAATG G-3′ (antisense), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) are 5′-AAGGTGAAGG TCGGAGTCAA CG-3′(sense) and 5′-TGGTGGTGCA GGAGGCATTG C–3′ (antisense), polymerase chain reaction (PCR) products were run on 1.5% agarose gel for 1.5 h at 100 V and quantified. Relative Ang1 band intensity was measured by normalization to GAPDH. Each experiment was repeated at least twice.

Apoptosis assay

Cells were grown to 50%–60% confluence and treated with different concentrations of Hsp in 5% FBS/DMEM for 24 h. Hsp-induced apoptosis was detected by Annexin V ELIZA kit (Mybiosource, Cat No: MBS940036) according to the manufacturer's protocol.

Induced laryngeal cancer in the animal model

Female mice (18–22 g, 6 weeks old) were housed in an animal house. The tested groups were given intraperitoneal injections of cyclophosphamide (Sigma, St. Louis) 100 mg/kg 5 days and 24 h before the start of the experiment to suppress immunity.[12] Laryngeal cancer cells (harvested by trypsinization and washed twice with DMEM medium) were injected intravenously into the tail vein of two groups of animals each consisted of 21 mice. One of them received intraperitoneal injections of Hsp on the 5th-day postinoculation and every other day (they received an Hsp-loading dose for a week). They were further subdivided into three groups (each consisting of seven animals and received 10, 20, and 40 mg/kg Hsp, respectively). A similar group of control animals was injected with the vehicle only. Venous blood samples were taken, Ang-1 and annexin V were measured by an ELISA kit (Lifespan Bioscience, Cat No LS-F2956-1, Mybiosource, Cat no: MBS940036). On day 45, the mice were sacrificed, and the livers and lungs were examined for metastasis.

Statistical analysis

Data were presented as mean ± standard deviation (SD) of the mean (SD). One-way analysis of variance was used to determine the difference in mean between groups. $P < 0.05$ was considered statistically significant.

Results

Anti-angiogenic and antimetastatic activities

We used a xenograft model to determine the value of Hsp as an anti-angiogenic and anti-metastatic agents. Mice were inoculated with 85020207 Hep2 cells. A dose of 40 mg/kg Hsp significantly reduced the number of liver and lung metastatic foci from 13.9 ± 0.3 in vehicle-treated control animals compared with 3.2 ± 0.6 in the treated group. The smallest dose of 10 mg/kg Hsp reduced the mean number of the malignant foci to 9.3 ± 0.7 (not significant) [Figure 1]. In addition, no significant difference in animal weights was evident among the tested groups. The serum levels of Ang1 were significantly lower and that of annexin V were significantly higher in the studied groups in a dose-dependent manner at variance compared with the control group [Figure 2].

Hesperidin induces apoptosis in Hep2 cells

Hep2 cells were treated with 10, 20, or 40 μM Hsp or DMSO for 48 h, after which annexin V levels (using ELISA kit) were measured in the supernatant. A concentration of Hsp as low as 10 μM significantly induced apoptosis and that was dose dependent [Figure 3]. This study shows that Hsp induces apoptosis in Hep2 cells even at low concentration.

Hesperidin reduces angiopoietin 1 expression in laryngeal cancer cells

Ang-1 increases the invasiveness of cancer cells.[4] Therefore, Hep2 cells were treated with 10, 20, or 40 μM Hsp or DMSO for 16 h, after which total mRNA was collected, and Ang1 mRNA levels were measured by real-time-PCR [Figure 4a and b]. 10 and 20 μM Hsp significantly reduced Ang1 expression. Ang1 expression was inhibited when cells were exposed to 40 μM Hsp [Figure 4a].

Discussion

Head-and-neck cancer is the 6th most common cancer in the world, 20% originate in the larynx, and the current treatment modalities did not accomplish disease control or improve survival that remained poor, resistant tumors often spread despite the use of chemotherapy,[13] hence the urgent need for

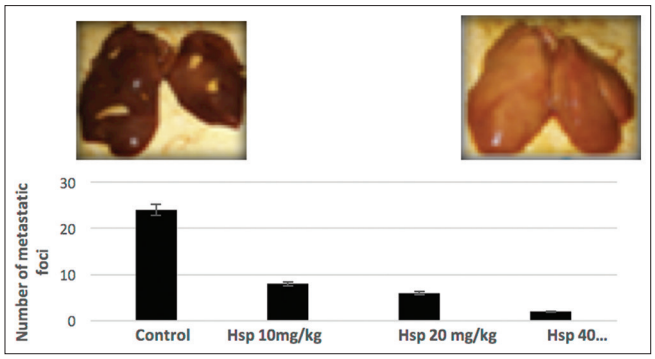

Figure 1: Hesperidin suppresses metastasis of laryngeal cancer cells (mean number of metastatic foci ± standard deviation, all are significantly different compared with control group [$P < 0.05$])

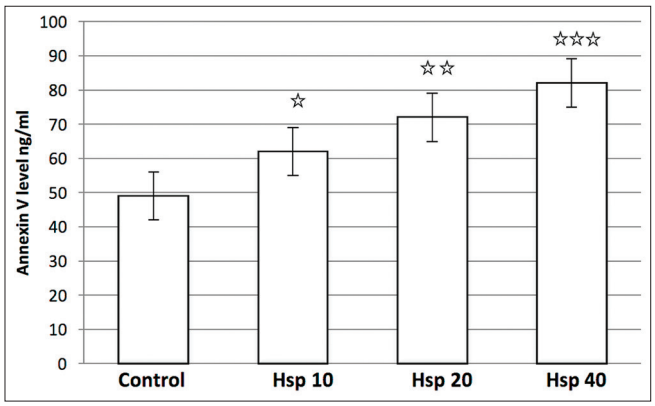

Figure 3: Hesperidin induces apoptosis Hep2 cells *in vitro*. Bars represent mean ± standard deviation ($n = 3$). *Significantly different compared with controls. **Significantly different compared with controls and 10 μM hesperidin, ***Significantly different compared with controls, 10 μM hesperidin and 20 μM hesperidin ($P = 0.001$)

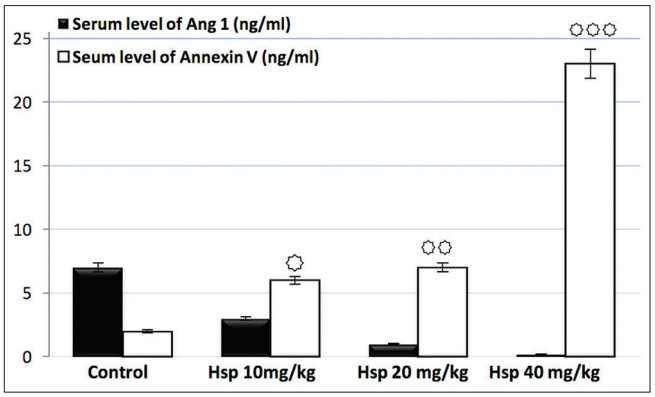

Figure 2: The mean ± standard deviation of the studied parameters in different groups. °Significantly different compared with controls, °°Significantly different compared with controls and 10 μM hesperidin, °°°Significantly different compared with controls, 10 μM hesperidin and 20 μM hesperidin ($P = 0.001$)

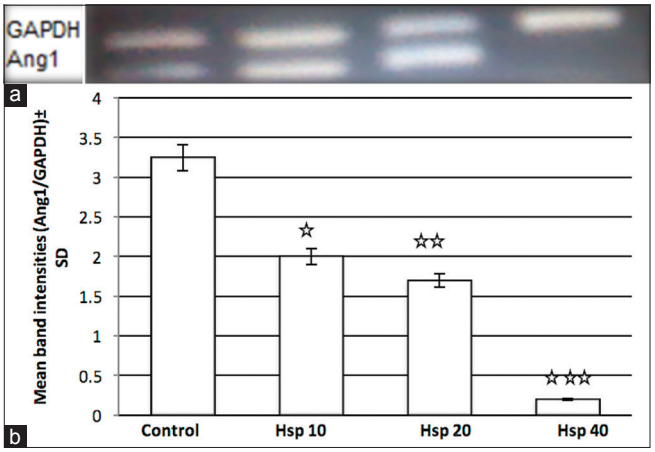

Figure 4: In the upper panel (a), cells were treated for 16 h with 10, 20, or 40 μM hesperidin or vehicle control, after which total mRNA was isolated and real-time-polymerase chain reaction for glyceraldehyde-3-phosphate dehydrogenase Ang1 was performed. In the lower panel, polymerase chain reaction-amplified Ang1 bands together with glyceraldehyde-3-phosphate dehydrogenase bands for normalization. In the lower panel (b) bar graph represents mean band intensities (Ang1/glyceraldehyde-3-phosphate dehydrogenase) ± standard deviation ($n = 3$). *Significantly different compared with controls, **Significantly different compared with controls and 10 μM hesperidin, ***Significantly different compared with controls, 10 μM hesperidin and 20 μM hesperidin ($P = 0.001$)

safer and more effective therapy for laryngeal cancer. Hsp is a nontoxic natural plant flavonoid that has a significant advantage over traditionally used aggressive drugs.[14]

The current study proved the effectiveness of Hsp to inhibit angiogenesis and metastasis of laryngeal cancer cells. Angiogenesis is an essential step for the spread and growth of tumor cells since new blood vessels facilitate the nutrition of the tumor and the spread of cancer cells to other sites.[15]

In this study, a xenograft model of lung and liver metastasis was used to test the ability of Hsp to decrease the metastatic ability of laryngeal cancer using Hep2 cell line, In the current study, Hsp significantly decreased and inhibited metastasis in a dose-dependent manner of Hep2 laryngeal cancer cells with no detected toxicity. To the best of our knowledge, it has never been reported that Hsp decreases the ability of laryngeal cancer cells to metastasize to the liver and lung.

The current findings proved that low concentrations of Hsp significantly reduced metastasis and higher levels almost abolished metastasis perhaps via suppression of Ang1 expression and secretion in tumor cells. Locally produced Ang1 is known to act via its receptors to promote tumor cell survival and migration. Ang1 serves a role in physiological neovascularization and an angiogenic role in tumor metastasis. Ang1 interacts with the Tie2 receptor expressed on endothelial cells. It plays an essential role in cell adhesion and endothelial cell sprouting from preexisting vessels and hence angiogenesis.[16] Ang1 regulates maturation of blood vessels in colon and prostate cancer cell line. In a xenograft tumor model, Ang1 enhanced angiogenesis and prostate tumor growth[16] but suppressed colon cancer growth.[17] No previous study evaluated its role in a xenograft laryngeal cancer animal model.

The current results showed that low concentrations of Hsp (10 and 20 μM) induced apoptosis of laryngeal cancer

cells efficiently in a dose-dependent manner. A higher Hsp concentration markedly increased apoptosis. Previous studies showed that Hsp at a high concentration (160 μM) induced apoptotic signaling including upregulation of Bax, cleavages of caspase-3 and down-regulation of Bcl-xl in mesothelioma cells.[18] Furthermore, Hsp inhibits glycogen synthase kinase-3 beta signaling cascades and induces apoptosis in experimental colon carcinogenesis,[19] but there are no previous reports about its effect on laryngeal cancer cells. The anticancer properties of hesperidin result from the C4'=C8' double bonds of the A and B rings, C5=C6, respectively, the 4-carbonyl group of the C ring and 3''–o-hydroxy, 4''-o-methoxy system in the B ring. Hsp serves as a hydrogen donor for α-tocopherol radical, thus regenerating α-tocopherol, scavenges superoxide radical (LOO), singlet oxygen ($1O_2$), hydroxyl radical (OH), nitrogen oxide (NO), superoxide anion radical (O_2-) and hesperidin radical is formed.[20,21] It is also proposed that the higher antioxidant activity is related to the greater number of hydroxyl groups on its flavonoid nucleus.[22]

Hsp induces relative oxygen species (ROS) generation that contributes to mitochondrial damage and cell death by acting as apoptotic signals[23,24] as reported in the current study. Induction of apoptosis in cancer cells is a key mechanism for anticancer therapy and chemotherapeutic agents induce apoptosis by generation of ROS and disruption of redox homeostasis.[25] Hsp induces intracellular Ca^{2+} mobilization too, depletion of endoplasmic reticulum Ca^{2+} stores result in growth arrest and apoptosis. Moreover, increase in intracellular Ca^{2+} induces mitochondrial dysfunction, which result in ROS overproduction.[26]

Several structural features were shown to be important for the effect of flavonoids on glutamate-mediated apoptosis including the presence of a hydroxyl group on C–3'' and a 4'-8' double bond in conjugation with a C–4 ketone function. The formation of hydrogen bonds between the ketonic oxygen and the hydroxyls at C–3'' and C–5 may have some influence on the scavenging power.[21,22,27-28]

Conclusions

These findings that Hsp inhibits laryngeal cancer cell metastasis reveal that it acts via multiple mechanisms as blockade of Ang1 expression and upregulation of Annexin V and induction of apoptosis. While its precise mechanism of action remains to be determined, this study supports the further investigation of Hsp as an antimetastatic agent that could be used to inhibit early-stage, as well as late-stage laryngeal cancer, including its metastasis with laryngeal preservation.

Authors' contribution

Both authors contributed adequately to the work reported in this article. They jointly drafted, revised, and approved the final version of the manuscript.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Compliance with ethical principles

The study was approved by the ethics committee of Assiut University, Assiut, Egypt.

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