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Original Article

Liushen Pills inhibit inflammation and growth of esophageal cancer by promoting the inflammatory cell infiltration

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Abstract: Background: Liushen pills (LSP) is a Chinese traditional medicine recorded by the Chinese medical dictionary. This study is aimed to investigate the effect of LSP on TNF-α/IL-1β and iNOS signaling pathways and their roles in the migration and invasion of esophageal cancer. Methods: By establishing the nude mice xenograft model of esophageal cancer, we evaluated the effect of LSP on esophageal cancer. The levels of chemokines were detected by Western blot and ELISA. Then, the role of LSP in cell proliferation, apoptosis, migration and invasion were validated by preparing the drug-containing serum. Results: LSP inhibited the tumor growth of esophageal cancer. LSP promoted the inflammatory cells infiltration around the tumor and induced the cell necrosis. LSP treatment significantly inhibited the levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2, IL-1β and IL-6 in esophageal cancer tissues. LSP serum induced cell apoptosis, and suppressed cell migration and invasion. Conclusion: LSP improves the microenvironment by inhibiting the inflammation and its contribution to metastasis via inhibition of esophageal cancer.

Keywords: Chinese medicine, Liushen pills, inflammation, metastasis, esophageal cancer

Introduction

Esophageal cancer is a malignant tumor that occurs in the epithelium of esophageal mucosa, which is one of the most common malignant tumors in the world [1]. The incidence of esophageal cancer in China ranks fifth among all the malignant tumors and the mortality rate ranks fourth [2]. Thus, it is one of the diseases that seriously threaten human life and health.

In 1889, Stephen Paget proposed the hypothesis of the metastatic "seed and soil". He believed that the growth of cancer cells must rely on the surrounding tissues to provide a specific environment [3]. In the process of tumorigenesis and development, the dynamic balance between the tumor cells and the surrounding tissue environment is constantly destroyed, causing the cell proliferation, differentiation, apoptosis, and expression and secretion of cytokines in cell surface are discarded, while the external tumor environment conductive to their own growth is constantly established, which leads to malignant transformation in the whole process of tumorigenesis and progression [4]. The tumor environment has the characteristics of hypoxia, low mesenchymal pressure, proteolytic fermentation and a large number of growth factors [3, 4]. It is the survival environment in which tumor cells grow and proliferate, and induces neovascularization, inhibits immune inflammatory reactions and inoculates cancer stem cells to facilitate the migration, invasion and metastasis of tumor cells [5-7]. In the tumor environment, a large number of cellular molecules (i.e. iNOS), growth factors, chemokines (i.e. TNF-α, IL-1β, and IL-6) and various proteolytic enzymes produced by the immune inflammatory response constituted the inflammatory microenvironment of tumors are particularly important [8, 9]. In recent years, more and more attention has been paid to the role of inflammatory microenvironment in the process of carcinogenesis [10, 11]. Antineoplastic drugs targeting tumor microenvironment such as non-steroidal anti-inflammatory drug COX-2 inhibitor celecoxib

have been emerging [12]. Therefore, exploring the mechanism of inflammatory microenvironment of tumors can provide a novel strategy for the treatment of tumors.

Liushen Pill (LSP) is recorded by the Chinese medical dictionary. It is composed of six medicines including bezoar, fragrance, pearl, borneol, toad venom and realgar, which is derived from Leiyun Shangyufeng Tang Fang [13]. LSP has shown good outcome in the treatment of liver cancer, gastric cancer, colon cancer, cervical cancer, and esophageal cancer [14]. Previous studies have shown that LSP inhibits the growth of esophageal cancer by inhibiting angiogenesis [13]. In this study, we aimed to investigate the effect of LSP on TNF-α/IL-1β and iNOS signaling pathways and their roles in the migration and invasion of esophageal cancer. The findings indicate that TNF-α pathway in the inflammatory microenvironment was involved in the metastasis of esophageal cancer.

Materials and methods

Cell culture

Human esophageal cancer cell line Eca-109 (ATCC, USA) was cultured in 50 mL flask. The cells were cultured in high glucose DMEM medium (Hyclone, USA) containing 10% newborn calf serum (Hyclone, USA) in a constant temperature incubator with 37°C, 5% CO_2 and saturated humidity.

Preparation of LPS solution

Liushen pills were thoroughly ground in a mortar and adjusted to 0.48 mg/mL with three distilled water for gastric lavage. The solutions were separately packed and stored at 4°C. Before use, the solution was warmed to room temperature and well mixed.

Animals and modeling

A total of 10 healthy, male, BALB/c nude mice, aged 6-8 weeks and weighted 18-22 g were purchased from Carvins Laboratory Animal Co., Ltd. (Changzhou, China). For drug-serum preparation, a total of 10 SD, male, rats weighted 200 g were randomly divided into normal serum group and LSP serum group. The mice and rats were fed with food and water freely, ventilated for 24 h in SPF animal room with 24-28°C, humidity 40% -50%. After 1 week of adaptive feeding, the xenograft mice model was performed under anesthetized by intraperitoneal injection of chloral hydrate (400 mg/k), and the rat drug-containing serum was prepared under anesthetized with sodium pentobarbital (50 mg/kg). At the end of experiments, the mice and rats were euthanized by increasing CO_2 levels to 33% over 1 min. Death was verified by decapitation. This study was approved by the Ethics Committee of Hainan Hospital of Traditional Chinese Medicine (no.2018.1.13, Haikou, China).

Xenograft model of esophageal cancer in nude mice

Healthy male BALB/c mice aged 6-8 weeks old and weighted 18-22 g were kept with isolator. The mice were anesthetized by intraperitoneal injection of chloral hydrate. 0.2 mL of human esophageal cancer cell suspension (1×10^7/mL) was extracted and inserted into the axillary subcutaneously. LSP treatment group was intragastrically administered with 4 mL LSP once a day for 20 days (n=5). The model group was intragastrically administered with normal saline (n=5). The tumor size was measured in vitro with Vernier caliper every 5 days, and the volume of tumor was calculated and the growth curve was drawn. After 20 days, the mice were sacrificed by spinal dislocation and the tumor were removed. After fixed with polyformaldehyde, the tumor tissue were dehydrated by gradient alcohol, embedded in paraffin, sectioned for routinely hematoxylin-eosin (HE) staining. The levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS and COX-2 were detected by Western blot. The levels of IL-1β and IL-6 were detected by ELISA.

Preparation of drug-containing serum

The SD male rats weighted 200 g were randomly divided into normal serum group and LSP serum group (n=5). The rats in LSP serum group were given 4 mL of LSP solution intragastrically twice a day for 3 days, while the normal serum group was given the same amount of saline daily. The rats were fasted for 12 h before the last intragastric administration and anesthetized with sodium pentobarbital after the last gavage for 1 h. Blood was collected from the posterior abdominal aorta and ventricle. Serum was aseptically separated, in activated and sterilized, and then placed in a vial and stored

at -20°C. The esophageal cancer cell was cultured using the normal serum and LSP serum for 48 h, respectively.

MTT assay

The cell proliferation was determined by MTT assay. The cells were inoculated in 96-well flat-bottom culture plate with 200 μL/well (2×10^4 cells per well) and cultured for 24 h. Then, the culture medium was removed and replaced with the normal serum and LSP serum, respectively for 24, 48, and 72 h. Four hours before the end of treatment, 20 μL MTT (5 mg/mL) was added into each well. At the end of treatment, 200 μL dimethyl sulfoxide was added to each well for 5 min. The absorbance was measured at 490 nm. Finally, the growth inhibition rate was calculated.

Detection of apoptosis by flow cytometry

The cells treated with normal serum and LSP serum were collected and suspended in binding buffer to adjust to 1×10^7/ml. 100 μL cells were taken and mixed with 5 μL annexin V-FITC and 5 μL PI for 15 min at room temperature in the dark. After adding 400 μL of binding buffer, the apoptosis was detected with a flow cytometer. The flow cytometry data was obtained by FACS Calibur flow cytometry and CELLQuest software, and the experimental data were analyzed using Winmidi 218 software. The normal cells are located at the lower left, Annexin V (-) and PI (-). The early apoptotic cells are located at the lower right, Annexin V (+) and PI (-). The late apoptotic cells are located at the upper right, Annexin V (+) and PI (+).

ELISA

Levels of IL-1β and IL-6 were measured suing the commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (Raybiotech Inc., GA, USA). The intra-assay and inter-assay coefficients of variation were less than 10% in enzyme immunoassays.

Transwell assay for migration and invasion

The cell migration and invasion were performed with QCM-96-well cell migration and invasion assay kits (Chemicon International, Temecula, CA, USA). The tumor cells were stained by Crystal violet and photographed under × 100 microscopy (Olymplus, Japan).

Western blot

Rabbit anti-TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2 (Abcam, 1:1000) and 1:3000 dilution of anti-rabbit IgG secondary antibody (ZSGB-BIO, China, 1:1000) were used to determine the protein expression. Rabbit anti-GAPDH (ZSGB-BIO, China, 1:1000) was used as an internal control. Images were acquired with a ChemiDoc Touch imager (Bio-Rad, USA) and quantification was done using Quantity One 4.4.0 software (Bio-Rad, USA).

Statistical analysis

All data are presented as the mean ± standard deviation of at least 3 independent experiments. Graph construction and statistical analysis were performed using SPSS 17.0 and GraphPad Prism 5.0. Differences between groups were evaluated with Student's t test. *P* values <0.05 were considered to be statistically significant.

Results

Effect of LSP on the growth of esophageal cancer

According to the change in transplanted human esophageal cancer in nude mice (**Figure 1A**), the growth curve was drawn (**Figure 1C**). LSP significantly inhibited the growth of esophageal cancer after 10 days treatment (P<0.05). The subcutaneous tumors were exfoliated (**Figure 1B**). The weight of tumors in model group were significantly higher than LSP treatment group (P<0.001) (**Figure 1D**). The tumors in model group were incomplete in envelope, while the LSP treatment tumors were intact in envelope (**Figure 1B**). These findings suggested that LSP inhibited the progression of esophageal cancer.

Histopathological observation of tumor tissues

In the model group, the cancer cells invaded the deep muscular layer, and the nucleus of the cancer cells was large and hyperchromatic (**Figure 2**). In the LSP group, inflammatory cells infiltrated around the tumor and the cancer cells invaded the muscular layer of the line

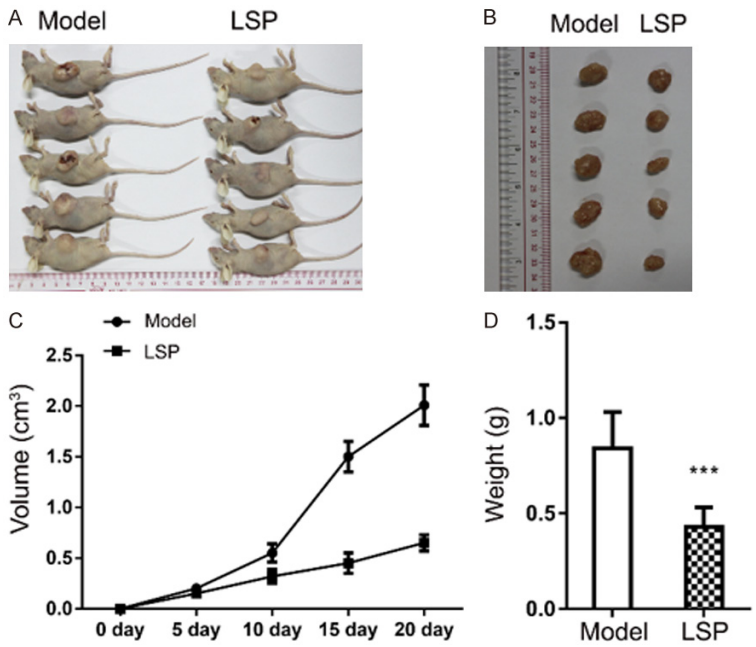

Figure 1. Effect of LSP on the growth of esophageal cancer. A. The xenograft model of esophageal cancer after treatment of LSP for 20 days. B. The tumors. C. The growth curve of esophageal cancer. D. The weight of tumors. *P<0.05, ***P<0.001.

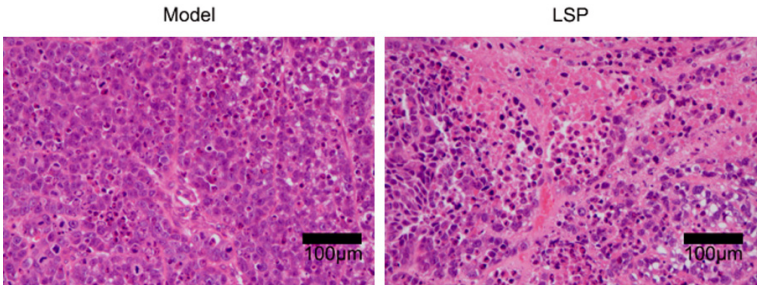

Figure 2. The histopathological change in tumor tissues in xenograft model after LSP treatment (100x). The scale bar is 100 μm.

(**Figure 2**). The abnormality of the tumor cells was obvious, and the necrosis surface was small. It showed hemorrhagic foci in LSP treated tumor tissues (**Figure 2**).

Effect of LSP on levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2, IL-1β and IL-6

The levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS and COX-2 were detected by Western blot (**Figure 3A-C**). The levels of IL-1β and IL-6 were detected by ELISA (**Figure 3D**). LSP treatment significantly inhibited the levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2, IL-1β and IL-6 in esophageal cancer tissues.

LSP serum induced cell apoptosis, and suppressed cell migration and invasion of esophageal cancer cells.

To further analyze the role of LSP in esophageal cancer cells, human esophageal cancer cells (line Eca-109) were treated with LSP serum (**Figure 4**). Compared with normal serum, LSP serum inhibited the proliferation of esophageal cancer cells at 48 h and 72 h ($P<0.05$). The apoptotic rate of the esophageal cancer cells was significantly increased. The apoptotic rate was mostly increased in the early apoptotic cells (**Figure 4B**). The migration and invasion of esophageal cells were inhibited by LSP treatment. Thus, the results suggested that LSP induces early apoptosis, and inhibited the migration and invasion of esophageal cancer cells.

Discussion

Metastasis are essential characteristics of malignant tumors and the leading causes of death in tumor patients [15, 16]. The occurrence, development, invasion and metastasis of tumors are complex processes involving cell-cell communication and multi-gene changes in microenvironment [17, 18]. Tumor invasion and metastasis are two different stages in the same process [19]. They are closely linked to each other. Invasion is the basis of metastasis, and metastasis is the consequence of invasion. The movement and migration of cancer cells ultimately achieve the invasion and metastasis of tumors [20]. In this study, we found that LSP promoted inflammatory cell infiltration, but inhibited the esophageal cancer cell migration, invasion and metastasis.

TNF-α, IL-6 and NF-κB are important cytokines in the inflammatory microenvironment of tu-

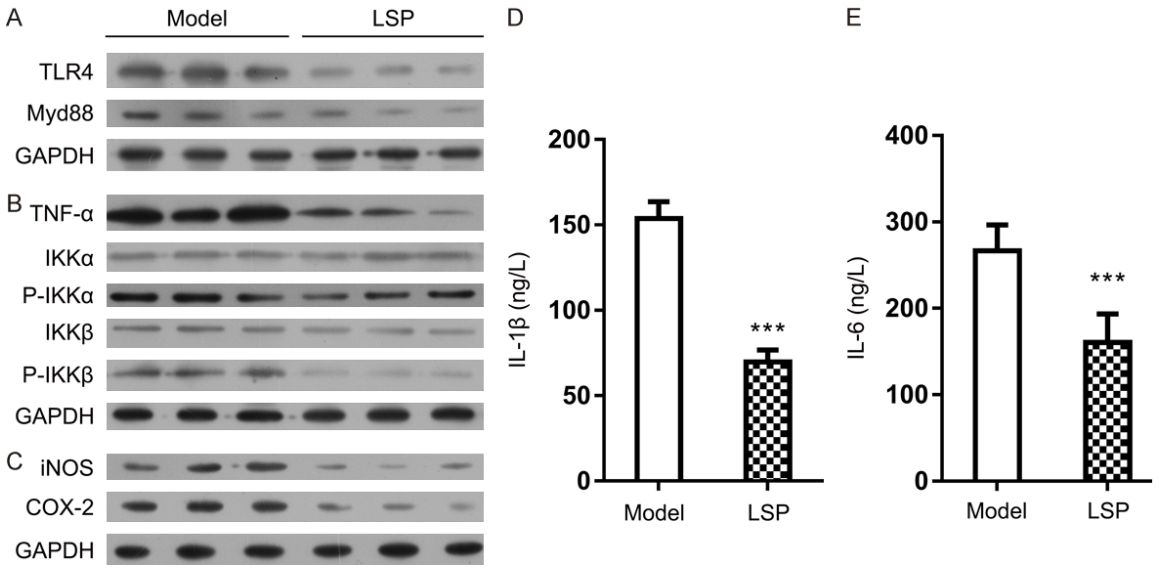

Figure 3. Levels of TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2, IL-1β and IL-6 in xenograft model after LSP treatment. TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS and COX-2 levels in tumor tissues in xenograft model after LSP treatment were performed by Western blot. The levels of IL-1β and IL-6 were detected by ELISA. ***P<0.001.

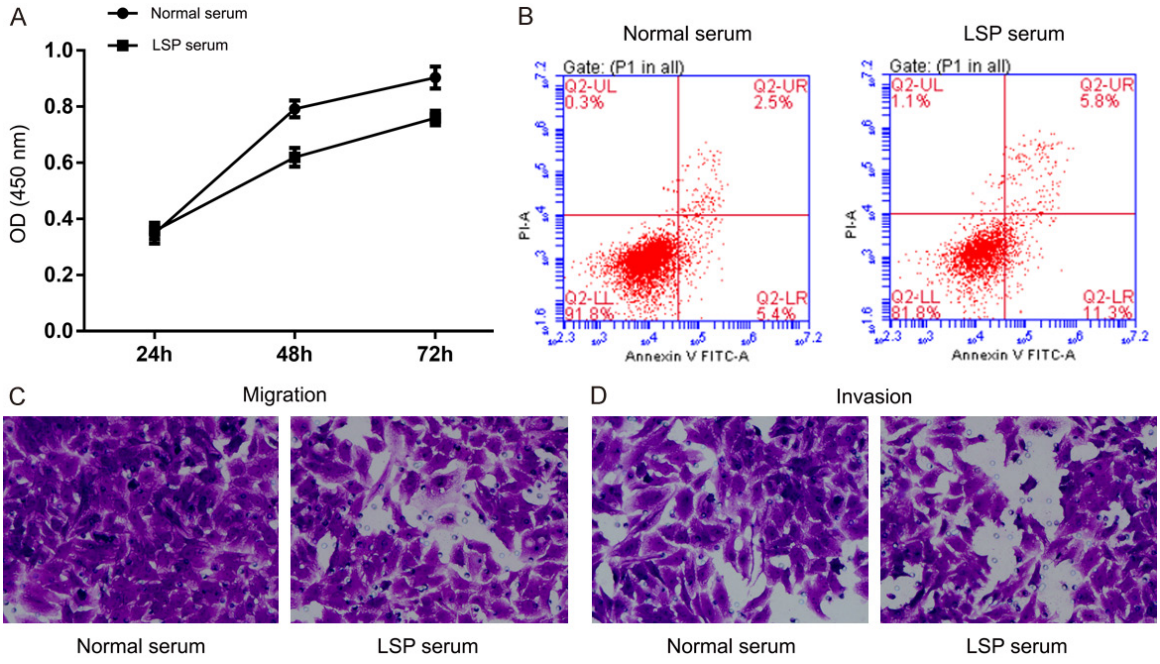

Figure 4. Effect of drug-containing serum on esophageal cancer cells. Serum was aseptically separated from SD rats after LSP or normal saline administration intragastrically twice a day for 3 days. (A) Cell proliferation was detected by MTT assay. (B) Cell apoptosis was detected by flow cytometry. (C) Migration and (D) invasion were detected by Transwell assays.

mors [21, 22]. IL-6 is an inflammatory factor, which is generally expressed in various tumor microenvironments and inflammatory diseases [23, 24]. It participates in the occurrence of various tumors, such as hepatocellular carcinoma, lung cancer, oral cancer, gall bladder carcinomas, gynecologic cancer and esophageal cancer [23-25]. Its function is to affect cell cycle, regulate angiogenesis and so on by activating multiple signaling pathways such as

JAKs/STAT3 [22]. TNF-α is an essential factor in the initiation of chronic inflammation and is one of the most influential inflammatory mediators in the early stage of inflammation [26]. TNF-α can activate the NF-κB pathway and triggers the transcription of many inflammatory cytokine genes, leading to tumorigenesis [27, 28]. It was found that TNF-α was expressed in tumor cells and stromal cells of breast cancer, prostate cancer, bladder cancer, colon cancer, liver cancer, lymphoma and leukemia, and the prognosis of patients with positive TNF-α was poor [26]. TNF-α could induce epithelial-mesential transition through NF-κB pathway mediated Snail stabilization, which can significantly promote the migration and invasion of tumor cells [28, 29]. TNF-α and IL-1β are not only the downstream target genes of NF-κB, but also the promoter of NF-κB. After activation of NF-κB pathway by TNF-α and IL-1β, NF-κB can be further activated, and the cycle is repeated, so that the inflammatory response is continuously expanded [30]. Most of the inflammatory reactions are self-limiting, but this process of inflammation self-amplification leads to excessive autologous tissue damage, which promotes the occurrence and development of tumors [31, 32]. In this study, we found that LSP treatment significantly inhibited the levels of inflammatory factors including TLR4, Myd88, TNF-α, IKK-α, p-IKKα, IKKβ, p-IKKβ, iNOS, COX-2, IL-1β and IL-6 in esophageal cancer tissues.

At present, the main methods of cancer treatment, such as surgery, radiotherapy and chemotherapy, cause damage to tissues and cells and then cause inflammation. This inflammatory environment is beneficial to tumor cells and increases the possibility of metastasis. Therapeutic effect will inevitably be discounted. Therefore, improving the microenvironment for treating inflammation can also improve the therapeutic effect and inhibit the occurrence and metastasis of tumors to a certain extent, which is of great significance for the treatment of tumors. LSP promoted the cell apoptosis but inhibited the inflammation, showing a promising role in improving the microenvironment for inhibition of tumor metastasis. However, due to the complexity of the inflammatory microenvironment, the role and mechanism of the inflammatory microenvironment are not yet fully elucidated and the establishment of tumor micrroenvironment model is still lack of certain standards. Nevertheless, we could conclude that LSP improves the microenvironment by inhibiting the inflammation and contribution to metastasis via inhibition of esophageal cancer.

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Disclosure of conflict of interest

None.

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