Journal of Ethnopharmacology 143 (2012) 680–685

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Antitumor activity and immunomodulatory effects of the intraperitoneal administration of Kanglaite in vivo in Lewis lung carcinoma

Pei Pan [a], Yan Wu [a], Zhu-Ying Guo [b], Rong Wang [a], Yu-Jie Wang [a], Yong-Fang Yuan [a,*]

[a] Department of Pharmacy, NO.3 People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine, No. 280, Mohe road, Baoshan District, Shanghai 201900, China
[b] Experimental Center, NO.3 People's Hospital affiliated to Shanghai Jiao Tong University School of Medicine, Shanghai 201900, China

ARTICLE INFO

Article history:
Received 26 March 2012
Received in revised form
18 June 2012
Accepted 23 July 2012
Available online 31 July 2012

Keywords:
Kanglaite
Coix seed
Lewis lung carcinoma
Immunomodulatory
NF-kappaB
IL-2

ABSTRACT

Aims of the study: Kanglaite (KLT) is a useful antitumor drug with proven effects when combined with chemotherapy, radiotherapy or surgery. We hypothesize that KLT has antitumor activity and immunomodulatory effects in Lewis lung carcinoma.
Materials and methods: C57BL/6 mice with Lewis lung carcinoma were divided into four groups: the control group (C), cisplatin group (1 mg/kg, DDP), low KLT group (6.25 ml/kg body weight [L]), and high KLT group (12.5 ml/kg body weight [H]). T cell proliferation was determined by the MTT assay. Nuclear factor-kappa B (NF-κB), inhibitor kappa B alpha (IκBα), IκB kinase (IKK) and epidermal growth factor receptor (EGFR) levels were measured by western blotting. An enzyme-linked immunosorbent assay was used to analyze the expression of interleukin-2 (IL-2).
Results: Intraperitoneal KLT significantly inhibited the growth of Lewis lung carcinoma, and the spleen index was significantly higher in the L and H groups than in the C group. KLT stimulated T cell proliferation in a dose-dependent manner. Treatment with KLT at either 6.25 or 12.5 ml/kg decreased the level of NF-κB in the nucleus in a dose-dependent manner, and KLT markedly decreased the expression of IκBα, IKK and EGFR in the cytoplasm of tumor cells and overall. IL-2 was significantly increased in the supernatant of splenocytes in the H group.
Conclusions: These results demonstrate that KLT has pronounced antitumor and immunostimulatory activities in C57BL/6 mice with Lewis lung carcinoma. These may affect the regulation of NF-κB/IκB expression, in addition to cytokines such as IL-2 and EGFR. Further work needs to investigate the relevant signaling pathway effects, but our findings suggest that KLT may be a promising antitumor drug for clinical use.

1. Introduction

Kanglaite (KLT) is an oily substance extracted from *Coix lacryma-jobi* (family Cramineae). The main active ingredient is a compound of triglycerides containing four types of fatty acid (Fig. 1) Yu et al. (2008). In clinical use in China, KLT has been proved to significantly improve the life span and quality of life of patients, when combined with chemotherapy, radiotherapy or surgery Li (2007), Wang et al. (2007), Wu et al. (2004). Coix seed is traditional Chinese medicine that is well known for its antitumor and immunomodulatory effects (The Pharmacopoeia Committee of China, 2005). Evidence has accumulated concerning the medical use of coix seed and its extracts in the treatment of diseases such as cancer metastasis, hypertension, arthritis, asthma and immunological disorders (Li, 2007). Research shows that KLT principally blocks the G2+M phase of the cell cycle, thereby reducing the mitotic division of cells and inhibiting the proliferation of tumor cells; at the same time, it can activate pro-apoptotic factors, leading to apoptosis Lu et al. (2008).

Nuclear factor kappa B (NF-κB) is an important regulator of transcription that controls the expression of various genes involved in the immune system, cancer and inflammatory functions (Wullaert et al., 2011; Porta et al., 2009; Karin et al., 2002). Since the identification of NF-κB and the cloning of the genes encoding NF-κB and inhibitor kappa B (IκB), much experimental evidence has accumulated demonstrating that this factor plays a major role in the development and progression of various human cancers. Many anticancer agents, including paclitaxel, vincristine, daunorubicin and resveratrol, activate NF-κB in cancer cells (Ikeda et al., 2010; Gupta et al., 2011). Solid tumors such as lung carcinoma, breast cancer, prostatic cancer, and stomach carcinoma are also characterized by constitutive and continuous NF-κB activity (Wang et al., 2010). NF-κB induces the expression of

* Corresponding author. Tel.: +86 2156786907; Mobile: +86 13621676746 fax: +86 21 56786907.
E-mail address: nmxyyf@yahoo.cn (Y.-F. Yuan).

0378-8741/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.07.025

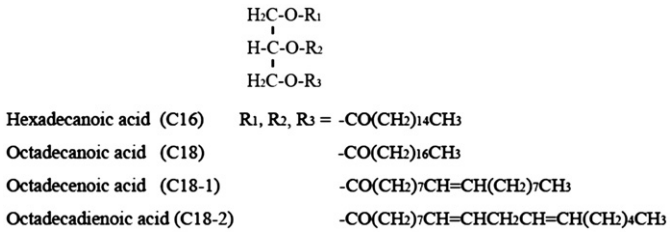

Fig. 1. Chemical structures of the main active ingredients in coix seeds.

diverse target genes that promote cell proliferation, regulate apoptosis, facilitate angiogenesis, and stimulate invasion and metastasis (Shen et al., 2009). Using a NF-κB dependent reporter assay, Woo et al. demonstrated the dose-dependent inhibition of NF-κB signaling by treating cell cultures with coix seed extracts. Immunofluorescence microscopy showed that these effects were associated with reduced translocation of the Rel-A/p65 subunit of NF-κB to the nucleus in xenografts of MDA-MB-231 breast cancer cells (Woo et al., 2007).

Epidermal growth factor receptor (EGFR), a member of the ErbB family of receptor tyrosine kinases with oncogenic potential, comprises an ectodomain juxtaposed with a transmembrane domain linked to the cytoplasmic kinase endodomain (Downward et al., 1984). High levels of expression of this receptor are often observed in malignancies, including non-small cell lung cancer, and it is implicated in the aggressive biological behavior of tumor cells and poorer prognosis Lin and Yang (2011).

Interleukin 2 (IL-2) is a pleiotropic cytokine produced primarily by mitogen- or antigen-activated T lymphocytes (Nelson et al., 1998). It plays a key role in promoting the clonal expansion of antigen-specific T cells (Nelson, 2004). T cell receptor signaling induces activator protein 1, increases the levels of active NF-κB, and causes calcineurin-mediated dephosphorylation of nuclear factor of activated T cells, promoting its translocation into the nucleus (Malek, 2008).

Although KLT injections have been successful in the treatment of various malignant tumors, it is unclear how this extract of coix seed acts in vivo. In this study, we used C57BL/6 mice with Lewis lung carcinoma to investigate the efficacy of treatment with KLT. Specifically, we assessed the tumor growth inhibition rate and spleen index, and the expression of NF-κB in nuclear and cytosolic proteins, IκBα, IκB kinase (IKK) and EGFR in tumor cytosolic protein, overall EGFR expression and IL-2 in the supernatant of splenocytes stimulated by concanavalin A (Con A).

2. Materials and methods

2.1. Materials

KLT injection was purchased from Zhejiang Kanglaite Pharmaceutical Co., Ltd (Hangzhou, China). Cisplatin (DDP) was purchased from Sigma Chemical Co. (St Louis, MO, USA). NF-κB p65 rabbit polyclonal IgG, IκBα rabbit polyclonal IgG, IKK rabbit polyclonal IgG, EGFR rabbit polyclonal IgG and horseradish peroxidase (HRP)-labeled goat anti-rabbit IgG were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse IL-2 immunoassays were purchased from R&D Systems (Minneapolis, MN, USA). Nuclear and cytoplasmic protein extraction kits were purchased from Kangchen Biotechnology (Shanghai, China). Lewis lung carcinoma cells were purchased from ATCC (Manassas, VA, USA).

2.2. Animals and lewis lung Cancer model

C57BL/6 male mice aged 6–8 weeks (weight, 18–22 g) were purchased from B&K Universal Group Ltd, Shanghai, China. The animals were kept under a 12 h light/12 h dark cycle at 25 °C and a humidity of 60% ± 10%. Forty mice were randomly divided into four groups of 10 animals in each: the control group (C), cisplatin group (1 mg/kg, DDP), KLT 6.25 ml/kg body weight (L) and KLT 12.5 ml/kg body weight (H). A subcutaneous injection of 2×10^6 Lewis lung cancer cells suspended in 0.1 ml phosphate buffered saline (PBS) was administered in the right axilla of each mouse. Starting the next day, the DDP group mice were injected peritoneally with DDP for 14 day. And the L and H group mice were injected peritoneally with KLT for 14 day. The mice in the C group were injected with saline at the same time. The mice were then sacrificed and the tumors were harvested. The tumors were weighed and the tumor growth inhibition rate was calculated using the following formula: [(mean tumor weight of C group − mean tumor weight of treatment group)/mean tumor weight of C group] × 100%. The spleen and the body weight were also measured, and the spleen index was calculated as splenic weight divided by body weight. The experiments were performed in accordance with local institutional and governmental regulations on the use of experimental animals.

2.3. MTT assay for T cell proliferation

Splenic cells were isolated from harvested mouse spleen tissue samples in a sterile environment. The isolated splenocytes (2×10^6/well) were planted in 200 μl of medium/well on in 96-well tissue culture plates, which were incubated for 48 h at 37 °C and 5% CO_2 in the presence of 5 μg/ml Con A. Then, 20 μl of 5 mg/ml 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) (Sigma, USA) was added to each well and the plates were incubated for 4 h. After the removal of MTT, the precipitate was solubilized in DMSO (100 μl/well) and the absorbance measured on a microplate reader (model 550; Bio-Rad, CA, USA) at a wavelength of 570 nm.

2.4. Preparation for cytosolic and nuclear protein determination

Cytosolic and nuclear proteins were isolated from harvested mouse tissues and prepared using a nuclear and cytoplasmic protein extraction kit (Kangchen Biotechnology, Shanghai, China) according to manufacturer's instructions. Briefly, tissue samples were dissected into small pieces (200 mg), placed in ice-cold PBS (1 ml), and homogenized in a tissue homogenizer (Fluko, China) at 16,000*g* for 1 min. Tissue homogenates were then pelleted by centrifugation at 500*g* for 3 min at 4 °C, and the supernatant was removed. The packed tissue homogenate volume was estimated for each sample, and the pellets were resuspended by vortexing vigorously for 15 s in 0.2 ml of the CER-A mix. After 10-min incubation on ice, 11 μl of ice-cold CER-B was added, and the tubes were vortexed vigorously for 5 s. After a further 1-min incubation on ice, the tubes were vortexed vigorously for 5 s, then centrifuged for 5 min at 16,000*g* at 4 °C. The supernatant, containing the cytoplasmic fraction, was removed into clean, pre-chilled tubes on ice and stored at −80 °C until use. The insoluble pellet containing the nuclear fraction was resuspended in 100 μl of ice-cold NER, vortexed vigorously for 15 s and allowed to incubate on ice for 40 min. During this incubation, the samples were vortexed for 15 s every 10 min for four times in total. The tubes were then centrifuged at 16,000*g* for 10 min. The supernatant, which contained the nuclear fraction, was transferred to clean, pre-chilled tubes on ice and stored at −80 °C until use.

2.5. Western blotting for NF-κB, IκB α, IKK and EGFR protein expression

Protein samples (50 μg) were boiled with 4 × Laemmli sample buffer containing 5% beta-mercaptoethanol for 3 min. Samples