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Inhibition of p38 and ERK1/2 pathways by Sparstolonin B suppresses inflammation-induced melanoma metastasis

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ABSTRACT

Background: Cancer related inflammation plays a fatal role in the metastatic process, which can foster tumor growth, angiogenesis and dissemination. Sparstolonin B (SsnB), derived from Chinese medicine of the tubers of *Scirpus yagara*, is a TLR2 and TLR4 antagonists. It has exhibited multiple activities of anti-inflammatory, anti-cancer, anti-obesity and anti-hepatitis. However, whether SsnB is involved in the regulation of inflammation-induced tumor metastasis is not well elucidated.

Purpose: The aim of this study was to investigate the effectiveness of SsnB as a treatment of inflammation-induced tumor metastasis and identify the underlying mechanisms of its anti-tumor metastatic activity.

Method: The anti-tumor metastatic activity *in vitro* was estimated by MTT, wound-healing assay, matrigel invasion analysis and extracellular matrix adhesion assay. Mice lung metastasis and hepatic metastasis experiments were performed to assess the activities *in vivo*. Lungs or livers were weighed and the number of metastatic nodules was determined after mice were sacrificed. The levels of pro-inflammatory cytokines in the serum, lungs and livers were detected by using enzyme-linked immunosorbent assay (ELISA). Micro-metastasis nodules in lungs or livers were analyzed by histological examination. Immunohistochemistry and western blot analysis were conducted to determine protein expression.

Result: Herein, SsnB dose-dependently inhibited cell migration and invasion in mouse melanoma B16 cells with or without stimulation of lipopolysaccharide (LPS), Pam3csk4 or molecules from damaged tumor cells (DTC-Ms). The expression of matrix metalloproteinases (MMP)-2 was also significantly abated by SsnB in LPS-modulated B16 cells. And SsnB reduced LPS-activated B16 cells adhesion to extracellular matrix components collagen I and fibronectin in a dose-dependent manner. *In vivo*, SsnB obviously attenuated LPS-activated pulmonary metastasis in mice by reduction the number of metastatic nodules on the lung surfaces, lung weight and levels of tumor necrosis factor (TNF)-α and interleukin (IL)-6 in serums and lungs. Moreover, in experimental hepatic metastasis model mice, SsnB remarkably repressed LPS-stimulated the number of metastatic nodules along with liver weight; and SsnB significantly suppressed LPS-activated increase levels of TNF-α and IL-6 in livers. Immunohistochemistry analysis indicated that SsnB inhibited the expression of TLR4 in livers. Furthermore, SsnB remarkably blocked p38 and ERK1/2 signaling pathway in LPS-induced B16 cells. P38 and ERK1/2 signaling silencing, using BIRB0796 (small molecular inhibitor of p38 MAPK) and PD184352 (inhibitor of MEK1/2 kinases that activate ERK1/2), significantly abated LPS-induced migration and invasion of B16 cells.

Conclusion: The present study reports a novel use of SsnB in mitigating TLRs ligands-induced melanoma metastasis by inhibition of p38 and ERK1/2 pathway.

1. Introduction

Tumor metastasis is the main cause of cancer death, which is formed by the spread of disseminated primary tumor cells to distant anatomic sites [1]. Chronic inflammation is a major contributor to carcinogenesis and metastasis of tumors [2,3]. Toll-like receptors (TLRs) are innate immune receptors involved in recognition of microbial and self-ligands associated with tissue damage and inflammation [4]. Activation of TLRs

Abbreviations: SsnB, Sparstolonin B; ELISA, Enzyme-linked immunosorbent assay; LPS, Lipopolysaccharide; DTC-Ms, Molecules from damaged tumor cells; MMP-2, Matrix metalloproteinases-2; TNF-α, Tumor necrosis factor-α; IL-6, Interleukin-6; TLRs, Toll-like receptors

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on tumor promotes chronic inflammation which stimulates cancer cell proliferation, migration, tumor angiogenesis and creates a tumor microenvironment which impairs the anti-tumor function of the immune system allowing tumors to develop and metastasize [5]. Previous researches have showed that over-expression of TLRs improved tumor metastasis in hepatocellular carcinoma, colorectal cancer and breast cancer [5–7].

Sparstolonin B (SsnB), a TLR2 and TLR4 antagonist, is isolated from Chinese herb *Scirpus yagara* [8]. It could selectively block TLR2- and TLR4- mediated macrophages' inflammatory responses [9], and could ameliorate brain edema and neurologic deficits in ICH model mice via inhibition of TLR2/TLR4 heterodimer formation [10]. In addition, SsnB could inhibit LPS-induced cytokines production in 3T3-L1 adipocytes and reduce HFD-induced obesity in rats [11]. However, whether SsnB is involved in the regulation of inflammation-induced tumor metastasis is not well elucidated. Herein, anti-tumor metastasis activity of SsnB *in vitro* and *in vivo* on inflammation- mediated melanoma, and the underlying molecular mechanism were investigated.

2. Materials and methods

2.1. Animals and reagents

BALB/c female mice (20 ± 2 g) were purchased from the Shanghai Jiesijie Laboratory Animal Center (certificate No. 2010002608097 Shanghai, China). The animals were maintained at 25 °C on a 12 h light/dark cycle, and provided standard commercial diet and water ad libitum. The animals were acclimated in room for 7 days before the initiation of the experiment. The mice experiments were under the guidelines of the committee for Animal Care and use of Laboratory Animals, College of Pharmacy, Nanjing University of Chinese Medicine.

LPS (*Escherichia coli* 055:B5) and MTT were purchased from Sigma (St .Louis, MO, USA). Matrigel was purchased from Becton Dickinson (Biosciences, San Jose, California). ELISA kits for TNF-α and IL-6 were provided by eBioscience (San Diego, CA); RPMI-1640 medium and fetal bovine serum were provided by Wisent Biotechnology Co., Ltd. (Nanjing, China). Lowry for proteins quantified were purchased from Multisciences Biotech Co., Ltd. (Hanzhou, China). TAK-242 (Ethyl (6R)-6-[*N*-(2-chloro-4-fluorophenyl) sulfamoyl] cyclohex-1- ene-1-carboxylate)) and antibodies for western blot analysis were purchased from Cell Signaling Technology (Beverly, MA, USA).

SsnB was prepared as described in supplementary materials.

2.2. Cell culture

Mouse melanoma cells B16 were purchased from the Cell Bank of the Chinese Academy of Sciences. B16 cells were cultured in RPMI-1640 medium with 10% fetal bovine serum (FBS). B16 cells were incubated at 37 °C in a humidified incubator with 5% CO_2.

2.3. Cell viability

Cell viability was determined using the MTT assay. Cells were seeded at 1×10^5 cells/well in 96-well plates. 4 h later, cells were washed and incubated with medium (FBS-free) containing different concentrations of SsnB. After 24 h, MTT (5 mg/mL in the medium) was then added to the medium, and the cells were incubated for an additional 4 h. The formazancrystals in each well were solubilized in dimethyl sulfoxide (DMSO, Sigma, St .Louis, MO, USA), and the absorbance was measured at 490 nm on a microplate reader.

2.4. Preparation of DTC-Ms from tumor cells

Tumor cells were washed with PBS and resuspended in PBS to a final cell concentration of 5×10^7/mL. After four-round frozen-thaw cycles followed by vortexing for 30 s, the cells were removed by centrifugation. The supernatant contained a mixture of molecules from damaged tumor cells (DTC-Ms). The concentration of DTC-Ms was defined by the concentration of protein, which was determined using lowry according to manufacturer's instructions.

2.5. Wound-healing assay

Wound-healing assay was used to evaluate the activity of SsnB suppressing the migration of B16 cells. In brief, B16 cells (8×10^5 cells/well) were seeded in a 6-well culture plate and grown to 80–90% confluence. After aspiration of the medium, the center of the cell monolayers was scraped with a yellow pipette tip to create a denuded zone of constant width. Subsequently, B16 cells were stimulated with LPS or Pam3csk4 or DTC-Ms in the presence or absence of SsnB. Wound closure was photographed at 0 h and 24 h with a Nikon inverted microscope (Nikon, Tokyo Japan), and cells migration into the scratch area was analyzed using Image-Pro Plus 6.0 software.

2.6. Matrigel invasion analysis

The invasion assays were carried out using transwell chamber with 10 mm diameter and 8 um pore size polycarbonate membrane (Corning Costar, Cambridge, Massachusetts) coated with matrigel. B16 cells were trypsinized and resuspended in serum-free medium. 200 μL the cell suspension (10^5 cells) with LPS or Pam3csk4 or DTC-Ms in the presence or absence of SsnB were added to the upper chamber of each well. The bottom chambers were filled with 500 μL RPMI 1640 medium supplemented with 20% FBS. The chamber was incubated for 24 h at 37 °C. At the end of incubation, the cells in the upper surface of the membrane were carefully removed with a cotton swab. Cells invading across the matrigel to the lower surface of the membrane were fixed with 4% paraformaldehyde and stained with 0.1% crystal violet. The invading cells on the lower surface of the membrane filter were counted with a light microscope.

2.7. Extracellular matrix adhesion assay

96–well plates were coated with 100 μL/well of 50 μg/mL collagen I, 50 μg/mL collagen IV and 20 μg/mL fibronectin overnight at 4 °C, and blocked by 1% BSA for 1 h at 37 °C before seeding cells. 3×10^4 cells resuspended in 100 μL media were seeded in each well and incubated at 37 °C for 1 h. Each well was washed gently with PBS twice and attached cells were fixed with 4% paraformaldehyde, stained in 10% crystal violet Images of adhered cells at five random fields were captured with a microscope objective of an inverted fluorescent microscope.

2.8. Experimental lung metastasis model

In order to evaluate lung metastasis of SsnB against B16 cells, three groups were considered as control group (B16 cells untreated), LPS group (B16 cells pre-treated with LPS for 4 h) and SsnB group (B16 cells pre-treated with LPS and 10 μM SsnB for 4 h). Then, these B16 cells (3×10^5 cells suspended in 200 μL physiological saline) were injected through the caudal vein into BALB/c female mice. Subsequently, the mice were sacrificed 21 days later and lung metastatic foci were identified. Half of the lung tissue was fixed with 10% neutral-buffered formalin, embedded in paraffin medium and sectioned (4 μm), and then stained with hematoxylineosin. Pathological changes were evaluated under a light microscope and digital images were obtained at magnifications of 200. The other half of the lung tissue was placed in homogenization buffer (0 °C), centrifuged at 12,000 rpm, 4 °C for 10 min and frozen at −80 °C until use. The blood samples of four groups were collected by retroorbital bleeding. Serum was separated by centrifugation at 3000 rpm for 15 min at 4 °C and frozen at −80 °C until use. The TNF-α and IL-6 concentrations in the lung tissue supernatant and serum were detected using ELISA according to the manufacturer's