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Osthole inhibits pancreatic cancer progression by directly exerting negative effects on cancer cells and attenuating tumor-infiltrating M2 macrophages

Botao Wang [a, b], Xin Zheng [a, b], Jing Liu [c], Zhen Zhang [a, b], Chongyang Qiu [a, b], Lei Yang [b], Lanqiu Zhang [b], Qi Zhang [b, *], Hongwei Gao [a, b, **], Ximo Wang [b, ***]

[a] Graduate School, Tianjin Medical University, Tianjin, 300070, China
[b] Tianjin Key Laboratory of Acute Abdomen Disease Associated Organ Injury and ITCWM Repair, Institute of Acute Abdominal Diseases, Tianjin Nankai Hospital, Tianjin, 300100, China
[c] School of Pharmacy, Jiangxi University of Traditional Chinese Medicine, Nanchang, 330004, China

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ABSTRACT

Pancreatic cancer has remained a major cause of cancer-related deaths. A hallmark of pancreatic cancer is extensive stromal reactions, resulting in a unique tumor microenvironment, especially the involvement of macrophages. These tumor-educated cells limit the efficacy of chemotherapy. Therefore, it is necessary to identify an effective treatment strategy. In this study, we aimed to explore the anti-tumor and immunomodulatory effects of osthole on pancreatic cancer. We found that osthole suppressed Panc 02 cell migration and proliferation and induced apoptosis as shown in vitro. Osthole also attenuated the development of pancreatic cancer in mice by inhibiting tumor-infiltrating M2 macrophages in our study. Additionally, osthole inhibited the polarization of primary bone marrow cells into M2 macrophages and inhibited the expression of *MRC1*, *CCL22* and *TGF-β* in the M2 polarization process in vitro. Detection of the related signaling pathways revealed that osthole exerted immunomodulatory effects on M2 macrophages by down-regulating p-STAT6 and the p-ERK1/2-C/EBP β axis. These results indicated that osthole has effective anti-tumor and immunomodulatory effects on pancreatic cancer.

1. Introduction

Pancreatic cancer is a malignant tumor that is extremely difficult to detect at an early stage. Its 5-year survival rate is only approximately 6%.[1] In the past few years, both the incidence and mortality of pancreatic cancer in men have increased in China.[2] The combination of immune-suppressive inflammation and oncogenic mutations is believed to trigger pancreatic cancer.[3] The pancreatic tumor microenvironment also promotes the development of pancreatic cancer.[4] Significant feature is that the main pancreatic tumor microenvironment comprises with a robust activated fibroblasts and immunosuppressive cells including vast majority of macrophages and regulatory T cells.[5,6] At present, the recognized treatment for pancreatic cancer is surgical resection combined with postoperative gemcitabine or S-1 chemotherapy.[7] However, long-term metabolic morbidity occurs in more than a third of patients after classic surgery, eventually leading to pancreatic malabsorption syndrome and seriously affects the survival rate of pancreatic cancer.[8] Meanwhile, tumor-associated macrophages have been shown to up-regulate the expression of cytidine deaminase, and result in the resistance of pancreatic cancer cells to gemcitabine chemotherapy.[9,10] Gemcitabine exhibits a certain immunoregulatory capacity against myeloid derived suppressor cells (MDSCs). It

* Corresponding author. Tianjin Key Laboratory of Acute Abdomen Disease Associated Organ Injury and ITCWM Repair, Institute of Acute Abdominal Diseases, Tianjin Nankai Hospital, No.6 Changjiang Road, Tianjin, 300100, China. Fax: +86 022 27435588.
** Corresponding author. Tianjin key Laboratory of Acute Abdomen Disease Associated Organ Injury and ITCWM Repair, Institute of Acute Abdominal Diseases, Tianjin Nankai Hospital, No.6 Changjiang Road, Tianjin, 300100, China. Fax: +86 022 27435588.
*** Corresponding author. Tianjin key Laboratory of Acute Abdomen Disease Associated Organ Injury and ITCWM Repair, Institute of Acute Abdominal Diseases, Tianjin Nankai Hospital, No.6 Changjiang Road, Tianjin, 300100, China. Fax: +86 022 27435588.
E-mail addresses: zhangqi8501@126.com (Q. Zhang), complement5a@163.com (H. Gao), wangximo@126.com (X. Wang).
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can reduce the granulocytic MDSCs in the peripheral mononuclear cells from patients with pancreatic cancer,[11] this may be achieved by induction of MDSCs apoptosis and necrosis.[12] However, M2 macrophages are increased unexpectedly, while MDSCs decreased significantly after gemcitabine treatment.[13]

Macrophages are critical phagocytic cells that equipped with wide of pathogen-recognition receptors, and play an effect in inflammation and the innate immune response.[14] Macrophages have a strong plasticity and significant functional diversity, and can be classified into classically activated macrophages (M1 macrophages) and alternatively activated macrophages (M2 macrophages) based on their inflammatory and functional status. They are produced after monocyte responses to lipopolysaccharide (LPS)/Interferon-γ (IFN-γ) and activation of Toll-like receptors (TLRs) and interleukin-4 (IL-4)/IL-13, respectively.[15,16] M1 macrophages can produce pro-inflammatory cytokines such as IL-1β, IL-6 and IL-12 through helper T cell 1 (Th1) cell responses. M2 macrophages exert anti-inflammatory effects, promote tissue repair and induce immunomodulatory effects through Th2 cell responses,[17] and these cells secrete epidermal growth factor (EGF), vascular endothelial growth factor (VEGF), transforming growth factor-β (TGF-β) and other factors that promote tumor neovascularization and play an immunosuppressive role,[18] thereby enhancing tumor growth, invasion and metastasis. Previous study have revealed that increased cluster of differentiation (CD)163^+ (M2 macrophage marker in human) tumor-associated macrophages secrete insulin-like growth factors 1 and 2, which activate chemoresistance of pancreatic cancer cells.[19] Therefore, it is important to identify more effective anti-pancreatic cancer drugs, especially with immunoregulatory effects on macrophages.

Coumarin, known as benzopyrone, has important antitumor effects and has even used as a tumor-targeted drug carrier.[20] Osthole [7-methoxy-8-(3-methyl-2-butenyl)-2H-1-benzopyran-2-one], an important natural member of the coumarin family, was extracted from *Cnidium monnieri* (Fructus Cnidii). Increasing evidence has shown that it exerts neuroprotective, osteogenic, anti-inflammatory and antitumor pharmacological effects.[21,22] Previous experiments showed that osthole induced HCC (human hepatocellular carcinoma) cell G2/M phase arrest, triggered DNA damage and suppressed migration in vitro[23]; inhibited osteosarcoma cell proliferation, migration and invasion via the PTEN/Akt signaling pathway[24]; and induced human nasopharyngeal cancer cell apoptosis through the Fas—Fas ligand and mitochondrial pathway.[25] Recently, researchers found that osthole displayed immunomodulatory effects on dendritic cells (DCs) and forkhead box P3-positive regulatory T cells, and inhibited the Th2 response in allergic asthma.[26] Osthole has also been found to promote anti-hepatocellular carcinoma immune responses through elevating tumor-infiltrating CD4+ T and CD8+ T cells.[27]

The role of osthole in pancreatic cancer has not been explored. In this study, we investigated the effects of osthole on cell proliferation, migration and apoptosis in vitro. Furthermore, we explored whether osthole has a regulatory effect on the pancreatic tumor microenvironment and the abundant pancreatic tumor-infiltrating macrophages.

2. Materials and methods

2.1. Cell lines and cell culture

Panc 02 (mouse pancreatic cancer cell) and RAW 264.7 (mouse macrophage) cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM) and RPMI 1640 medium, respectively, containing 100 U/ml streptomycin, 100 U/ml penicillin, and 10% fetal bovine serum (FBS) in a humidified atmosphere of 5% CO_2 at 37 °C. RAW 264.7 cells were stimulated with 20 ng/mL IL-4 (Peprotech, USA) for M2 polarization.

2.2. Animals and an orthotopic model of pancreatic cancer

Six-to eight-week-old female C57BL/6 mice were obtained from the Experimental Animal Center of Military Medical Sciences (Beijing, China), and acclimated for at least 1 week in specific pathogen-free cages before experimentation. All animal experiments were approved by the Animal Ethics Committee of Tianjin Medical University.

Pancreatic cancer orthotopic model: The mouse abdomen was disinfected, and an approximately 1 cm longitudinal incision was made in the left upper abdomen. The tail of the pancreas was fully exposed. Panc 02 cells (5×10^5 cells suspended in 50 μl of PBS) were injected into pancreas using a 1 mL syringe with a 27-gauge needle. Then, the organ was returned, and the abdominal cavity was sutured. Osthole was purchased from Aladdin Biochemical Technology Co., Ltd. (Shanghai, China) and was dissolved in corn oil. Mice were randomly divided into the drug treatment group (n = 9) and the control group (n = 9). From the third day after operation, the mice received daily intraperitoneal injections of osthole (120 mg/kg) or solvent. Mice were sacrificed after 14 days of treatment, and the tumor and spleen were harvested for further study.

2.3. Tissue mononuclear cell isolation

The spleen was ground and filtered through a 40 μm cell strainer (BD Biosciences, USA), and the filtrate was collected and centrifuged at 2000 rpm for 5 min at 4 °C. Erythrocytes were lysed and subjected to flow cytometry.

The tumor tissue was cut into pieces of approximately 1 mm^3 and shaken with digestion solution (0.05 mg/mL each of type-IV collagenase, hyaluronidase and DNase I) (Sigma, USA) at 37 °C for 60 min. After grinding with 200 mesh stainless steel screen, the filtrate was collected and centrifuged at 2000 rpm for 5 min at 4 °C. Tumor infiltrating lymphocytes and monocytes were isolated by Percoll (GE Healthcare, USA) density gradient centrifugation.

2.4. Isolation and induced polarization of primary bone marrow macrophages

The macrophage polarization process was assessed according to a previous study.[28,29] Bone marrow cells were extracted from the tibia and femur of healthy C57BL/6 mice, and were cultured in DMEM with 100 U/ml streptomycin, 100 U/ml penicillin and 10% FBS. Meanwhile, incubated with 10 ng/mL recombinant mouse monocyte colony-stimulating factor (M-CSF) (PeproTech, USA) for 5 days and replaced with fresh media on the third day. IL-4 (20 ng/mL) was then added and incubated for 24 h to induce M2 macrophage polarization. During the polarization process, different concentrations of osthole were added. Macrophage marker expression was detected by flow cytometry through labeling by a FITC anti-mouse CD11b monoclonal antibody, APC anti-mouse F4/80 monoclonal antibody and PE anti-mouse CD206 monoclonal antibody (Sungene Biotech, China).

2.5. Flow cytometry

The isolated immune cells and in vitro differentiated macrophages were stained with antibody at 4 °C in dark for 30 min. NovoCyte flow cytometry and NovoExpress software (ACEA Biosciences, USA) were used for detection and analysis. Cell surface markers were labeled with the following antibodies: FITC anti-

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