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Original Article

Metformin suppresses gastric tumorigenesis by regulating chemokine CXCR4

Weiqiang Wu[1], Heng Xi[2], Feng Gao[1], Feng Song[1], Yong Zhao[1], Ming Xu[1], Xin Zhang[1], Zengqiang Yang[1]

[1]*Department of Colorectal Surgery, Lanzhou General Hospital of Lanzhou Command PLA, Lanzhou, China;*
[2]*Department of Pharmacy, The Third People's Hospital of Chengdu, Chengdu, China*

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Abstract: Objective: To investigate the role of metformin in the treatment of gastric cancer. Methods: *In vitro*, following metformin exposure, the proliferation of MFC cells was determined using MTT assay, and the mRNA and protein levels of CXCR4 were detected by real-time quantitative PCR and western blot, respectively. *In vivo*, thirty-six mice were randomly divided into two groups, the experimental group and the control group (n = 18), followed by i.p. injection of gastric cancer cells. The experimental group was treated with metformin (10 mg/kg), while the control group was injected i.v. with saline. The tumor volume was measured. Results: Results from real-time quantitative PCR and western blot showed that both CXCR4 mRNA and protein levels were significantly suppressed by metformin, which resulted in reduced gastric cancer cell viability *in vitro*. Moreover, metformin inhibited tumor growth *in vivo*. Conclusion: Our findings suggest that metformin inhibits gastric tumor cell growth via decreasing CXCR4 both *in vitro* and *in vivo*.

Keywords: Metformin, gastric cancer, CXCR4

Introduction

Gastric cancer is now the second-leading cause of cancer-related deaths worldwide, and the prognosis of advanced gastric cancer is poor [1, 2]. Apart from potentially curative surgery, chemotherapy and radiochemotherapy may be applied at advanced stages of gastric cancer but neither of these can be curative [3, 4]. Thus, there is a strong demand for new effective approaches to advanced gastric cancer.

Metformin is a biguanide family member commonly used in the treatment of type 2 diabetes. It increases liver and peripheral tissue sensitivity to insulin and reduces hepatic glucose production [5]. According to a recent epidemiologic survey, metformin inhibits tumorigenesis. However, the molecular mechanism underlying the suppression of cancer growth by metformin remains relatively unknown.

Recent data suggest that certain chemokines and their receptors, in particular stromal cell-derived factor (SDF)-1 and CXC chemokine receptor 4 (CXCR4), play an important role in the behavior of cancer cells and modulate cell migration, proliferation, and survival. It has been shown that neutralizing the interaction of SDF-1 and CXCR4 significantly impairs the metastasis of breast cancer cells to regional lymph nodes and lung *in vivo*, suggesting that chemokines and their receptors have a critical role in determining the metastatic destination of tumor cells [6].

Here, we showed that metformin inhibits gastric cancer cell growth by decreasing CXCR4 expression.

Materials and methods

Animals

Total 36 female 615 inbreeding mice (6-8 weeks) were purchased from Dalian Medical University (Dalian, China). Animals were fed with a commercial diet and maintained in standard conditions of 12 h light/darkness and at room temperature (23-25°C). All the protocols were approved by the animal care and use committee, and guidelines for the care and use of laboratory animals were based on published by the National Academy Press.

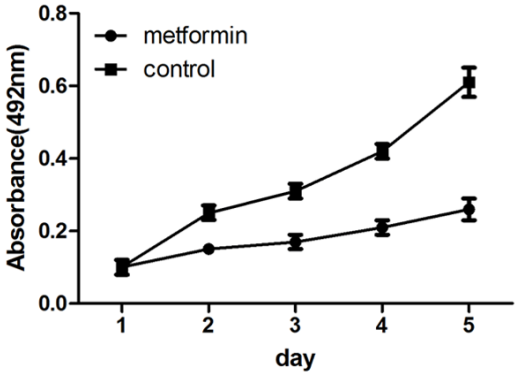

Figure 1. Metformin inhibits MFC cell proliferation. The effect of metformin on MFC cell proliferation was determined by MTT assay. Control, saline.

Cell line

Mouse gastric cancer cells MFC were obtained from Chinese Academy of Sciences Typical Culture Preservation Commission Cell Bank (Shanghai, China). MFC cells were routinely cultured in RPMI-1640 (GIBCO) supplemented with 10% fetal bovine serum (Hyclone Laboratories) at 37°C in an incubator with 5% CO_2. Culture medium was changed every 1-2 days.

Real-time PCR

Total RNA was isolated from MFC cells using Trizol reagent (Invitrogen), and was reverse-transcribed to cDNA. The forward primer of CXCR4 gene was 5'-GCCTGAGCTACAGATGCCCA-3', and the reversed primer was 5'-TTCGGGTCAATGCACTTGT-3'. The glyceraldehydes phosphate dehydrogenase (GAPDH) gene was used as internal reference, and the primers were: 5'-GAAGGTGAAGGTCGGAGTC-3' and 5'-GAAGATGGTGATGGGATTTC-3'. The real time-PCR (RT-PCR) was performed on ABI Prism 7700 PCR instrument (Applera Corporation Norwalk). The amplification conditions were 95°C for 5 min; 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; and a terminal extension step at 72°C for 10 min. After the reaction, 5 μL DNA was analyzed by agarose gel electrophoresis, and the gel image was scanned and the gray scale was measured. The ratio of CXCR4 and GAPDH was expressed as their relative expression.

Western blotting

The protein was extracted from MFC cells, and the protein concentration was measured using Brandford method. Samples were separated using SDS-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to polyvinylidene difluoride (PVDF) membrane. After blocking with 5% defatted milk, the membrane was incubated with mouse anti-CXCR4 monoclonal antibody (1:1000) at 4°C overnight, followed by incubation with HRP-labeled goat anti-mouse IgG (1:5000) at 37°C for 45 min. Protein bands were visualized with ECL development. The images were analyzed by measuring the gray scale, and the ratio of CXCR4 and GAPDH was expressed as their relative protein expression.

MTT assay

Cell growth inhibition was determined by modified microculture tetrazolium (MTT) assay based on the enzymatic reduction of 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (Sigma) to form formazan crystal by mitochondria and cellular dehydrogenase enzymes. The OD value (A) was measured at a wavelength of 570 nm on the Microplate Reader EXL2800. Inhibition rate (%) = control group A-experimental group A/control group A × 100%.

In vivo assay

The 615 inbreeding mice were randomly assigned to two groups (n = 18 per group), the experimental group and the control group. MFC cells in logarithm growth phrase were harvested and washed twice with PBS. A total of 5.0 × 10^6 cells in 200 μL serum-free medium with a viability of > 95% tested by staining with try-pan blue were injected into the back of each mouse in these two groups. The mice were observed every two days, and were sacrificed 28 days post-inoculation.

Statistic analysis

The data were expressed as means ± standard deviation ($\bar{x}$±s). Statistical comparisons were performed by student's t-test. $P < 0.05$ was considered statistically significant.

Results

Metformin inhibits gastric cancer cell proliferation

The effect of metformin on gastric cancer cell growth was determined using MTT assay. Sig-

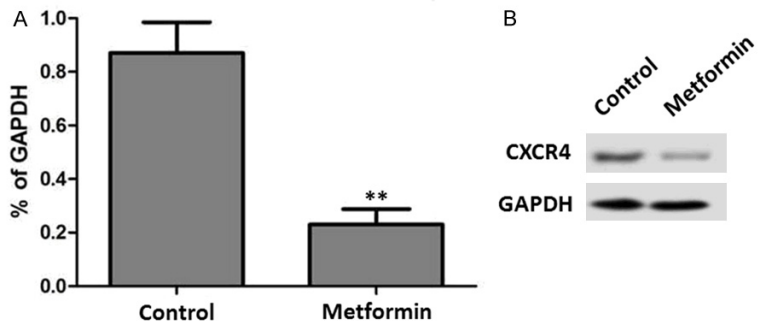

Figure 2. Metformin inhibits CXCR4 expression. A. The effect of metformin on CXCR4 mRNA expression. CXCR4 mRNA levels were determined by real-time quantitative PCR. B. The effect of metformin on CXCR4 protein expression. CXCR4 protein levels were determined by western blot. Control, saline.

nificant time and dose-dependent growth inhibition was observed in gastric cancer cells. The result of MTT test showed that metformin significantly suppressed cell proliferation of MFC cells ($P < 0.01$) (**Figure 1**).

Metformin inhibits CXCR4 expression

The RT-PCR result showed that metformin exposure resulted in a significant reduction of CXCR4 mRNA levels in MFC cells ($P < 0.05$) (**Figure 2A**). In agreement with this result, western bolt analysis showed that the CXCR4 protein expression was also significantly decreased after metformin treatment ($P < 0.05$) (**Figure 2B**).

Metformin inhibits the tumor growth and prolongs the survival time

To further examine the effect of metformin on gastric tumor growth *in vivo*, MFC cells were injected subcutaneously to the back of inbreeding mice. After inoculation for 7 days, the experimental group (n = 18) was injected i.v. with 10 mg/kg metformin, while the control group (n = 18) received an injection of saline. The tumor volume was significantly decreased after metformin exposure, as compared to the control group. In contrast, the survival time was significantly increased in the metformin-treated group, compared with the control group ($P < 0.05$) (**Figure 3A** and **3B**).

Discussion

In this study, we demonstrated that metformin reduced the gastric cancer cell proliferation and tumor growth by inhibition of CXCR4 gene and protein expression.

Metformin, one of most widely prescribed oral hypoglycemic agents, enjoys wide use in the management of type 2 diabetes. Clinical trials with metformin have indicated improvement in cardiovascular events in diabetic patients apparently beyond its glucose-lowering properties [7, 8]. Recently, metformin has received increased attention because of its potential antitumorigenic effects. Several potential mechanisms have been suggested for the its suppressive effects on cancer growth: (1) activation of LKB1/AMPK pathway; (2) induction of cell cycle arrest and/or apoptosis; (3) inhibition of protein synthesis; (4) reduction in circulating insulin levels; (5) inhibition of the unfolded protein response (UPR); (6) activation of the immune system; and (7) eradication of cancer stem cells [9]. Earlier studies showed that phenformin, a biguanide that was only briefly used in humans secondary to its increased propensity to cause lactic acidosis, could result in better inhibition of tumor cell growth, when added to conventional chemotherapeutic agents [10-12]. Epithelial tumor cells exploit several mechanisms including chemokine systems that normally regulate leukocyte trafficking and homing. The distinct pattern of chemokine receptor expression by tumor cells plays a critical role in tumor biology. In particular, CXCR4 expression is associated with more aggressive behaviors of various tumor cells [13-15]. CXCR4 is widely expressed on hematopoietic cells including $CD34^+$ HSC, T-lymphocytes, B-lymphocytes, monocytes and macrophages, neutrophils and eosinophils as well as by brain, lung, colon, heart, kidney, and liver, and endothelial and epithelial cells, microglia, astrocytes and neuronal cells, and progenitor cells including endothelial and smooth muscle progenitors. Functional CXCR4 is expressed on embryonic pluripotent stem cells and several types of tissue-committed stem cells, for example, neural tissue, skeletal muscles, heart, liver, endothelium, and renal tubular- and retina pigment-epithelium [16]. The expression of CXCR4 on malignant epithelial cells and on cells from several hematopoietic malignancies implies that the CXCL12/CXCR4

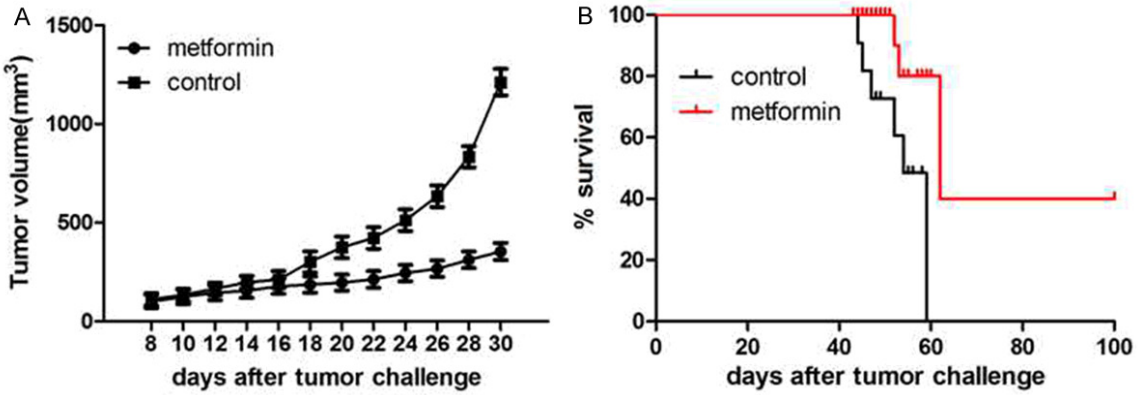

Figure 3. Metformin inhibits gastric tumor growth *in vivo*. A. The effect of Metformin on gastric tumor growth. B. The effect of Metformin on mouse survival rate. Control, saline.

pathway may influence cancer biology and play a pivotal role in directing the metastasis of CXCR4+ tumor cells to organs that express CXCL12 (e.g., lymph nodes, lungs, liver, or bones). Several CXCR4+ cancers metastasize to the bones and lymph nodes in a CXCL12-dependent manner, in which the bone marrow provides a protective environment for tumor cells [17]. Therefore, we investigated the expression and function of CXCR4 in gastric cancer cells, and attempted to correlate clinicopathological factors of gastric cancer with CXCR4 expression.

We found that MFC cells contained CXCR4 transcripts and cytosolic CXCR4 protein, but did not express membrane CXCR4. CXCR4 plays an important role in the spread and progression of sarcomas, neuroblastomas, and melanomas, and cancers of the breast, ovary, bladder and cervix [18, 19]. Genetic lesions and aberrant signaling networks within cancer cells have been the main focus in cancer biology over the past decades. As a consequence of this oncogene- and tumor suppressor-centric view, most current cancer therapies target the tumor cells, which frequently acquire resistance owing to their inherent genomic instability. Signals from the tumor microenvironment may make pivotal contributions to the progression of hematopoietic and epithelial malignancies, thus increasing emphasis is being placed on targeting the tumor cell microenvironment. The CXCR4-CXCL12 axis plays a central role in the spread and progression of many different types of tumors [20, 21]. Therefore, the CXCR4-CXCL12 axis may be involved in the role of metformin in the treatment of gastric cancer. However, more evidence should be explored.

In the present study, we showed that metformin inhibits the gastric cancer cell proliferation and tumor growth, and prolongs the survival time by downregulating CXCR4 expression.

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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Weiqiang Wu, Department of Colorectal Surgery, Lanzhou General Hospital of Lanzhou Command, PLA, 333 Nanbin Road, Qilihe District, Lanzhou 730050, China. Tel: +86-15117202099; E-mail: weiqiangewu@126.com

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