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Tumor-associated macrophages promote the metastasis of ovarian carcinoma cells by enhancing CXCL16/CXCR6 expression

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ABSTRACT

This study investigated the underlying mechanism by which C-X-C motif chemokine ligand 16 (CXCL16)/C-X-C motif chemokine receptor 6 (CXCR6) signaling is activated by tumor-associated macrophages and assists in regulating the metastasis of ovarian carcinoma. Specimens of ovarian carcinoma tissue and adjacent tissue were collected from 20 ovarian carcinoma patients. Human THP-1 cells were induced to differentiate into macrophages, which were then co-cultured with SKOV3 cells and low concentrations of tumor necrosis factor-α (TNF-α) to simulate the inflammatory microenvironment of ovarian carcinoma. Additionally, small interfering RNA (siRNA) targeting CXCR6 was transfected into SKOV3 cells; after which, the levels of nuclear factor kappa B p65 (NF-κB p65) protein and phosphorylated PI3K and Akt were measured. The migration and invasion abilities of the SKOV3 cells were also tested. The levels of TNF-α, interluekin-6 (IL-6), NF-κB p65, CXCL16, and CXCR6 expression in the ovarian carcinoma tissues were higher than those in the precancerous tissues. CXCR6 expression was positively correlated with TNF-α, IL-6, and CXCL16 expression. Co-culture of SKOV3 cells with macrophages significantly promoted CXCL16, CXCR6, NF-κB, and p65 expression by the SKOV3 cells, increased their levels of phosphorylated PI3K and Akt, and increased the migration and invasion abilities of SKOV3 cells. Silencing of CXCR6 or blocking the PI3K/Akt signal pathway markedly attenuated the expression of NF-κB p65 and phosphorylation of PI3K and Akt, as well as the migration and invasion abilities of SKOV3 cells. These findings demonstrate that macrophages can promote the migration and invasion of ovarian carcinoma cells by affecting the CXCL16/CXCR6 pathway.

1. Introduction

Ovarian carcinoma is a common gynecological cancer worldwide; however, it is usually diagnosed at an advanced stage and has a poor prognosis [1]. Although great advances have been made in medical technology, the overall survival rate of patients with advanced ovarian carcinoma has shown no obvious improvement [2], and patients with stage III or IV disease have only a ~ 20% survival rate [3]. The incidence of clinically diagnosed ovarian carcinoma is rapidly increasing, and in 2015, approximately 225,000 women in China died from the disease [4]. Due to the limited diagnostic capabilities of many medical facilities in China, the survival rate of ovarian carcinoma patients in China might be lower than the world-wide average. Therefore, there is an urgent need to understand the underlying pathological mechanism of ovarian carcinoma and explore new methods of treatment. Metastasis is an important characteristic of carcinomas, and the tumor's microenvironment plays a critical role in enabling the migration and invasion of carcinoma cells [5,6]. Macrophages are an important components of a tumor's microenvironment, and play key roles in regulating the growth and metastasis of cancers [7]. Zhou et al. [8] reported that increased levels of the autocrine growth factor HB-EGF can short-circuit the tumor cell/macrophage paracrine regulatory loop and thereby promote the invasion of breast cancer cells via macrophage signaling [8]. Macrophage recruitment also enhanced ovarian carcinoma growth [9]. Macrophage M2 polarization was shown to enhance the metastasis of non-small cell lung cancer cells by activating the ERK signaling pathway [10]. Moreover, the targeting of NF-κB suppressed colon cancer peritoneal metastasis by enhancing the M1-like macrophage phenotype [11]. Additionally, interleukin-34 was shown to facilitate macrophage recruitment and angiogenesis to promote the growth and metastasis of osteosarcoma [12]. Toy et al. [13] demonstrated that activated macrophage colony-stimulating factor receptors

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are a biomarker for the poor prognosis of advanced ovarian cancer patients [13], and Robinson-Smith et al. [14] reported that macrophages mediate the metastasis of mouse ovarian tumors in an inflammation-dependent manner [14]. However, the exact role played by macrophages in the metastasis of ovarian cancer is not fully understood.

CXCR/CXCL16 plays crucial roles in regulating the development and metastasis of cancers [15]. CXCL16 is one of the few scavenger receptors that exists in both membrane-bound and soluble forms [16]. Specifically, the membrane-bound form promotes cell adhesion to express its cognate receptor, CXCR6 [17]. Upregulation of CXCR6 drives the metastasis of hepatocellular carcinoma by regulating its pro-inflammatory tumor microenvironment [18]. Targeting of the CXCR/CXCL16 axis inhibits the metastasis of prostate cancer [19]. Furthermore, the CXCR6/CXCL16 axis drives the progression of breast cancer by activating the ERK1/2 signaling pathway [20]. In addition, elevated CXCL16 levels are significantly correlated with a poor prognosis for ovarian cancer patients [21], and the CXCR6/CXCL16 axis is significantly correlated with the lymph node metastasis in ovarian carcinoma [22]. Despite all these findings, the underlying pathological mechanism of CXCR6/CXCL16 in ovarian carcinoma remains unclear.

Our current study explored the expression of CXCR6 and CXCL16 in ovarian carcinoma tissue and ovarian carcinoma cells that were co-cultured with macrophages. Moreover, we also investigated how CXCR6/CXCL16 influences cell behavior and the underlying mechanism for those affects. We hope that our findings provide new insights into the pathology and treatment of ovarian carcinoma.

2. Materials and methods

2.1. Clinical samples

From October 2016 to December 2017, 20 pairs of ovarian cancer tissue samples and matched adjacent normal tissue samples were collected at the Hainan General Hospital for use in this study. All samples were confirmed by biopsy, and none of the donors had received chemoradiotherapy. The study protocol was approved by the Ethics Committee of Hainan General Hospital, and all enrolled patients provided their signed written Informed Consent.

2.2. Cell culture

Human monocyte THP-1 cells were cultured in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) containing 10% of fetal bovine serum (FBS, Gibco), 10 mM Hepes (Gibco), 1 mM pyruvate (Gibco), 50 pM β-mercaptoethanol (Gibco), and 2.5 g/L D-glucose (Merck, Kenilworth, NJ, USA). The THP-1 monocytes were then incubated with 100 ng/mL phorbol ester (Sigma, St. Louis, MO, US) for 72 h at 37 °C with 5% CO_2 to stimulate their differentiation into macrophages. SKOV3 ovarian cancer cells were purchased from the Institute of Biochemistry and Cell Biology of the Chinese Academy of Sciences (Shanghai, China). The cells were maintained in PRIM 1640 medium that contained 10% FBS (Gibco), 100 mg/mL streptomycin (Invitrogen, Carlsbad, CA, USA) and 100 U/mL penicillin in a humidified 37 °C incubator with 5% CO_2. Next, the cells were divided into three groups: SKVO3, SKOV3 + TNF-α (100 ng/L), and SKOV3 + macrophages (THP-1 induced for 72 h) + TNF-α (100 ng/L) for use in the following investigations. Macrophages were cultured in the upper chambers of a Transwell plate and SKOV3 cells were added to the lower chambers. Additionally, a PI3K inhibitor (LY294002, Sigma) and an AKT inhibitor (MK-2206, Sigma) were added during the treatment of SKOV3 cells with macrophages and TNF-α for 24 h to further confirm our findings regarding the PI3K/Akt signaling pathway.

2.3. CXCR6 silencing in SKOV3 cells

SKOV3 cells were seeded into a 6-well plate at a density of 2×10^5/cells per mL and incubated overnight. Cells at approximately 70–75% confluence were transfected with a scrambled or CXCR6-shRNA plasmid by using Lipofectamine 2000 (Invitrogen). The following two CXCR6-shRNA sequences were designed for use in the study: CXCR6-siRNA1, 5′-CTGAGGACAATTCCAAGACT-3′; CXCR6-siRNA2, 5′-GCTTTGCTCATCTGGGTGATAT-3′ (GENECHEM, Shanghai, China). After transfection, the SKOV3 cells were divided into the following three groups for use in subsequent investigations: control SKOV3 cells + macrophages + TNF-α, siRNA1-SKOV3 cells + macrophages + TNF-α, and siRNA2-SKOV3 cells + macrophages + TNF-α.

2.4. Quantitative real-time polymerase chain reaction (RT-qPCR)

The total RNA in tissue samples or cells was extracted using TRIzol regent (TakaRa, Dalian, China) according to manufacturer's protocol, and then reverse transcribed into cDNA using a PrimeScript™ RT-PCR kit (TakaRa, Dalian, China). The expression levels of various genes were examined using an iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA) and SYBR Green Master Mix according to the manufacturer's instructions. The reaction conditions were as follows: 95 °C for 5 min, and 40 cycles of 95 °C for 10 s, 60 °C for 20 s, and 72 °C for 10 s. GAPDH was used as an internal control. The relative levels of gene expression were analyzed using the $2^{-\Delta\Delta CT}$ method [23]. The primers used in the RT-qPCR studies are shown in Table 1.

2.5. Western blotting

Tissue or cell samples were lysed with RIPA lysis buffer (Millipore, Billerica, MA, USA) containing 10% PMSF (Sigma) and a protease inhibitor cocktail (Calbiochem) at 4 °C for > 30 min. The lysates were centrifuged at 12,000 rpm and 4 °C for 15 min, and the supernatant fractions were harvested and concentrated. The amount of protein in each supernatant was quantified using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Next, each supernatant was boiled with loading buffer for 10 min, and 20 μg of total protein was loaded onto a SDS polyacrylamide get and separated by electrophoresis. The separated protein bands were transferred onto a polyvinylidene (PVDF) membrane, which was then blocked with 5% bovine serum albumin (BSA) at room temperature for 1 h. The membrane was incubated with the following antibodies: anti-CXCR6, 1:2000; anti-CXCL16, 1:1000; anti-NF-κB p65, 1:1000; anti-p-PI3K, 1:1500; anti-p-Akt, 1:500; anti-GAPDH, 1:10,000 (all purchased from Abcam, Cambridge, MA, USA). Next, the membrane was rinsed 3 times (5 min per rinse) with TBST (10 mL of 1 mol/L Tris-HCL, pH 7.5, 8.8 *g* NaCl, and 1 mL of Tween-20 in a total volume of 1 L) and then incubated with anti-rabbit or anti-mouse secondary antibody (1:20,000) at room temperature for 40 min. Thereafter, the membrane was rinsed three more times with BST (10 min per rinse). Finally, the protein bands in the membrane were visualized with enhanced chemiluminescence (ECL) reagent (Beyotime Institute of Biotechnology, Jiangsu, China).

Table 1
Primer sequence used for RT-qPCR.

ID	Sequence(5'- 3')
GAPDH Former	TGTTCGTCATGGGTGTGAAC
zGAPDH Reverse	ATGGCATGGACTGTGGTCAT
IL-6 Former	ACTCACCTCTTCAGAACGAATTG
IL-6 Reverse	CCATCTTTGGAAGGTTCAGGTTG
TNF-α Former	AGCCTGTAGCCCATGTTGTA
TNF-α Reverse	GAGGTACAGGCCCTCTGATG
CXCL16 Former	CCCGCCATCGGTTCAGTTC
CXCL16 Reverse	CCCCGAGTAAGCATGTCCAC
CXCR6 Former	GACTATGGGTTCAGCAGTTTCA
CXCR6 Reverse	GGCTCTGCAACTTATGGTAGAAG