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Th17 cells regulate the production of CXCL1 in breast cancer

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ABSTRACT

Recently, the link between inflammation and cancer has been targeted for the prevention or treatment of malignant tumours. We aimed to investigate the relationship between Th17 cells and CXCL1 in breast cancer and the biological effects of CXCL1 on breast cancer. *In vivo*, the Th17 cell frequency in the peripheral blood was determined by flow cytometry. Secretion of IL-17 and CXCL1 in the blood serum was determined by enzyme-linked immunosorbent assay (ELISA). Expression of IL-17A and CXCL1 mRNA was determined by qRT-PCR. *In vitro*, the effects of Th17/CXCL1 during breast cancer were assessed in the human breast cancer cell lines MCF-7 and MDA-MB-231. Cell proliferation was measured using the CCK8 assay. Cell invasion and migration ability were assessed using a transwell cell invasion and wound- healing assay. *In vivo*, Th17 cells and CXCL1 were increased in breast cancer patients. Moreover, their changes were correlated in breast cancer cells. Th17 cells upregulate the production of CXCL1 during breast cancer progression. CXCL1, which is produced by breast cancer cells, can promote cancer growth and development, and may also point to a specific histogenetic pathway.

1. Introduction

Cancer is a disorder of hyperproliferation involving morphological cell transformation, uncontrolled proliferation, dysregulated apoptosis, invasion, metastasis and angiogenesis [1]. Breast cancer is the most common malignant tumour and the most frequent cause of cancer-related death among women in the global world, leading to an estimated 1.7 million cases and 521,900 deaths in 2012 [2]. As the morbidity and mortality increase, breast cancer has drawn substantial attention worldwide.

Recently, the link between inflammatory microenvironment and cancer has been targeted for the prevention or treatment of malignant tumours [3]. The inflammatory response plays a critical role at different stages of tumour progression, including initiation, proliferation, invasion, metastasis and angiogenesis, and it also affects immune surveillance. Immune cells infiltrating into tumours engage in a dynamic and complex crosstalk with tumour cells; many molecular events that participate in this process have been revealed [4]. The tumour microenvironment, which is formed largely by inflammatory cells, is a key component in the survival, proliferation and migration of cancer cells [3]. Many factors that function as tumour promoters activate immune effector programmes, leading to the infiltration of tissues by immune cells; when sustained without resolution long-term, the damage of these tissues becomes chronic and fuels tumour development by various mechanisms [5]. In addition, tumour cells have cooperated with some signalling molecules in the innate immune system such as cytokines, selectins, chemokines and their receptors for proliferation, migration, invasion and metastasis [6]. The inflammatory response represents a correlation between intrinsic factors (genome stability genes, oncogenes, and tumour suppressors) and extrinsic factors (immune and stromal elements) that contribute to tumour development [5]. Currently, the relationship between inflammation, the immune response and cancer is increasingly accepted, but the clear mechanism underlying this relationship is not well understood [4].

Recently, an independent subset of effector T helper cells termed Th17 cells has been regarded as a potent inducer of tissue inflammation. These cells exhibit different effector functions from Th1 and Th2 cells, which was first identified by their ability to produce interleukin 17A (IL-17A) specifically and thus they are, associated with chronic inflammation and the immune response [7–9]. During Th17 cell

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differentiation programmes, both transforming growth factor-β (TGF-β) and IL-6 are required to activate the unique transcription factor named retinoid-related orphan receptor-γt (RORγt). RORγt cooperates with other transcription factors to increase the expression of both IL-23R and IL-17A in Th17cells. It induces the transcription of the genes encoding IL-17 and other Th17 cytokines in naive $CD4^+$ T helper cells and is required for their expression [10–12]. Th17 cells mainly produce IL-17A, IL-17F, IL-21, IL-22, IFN-γ and GM-CSF as well as TNF-α [13–15]. Th17 cells are primarily considered to be important for the clearance of pathogens that are not handled completely by Th1 or Th2 cells [11]. As the main effector cytokine of Th17 cells, IL-17 is the major regulator of Th17-mediated inflammation. Thus, IL-17 plays a critical role in bridging the innate and adaptive immune responses by regulating the production of various cytokines, chemokines and other pro-inflammatory mediators to sustain chronic inflammation [16]. Chronic activation of leucocytes provides direct or indirect mitogenic growth factors which stimulate development of cancer cells [17]. Chemokines are chemotactic cytokines that direct leucocytes to move into tissues and therefore engage in a series of biological processes, including organ development, homeostasis, angiogenesis, immune activation and regulation [18].

Solid tumours not only contain tumour cells and kinds of stromal cells (such as fibroblasts and endothelial cells), but are also infiltrated by many inflammatory cells (such as macrophages, neutrophils and lymphocytes), tumour cells, stromal cells, and tumour-associated leucocytes that contribute to the local production of tumour-derived chemokines that further determine the infiltration of leukocytes into the tumour [19,20]. They activate a subset of seven-transmembrane, G protein-coupled receptors (GPCRs) and regulate the recruitment of inflammatory cells to the site of the injury or immune response [21,22]. Chemokines are divided into four subfamilies, CXC, CC, (X)C, and CX3C, according to the arrangement of the N-terminal two cysteine residues [23]. Based on the tripeptide glutamic acid-leucine-arginine (Glu-Leu-Arg; ELR) motif that precedes the CXC domain, the CXC chemokines can be further divided into ELR^+ and ELR^- chemokines [24]. An ELR^+CXC chemokine named CXC chemokine ligand 1 (CXCL1), also termed growth-related oncogene α (GROα), was initially identified from culture supernatants of melanoma cell lines [25]. Many studies have suggested that CXCL1 binding to its receptor CXCR2 plays a pro-tumour role in the initiation and promotion of malignant tumours [26–28]. However, whether CXCL1 could promote breast cancer development and the detailed mechanism involved is unclear, and it remains unknown whether a relationship exists between CXCL1 and inflammation modulated by Th17 cells? The sequestions will be investigated in this study.

2. Materials and methods

2.1. Tissue and blood acquisition

Our study included 46 patients who were diagnosed with breast cancer at the Breast Centre at the Affiliated Hospital of Qingdao University Medical College from January 2014 to December 2015. All patients were women with an age ranging from 23 to 65 (mean age: 52.90 ± 6.94) years. We collected fresh breast cancer (BC) tissues, the adjacent non-cancerous breast (AN) tissues (3–5 cm) and distal normal breast (DN) tissues (> 5 cm). Peripheral blood samples were collected from the above patients and 20 healthy donors.

2.2. Breast cancer cell culture and treatment

The breast cancer cell lines MCF-7 and MDA-MB-231 were supplied by the Department of Biochemistry, Qingdao University Medical College. Two cell lines were cultured in RPMI 1640 (HyClone, USA) supplemented with 10% Fetal Bovine Serum (FBS) (HyClone, USA) and 1% penicillin-streptomycin (Solarbio, Beijing, China) in a 37 °C, 5% CO_2 incubator. The cells were treated with 80 ng/ml recombinant human CXCL1 (rhCXCL1) (PeproTech, USA) and anti-CXCR2 neutralizing antibody (Boster, Wuhan, China). The control group was cultured without any treatment.

Table 1
Primer sequences.

Gene	Primer sequence
IL-17A	F:5′-GCCATAGTGAAGGCAGGA-3′ R:5′-CTCATTGCGGTGGAGATT-3′
CXCL1	F:5′-CATCCAAAGTGTGAACGTGAA-3′ R:5′-GATGCAGGATTGAGGCAAG-3′
RORγT	F:5′-AGGCTCTCAGGCTTTATGGAG-3′ R:5′-TGCGGTTGTCAGCATTGTAG-3′
GAPDH	F:5′-TCTCTGCTCCTCCTGTTC-3′ R:5′-ACTCCGACCTTCACCTTC-3′

2.3. Ethics statement

This project was approved by the Ethics Committee of Qingdao University Medical College. All experiments involving humans were performed in accordance with the principles of Declaration of Helsinki. Written consents were obtained from each patient and healthy donor.

2.4. Histopathologic analysis and haematoxylin-eosin (HE) staining

Clinical data were collected from the patients. Fresh tissue samples were washed with PBS, followed by 4% paraformaldehyde. The paraformaldehyde-fixed samples were dehydrated in increasing concentrations of ethanol-xylene and embedded in paraffin. The obtained paraffin blocks were cut into 4 μm slices, and placed on glass, and then the samples were deparaffinized and rehydrated by passing through a series of xylenes and alcohols solutions. The sections were stained with haematoxylin and eosin (HE) by the standard procedures. Stained sections were examined under the microscope at 400× magnification and photographed.

2.5. Immunohistochemistry (IHC) evaluation of protein expression in tissue samples

IHC is the primary technique used to determine the protein expression status in tissue samples. It is a simple, fast, easy and relatively inexpensive method for protein detection. Formalin-fixed and paraffin-embedded tissues were examined using the avidin-biotin complex method. The sections were incubated primary antibody at 4 °C for 40 h. Subsequently, the sections were incubated with a biotin-labelled secondary antibody at 37 °C for 60 min. Colour development was accomplished by exposure to 3, 3′-diaminobenzidinetetrahydrochloride (DAB) for 40 s to 1 min. Finally, the sections were dehydrated and mounted for microscopic observation. Three to five fields (100× and 400×) were selected in each sample. Finally, the expression of IL-17, CXCL1, CXCR2, EGFR, Cyclin D1, Bcl-2, MMP-9, VEGF, pAKT and pNF-κB were analysed and evaluated by the average optical density (AOD), as measured by ImageJ software.

Rabbit anti-human IL-17 antibody, rabbit anti-human CXCL1 antibody, rabbit anti-human CXCR2 antibody, rabbit anti-human EGFR antibody, rabbit anti-human CyclinD1 antibody, rabbit anti-human Bcl-2 antibody, rabbit anti-human MMP-9 antibody, rabbit anti-human VEGF antibody, rabbit anti-human pAKT antibody, rabbit anti-human pNF-κB antibody and sheep anti-rabbit IgG were purchased from Bioss Biological Technology (Beijing, China).