Biomedicine & Pharmacotherapy 77 (2016) 142–149

Available online at
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Original article

CCL5 secreted by tumor associated macrophages may be a new target in treatment of gastric cancer

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ARTICLE INFO

Article history:
Received 22 October 2015
Accepted 11 December 2015

Keywords:
Tumor associated macrophages (TAMs)
CD68
CCL5
Gastric cancer
AGS

ABSTRACT

Aim: To investigate the role of CCL5 secreted by tumor associated macrophages (TAMs) in gastric cancer, and to explore how CCL5/CCR5 axis modulates phenotypes of gastric cancer cells.
Methods: Expression of CCL5 and TAM surface marker CD68 in gastric cancer tissues was examined using SP immunohistochemistry. Serum CCL5 levels of patients were assessed using ELISA. Cross-analyses of CCL5 and CD68 expression with clinicopathological data were done. Correlation between CCL5 and CD68 in gastric cancer tissues was also studied. In vitro functional characterization of CCL5 in gastric cancer was done in co-culture of AGS and THP-1 derived macrophages using MTS assay, plate clone formation assay, and transwell experiment. Expression of chemokines and its receptors were detected by RT-PCR, while Stat3 phosphorylation and downstream target proteins were studied using western blot.
Results: CCL5 and CD68 were both highly expressed in tissues gastric cancer, of which the expressions were positively correlated with each other, and of clinical importance, were associated with the depth of invasion, lymph node metastasis, TNM staging and tumor differentiation. Serum CCL5 was also elevated in patients with gastric cancer comparing to healthy volunteers. Co-culture of AGS cells with THP-1 derived macrophages increased cell proliferation, clone forming ability as well as migration of AGS cells. Migration of AGS cells across transwell membrane was also enhanced by increasing exogenous CCL5. Meanwhile, mRNA expression of CCL5, MMP2, MMP9, and CCR5 was also highly expressed in the cells. Stat3 signaling as reflected by its phosphorylation was also increased in AGS cells upon co-culture with THP-1 derived macrophages.
Conclusion: CCL5 secreted by TAMs may promote the proliferation, invasion and metastasis of gastric cancer cells, in which Stat3 signaling pathway is likely to play an important role. The correlation of CCL5 with clinicopathological parameters suggested CCL5 holds promise as important molecular marker of gastric cancer staging and disease progression.

1. Introduction

Gastric cancer is one of the most common malignancies in China, of which the incidence accounts for 47.8% of the gastric cancer-related deaths worldwide, ranking the first among common cancers in East Asia [1]. At present, chemotherapy and surgical intervention are available for patients with gastric cancer, but the 5-year survival rate is only around 50%. The relatively low survival rate of gastric cancer comparing to other malignant diseases is due to its high rate of invasion and metastasis [2]. Therefore, it is essential to study the specific mechanisms of gastric cancer development in order to allow early prediction and intervention. Tumor associated macrophages (TAMs) has been proposed to play important roles in tumorigenesis and disease progression. Wu et al. reported that TAMs promoted angiogenesis and lymph node metastasis of gastric cancer through the expression of VEGF and VEGF-C [3]. Despite the finding, the underlying mechanisms by which TAMs promote proliferation, migration and invasion of gastric cancer cells remain to be studied.

Evidences from accumulating studies have implicated the role of chemokines and their respective receptors in the regulation of tumor local inflammation and inhibition of immune system upon anti-tumor response [4]. Chemokine ligand 5 (CCL5/RANTES) belongs to the CC chemokine family, and is recognized by CCR1, CCR3 and CCR5 receptors [5]. CCL5 is mainly expressed in T cells, macrophages, platelets, synovial fibroblasts, renal tubular epithelial cells and some types of tumor cells [6]. CCL5 plays an important role in cancers and inflammatory diseases. Expression of CCL5 was directly related to the disease progression in breast cancer patients [7–12]. In addition, CCL5 levels in tissue and serum were

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http://dx.doi.org/10.1016/j.biopha.2015.12.004
0753-3322/

prognostic to the progression of cervical cancer, ovarian cancer, prostate cancer, pancreatic cancer, lung cancer and melanoma [8–10]. There are few studies suggesting the relationship between CCL5 and gastric cancer, but how CCL5 would contribute to gastric cancer development has yet to be elucidated [13–18].

The present work aimed to delineate the relationship between CCL5 and TAMs in gastric cancer, and to characterize the functional role of CCL5 secreted from TAMs in gastric cancer cell. We first showed that CCL5 and CD68 were highly expressed in gastric cancerous tissues in which both positively correlated with each other and were associated with cancer phenotypes. CCL5 was elevated in serum samples of patients with gastric cancer as well. The level was closely related to the clinical stage, prognosis and distant metastasis of gastric cancer. In vitro studies demonstrated that CCL5 from TAMs modulated proliferation, invasion and metastasis of gastric cancer cells through STAT3 signaling. Our work collectively provides new insights into therapeutic target for treating gastric cancer.

2. Materials and methods

2.1. Clinical gastric cancer samples

Cancer tissues were surgically harvested from 48 patients admitted in The Fourth Hospital of Hebei Medical University in 2013. The cohort included 28 males and 20 females with ages ranged from 50 to 70 year old. Serum samples were collected from 50 patients (26 males and 24 females) admitted in the same medical center from October 2013 to March 2014. Gastric cancer was confirmed in all cases by histological examination on resected tissues, and no other diseases were diagnosed. All patients received no radiotherapy, chemotherapy, or biologic treatment. Pathologic grading and tumor staging followed AJCC guidelines. Control serum samples were also collected from 16 healthy individuals who had medical check up in the Physical Examination Center of the Fourth Hospital of Hebei Medical University. Healthy subjects included 7 males and 9 females with ages ranged from 25 to 80 year old (median 52.5 year old).

2.2. Chemicals and reagents

Mouse anti human CCL5 and CD68 monoclonal antibodies, SP immunohistochemistry kit and DAB kit were purchased from Santa Cruz (CA, USA). Human CCL5 ELISA kit was obtained from Ocean Science (Beijing, China). RPMI 1640 and FBS were purchased from Gibco (New York, United States). GAPDH antibody was purchased from Epitomics (Zhejiang, China). PVDF membrane was purchased from Millipore (Massachusetts, United States). SDS-PAGE, ECL kit, Crystal violet stain were obtained from Beyotime (Beijing, China). IL-4 was purchased from eBioscience (Shanghai, China). Transwell was purchased from Corning (New York, USA). MTS, anti-CCL2, CCL4, CCL5, CCL17, CCL22, MMP2, MMP9 were purchased from Promega (Shanghai, China). Polyclonal antibodies against p-Stat3 (S727), Stat3 (T721), and p-Stat3 (Y705) were obtained from Bioworld (Minnesota, United States)

Table 1
Primers used in the study.

Targets	Primers	Length of products (bp)
GAPDH	5′-CGGATTTGGTCGTATTGGG-3′ 5′-TGCTGGAAGATGGTGATGGGATT-3′	283 bp
CCL2	5′-CACCTTCATTCCCCAAGGGC-3′ 5′-GGAGTTTGGGTTTGCTTGTCC-3′	246 bp
CCL4	5′-TGCTAGTAGCTGCCTTCTGC-3′ 5′-TCACTGGGATCAGCACAGAC-3′	202 bp
CCL5	5′-GGATCAAGACAGCACGTGGA-3′ 5′-CTTGTTCAGCCGGGAGTCAT-3′	311 bp
CCL17	5′-TGCTGATGGGAGAGCTGAAT-3′ 5′-GCAGTCCTCAGATGTCTGGT-3′	260 bp
CCL22	5′-CACTCCTGGTTGTCCTCGTC-3′ 5′-GAGAGTTGGCACAGGCTTCT-3′	368 bp
MMP2	5′-TTTTGGACACATCTGGGCAGT-3′ 5′-GGTCACATCGCTCCAGACTT-3′	360 bp
MMP9	5′-TTCAGGGAGACGCCCATTTC-3′ 5′-AGAAGCCGAAGAGCTTGTCC-3′	393 bp
CCR5 [19]	5′-AAACTCTGCTTCGGTGTCG-3′ 5′-CAGCCCACTTGAGTCCGT-3′	423 bp

2.3. Immunohistochemistry (IHC)

Cancer and adjacent non-cancer tissues were differentiated using Hematoxylin and eosin staining. CCL5 and CD68 expression in both tissue types were then examined using SP immunohistochemistry following the instructions of the manufacturer. Expression of both targets was determined through integral comprehensive measurement on the intensity of positive staining and the number of positive cells of at least 5–10 random high-power fields.

2.4. Enzyme-linked immunosorbent assay (ELISA)

Blood samples collected from patients with gastric cancer and healthy volunteers were placed at room temperature for 4 h before they were centrifuged at 2000 × *g* for 20 min. The serum samples obtained were stored at −80 °C until use for CCL5 measurement by CCL5 ELISA kit (Ocean Science,Beijing, China) following the instructions of the manufacturer.

2.5. Co-culture of AGS and THP-1 derived macrophage

AGS was purchased from the cell bank of Chinese Academy of Sciences (Beijing, China). THP-1 was purchased from American Type Culture Collection (Manassas, USA). To perform co-culture experiment, AGS cells were maintained in 6-well plates with complete medium, while THP-1 cells were differentiated into macrophages by IL-4 for 24 h. THP-1 derived macrophages were then seeded into chambers, which were subsequently inserted into each well of the 6-well plate with AGS cells. Co-culture was maintained in RPMI-1640 medium supplemented with 10% fetal calf for 24 h. After incubation AGS cells were used for subsequent experiment.

2.6. Cell proliferation assay

AGS cells were plated in 96-well plates at a density of 2 × 103 cells/well, and were treated with conditioned medium harvested from THP-1, or THP-1 derived macrophage cultures. AGS cells received no conditioned medium served as the baseline. At every 24 h for 5 consecutive days, a volume of 10 μL MTS solution (500 ug/ml) was added to each well, and was allowed to incubate for 2.5 h at 37 °C. After incubation the resulting absorbance was measured at 492 nm using an automated microplate reader. For each treatment, triplicate data were recorded at each time point. The assay was repeated for three times.

2.7. Cell migration assay

Cell migration assay was performed using 12 mm transwell with pore size of 12 μm. AGS cells were first treated with conditioned medium of AGS, THP-1 or THP-1 derived macrophage culture. To test the migration ability of treated AGS, the cells (1×10^5 cells/well) were seeded into the upper chamber of the transwell, while 10% FCS was placed in the lower chamber as the