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Chemokine CXCL13 expression was up-regulated in *Clostridium difficile* infection

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ABSTRACT

Clostridium difficile infection (CDI) is the leading cause of antibiotic- and healthcare-associated diarrhea. CXCL13 is a well-known CXC chemokine involved in inflammation, but its role in CDI remains unknown. In this study, serum and fecal samplings were collected from 51 CDI patients, 50 diarrhea patients without CDI and 50 healthy control subjects to determine the CXCL13 levels by enzyme-linked immunosorbent assay (ELISA). Besides, a mouse model of *C. difficile* infection was established, and murine serum and colon tissues were collected for detection of CXCL13 expression using quantitative real-time RT-PCR, ELISA, Western blot, or immunohistochemistry. We found that CXCL13 concentration in serum and fecal samples from CDI patients was significantly higher compared with that from diarrhea patients without CDI and that from healthy controls. Elevated serum CXCL13 positively and significantly correlated with blood markers of inflammation and yielded an increased area under the ROC curve of 0.929. In murine *C. difficile* infection, CXCL13 were also dramatically increased in serum and infected colon tissues at the transcriptional and protein levels. The elevated CXCL13 levels positively and significantly correlated with inflammatory scores. Therefore, CDI is associated with enhanced release of CXCL13. This study indicated that CXCL13 may be pathogenically involved in CDI and served as a potential new biomarker for diagnosis and prognosis in CDI.

1. Introduction

Clostridium difficile, a Gram-positive anaerobic spore-bearing rod bacterium, is currently the leading cause of antibiotic- and health care-associated infectious diarrhea and pseudomembranous colitis in humans [1,2]. It is currently reported that *C. difficile* caused 250,000 infections and 14,000 deaths per year and annual excess medical costs totaling $1 billion in North America and Europe [3], and the emergence of a new, hypervirulent strain of C. Difficile (BI/NAP1) has further compounded this problem [4]. Developing fast and efficient diagnosis methods has been particularly frustrating [5], highlighting the need for identification of new targets for early rapid diagnosis to provide prompt effective treatment of infection.

C. difficile is a formidable nosocomial and community acquired pathogen, causing clinical manifestation ranging from asymptomatic colonization to toxic megacolon, pseudomembranous colitis, sepsis and even death [6]. Over the last 2 decades, the methods used to diagnose *C. Difficile* infection by clinical laboratories have evolved from cell culture cytotoxicity neutralization assay (CCCNA) to nucleic acid amplification techniques (NAATs) [7–9]. For many years, the mainstay of *C. difficile* disease diagnosis was enzyme immunoassays for detection of the *C. difficile* toxins, although it is now generally accepted that these assays lack sensitivity [5]. However, the lack of sensitivity of these methods for the diagnosis of *C. difficile* has limited their routine use for screening of patients and has ushered in a new era of molecular assays, such as fecal lactoferrin, calprotectin, IL-1 and IL-8, which were commercially available for the detection of *C. difficile* [10–12]. However, these assays do not solve the diagnostic uncertainty in *C. difficile* infection, as detection of *C. difficile* in a fecal specimen does not automatically imply infectious diseases.

CXCL13, also known as B cell-attracting chemokine-1 (BCA-1) or B-lymphocyte chemoattractant (BLC), is mainly expressed within follicles of secondary lymphoid tissues which include stromal cells in B-cell follicles, dendritic cells, and T follicular helper cells [13,14]. When binds to its exclusive receptor CXCR5, CXCL13 can regulate homing of B cells and subsets of T cells to lymphoid follicles [15,16]. Earlier reports have demonstrated that CXCL13 was implicated in the formation of lymphoid tissue in chronic inflammation such as multiple sclerosis, rheumatoid arthritis or

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neuroinflammation [17–19]. Recent work of detection CXCL13 expression in *Helicobacter pylori* gastritis, pulmonary tuberculosis or pneumococcal meningitis indicated a role of this chemokine in bacterial infections [20–24]. Although CXCL13 displays some important activities in inflammatory diseases, its role in CDI is largely unknown.

In this study, we hypothesized that CXCL13 may participate in CDI. We aim (1) to study CXCL13 expression in serum and fecal sampling obtained from patients with CDI, (2) to evaluate the clinical values of CXCL13 for diagnosis CDI, and (3) to explore the correlation between inflammatory severity and CXCL13 expression by using a clinically relevant model of *C. difficile* infection.

2. Materials and methods

2.1. Study population

We initially recruited 63 patients of 18 years or older, hospitalized in The First Affiliated Hospital of Chongqing Medical University between January 2014 and May 2016, who had been diagnosed as CDI with diarrhea and positive *C. difficile* anaerobic culture performed in the clinical microbiology laboratory. Exclusion criteria included autoimmune diseases, neuroinflammatory diseases and cancer, and patients with little fecal specimen or that we could not obtain their serum seasonably. Finally 51 CDI patients were eligible to enroll in our study. Meanwhile, 50 diarrhoea patients (three or more unformed stools per 24-h period) who were negative for *C. difficile* and 50 healthy subjects without any aforementioned symptoms served as controls. The study was approved by the Clinical Research Ethics Committee of The First Affiliated Hospital of Chongqing Medical University according to guidelines for the protection of human subjects and informed consent was obtained from all participants.

2.2. Sampling and clinical data collection

Fecal samples were obtained for *C. difficile* toxin tests and anaerobic incubation following the doctor's advice. Subsequently, the remaining fecal smears were stored at −80 °C as soon as possible. Ethylene diamine tetra acetic acid (EDTA) anticoagulant venous peripheral blood was also collected to obtain serum. All sera and fecal specimens were stored at −80 °C until analysis. At the time of enrollment, all data were collected according to the Laboratory Information System (LIS) and Clinical Electronic Medical Record of our center. We recorded demographics including age and gender, and some blood biomarkers involving leukocyte (WBC), neutrophil counts (NC) and hemoglobin (Hb), albumin (ALB), creatinine (Cr), C-reactive protein (CRP), procalcitonin (PCT) and Blood Urea Nitrogen (BUN), which may be potentially related to the diagnosis and prognosis of disease.

2.3. Mice

C57BL/6 mice aged 6–8 weeks were obtained from and kept at Chongqing Medical University. All mice were housed, bred and maintained under humidity and temperature-controlled specific pathogen-free conditions in the animal facility. All animal experiments were prior approved by the Animal Care and Use Committee of the Chongqing Medical University and carried out according to the recommendations in the guide for the care and use of laboratory animals conformed to animal protection laws of China and applicable guidelines.

2.4. Murine model of C. difficile infection

A murine model of *C. difficile* infection was previously established [25]. Briefly, wild-type female C57BL/6 mice were treated with antibiotic drinking water containing kanamycin (0.4 mg/mL), entamicin (0.035 mg/mL), vancomycin (0.045 mg/mL), colistin (850 U/mL), and metronidazole (0.215 mg/mL) for 3 days. After the treatment all mice were given regular sterile water for 2 days and all mice received a single dose of clindamycin (10 mg/kg) intraperitoneally 1 day before *C. difficile* challenge. Afterwards, all mice were randomly divided into two groups. For the experimental group, all animals were infected by oral gavage with 10^8 colony-forming units (cfu) of *C. difficile* strain VPI 10463 (ATCC 43255). Mice were given by equivalent does of sterile phosphate buffer served as controls. Subsequently, all mice were monitored and recorded daily for symptoms of disease such as diarrhea, hunched posture, wet tail, and weight loss, and any mice that became moribund, 15% loss in body weight, were humanely killed.

2.5. Enzyme-linked immunosorbent assay

For human sera and fecal samples, CXCL13 levels were determined with commercially available ELISA kits from R&D Systems in duplicate. CXCL13 in the murine serum and colon homogenate was also measured using ELISA regents from Biolegend Systems in duplicate. All procedures were completed according to the manufacturer's instructions.

2.6. Histologic pathology evaluation

Colon tissues were removed from mice, fixed overnight at 4 °C in freshly made ice-cold 4% paraformaldehyde. Tissues were embedded in paraffin, sectioned, and stained with hematoxylin (H&E). The severity of enteritis was evaluated by using a grading system that includes evaluation of neutrophil margination and tissue infiltration, vascular congestion and exudates, mucosal edema and epithelial cell damage [25]. Each parameter was scored on a scale from 0 to 3, with higher clinical scores indicative of more severe morbidity.

2.7. Western blot analysis

At designated time points, the mice were euthanized by CO2 inhalation. Colon tissue were then removed from euthanized animals. After fully milling in liquid nitrogen, total proteins were extracted using the ice-cold RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) containing 1 mM PMSF and protease inhibitor cocktail (Roche Applied Science). The protein concentration was determined by the BCA method and separated by 12% SDS polyacrylamide gels and transferred to a nitrocellulose (Amersham). In the next step, the membranes were blocked with 5% skim milk in Tris–Tween-buffered saline (TBST) for 1 h at room temperature, and incubated overnight at 4 °C with rabbit antimouse anti-CXCL13 (Bioss, Beijing, China, 1:100 dilutions in TBST). After washing, the membranes were incubated with peroxidase-conjugated secondary anti-rabbit antibody (1:5000 dilutions in TBST). Antibody–antigen complexes were then detected using an enhanced chemiluminescent (ECL) detection system according to the manufacturer's instructions (Amersham and Pharmacia Biotech).

2.8. Immunohistochemical Staining

Tissues obtained from mice were fixed with 4% Paraformaldehyde, embedded in paraffin and cut into 4 μm sections for immunohistochemical staining. The sections were dewaxed into