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Chemokine and cytokine levels in inflammatory bowel disease patients

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Abstract

Crohn's disease (CD) and ulcerative colitis (UC), two forms of inflammatory bowel disease (IBD), are chronic, relapsing, and tissue destructive lesions that are accompanied by the uncontrolled activation of effector immune cells in the mucosa. Recent estimates indicate that there are 1.3 million annual cases of IBD in the United States, 50% of which consists of CD and 50% of UC. Chemokines and cytokines play a pivotal role in the regulation of mucosal inflammation by promoting leukocyte migration to sites of inflammation ultimately leading to tissue damage and destruction. In recent years, experimental studies in rodents have led to a better understanding of the role played by these inflammatory mediators in the development and progression of colitis. However, the clinical literature on IBD remains limited. Therefore, the aim of this study was to evaluate systemic concentrations of key chemokines and cytokines in forty-two IBD patients with a range of disease activity compared to levels found in ten healthy donors. We found a significant increase in an array of chemokines including macrophage migration factor (MIF), CCL25, CCL23, CXCL5, CXCL13, CXCL10, CXCL11, MCP1, and CCL21 in IBD patients as compared to normal healthy donors ($P < 0.05$). Further, we also report increases in the inflammatory cytokines IL-16, IFN-γ, IL-1β and TNF-α in IBD patients when compared to healthy donors ($P < 0.05$). These data clearly indicate an increase in circulating levels of specific chemokines and cytokines that are known to modulate systemic level through immune cells results in affecting local intestinal inflammation and tissue damage in IBD patients. Blockade of these inflammatory mediators should be explored as a mechanism to alleviate or even reverse symptoms of IBD.

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Keywords

Inflammatory bowel disease (IBD); Chemokine; Inflammation; Cytokine; Ulcerative colitis (UC); Crohn's disease (CD)

1. Introduction

Inflammatory bowel disease (IBD), which includes Crohn's disease (CD) and ulcerative colitis (UC), can be classified as an inflamed state of the gastrointestinal tract that is not caused by infection or cancer. IBD affects millions of people globally [1] and the prevalence is increasing annually. The precise mechanism for the development and progression of IBD remains unclear, but accumulating evidence suggests that the pathology is driven by the advancement of immunological lesions that are accompanied by a prominent infiltrate of cells including T lymphocytes, macrophages, neutrophils, and plasma cells [2]. Based on several recent studies in rodents, an imbalance in T helper cells [3–5], IFN-γ overproduction by lamina propria (LP)-macrophages and -T cells [6,7], and domination of Th1 cells producing inflammatory cytokines [8] have all been implicated as major contributors to IBD progression. Further, it is well known that the mucosa of CD patients is dominated by the Th1 phenotype and is characterized by the production of IFN-γ by lamina propria (LP) T cells and IL-12 by LP macrophages [9,10].

Chemokines are a recently discovered family of small (8–10 kDa) proteins that play an important role as potent chemoattractants for activation and recruitment of leukocytes [11,12]. They are divided into four subfamilies, C, CC, CXC, and CX3C depending on the position of the first two-cysteine residues [13]. Chemokines have the ability to attract inflammatory cells to IBD lesions and in fact are also involved in the activation of these cells. In addition to T cells, neutrophils, and macrophages, the gut of IBD patients is also infiltrated by an abundance of fibroblasts, endothelial cells, and epithelial cells, which in addition to being recruited by chemokines, can themselves produce many chemokines with prominent roles in inflammatory processes [14]. Thus, chemokines and their receptors orchestrate tissue specific and immune cell selective trafficking and retention of leukocytes at the site of inflammation, a process that is known to contribute to the development and progression of IBD.

Both experimental studies in mice and evidence from clinical investigations support a role for various chemokines in IBD pathogenesis. For example, polymorphisms in macrophage inhibitory factor (MIF), a chemokine responsible for recruitment of distinct macrophage populations is associated with risk of IBD [15,16]. Further, we have recently shown that CXCR3 ligands (CXCL9, CXCL10, and CXCL11) expressed by activated T cells and NK cells are upregulated at sites of colitis [17]. Similarly, CXCL10 has been shown to be upregulated during UC [18], while CD tissues have been shown to express CXCR3, CXCL10, and CXCL9 [19,20]. Chemokine CCL25, also known as thymus-expressed chemokine (TECK), a key regulator of leukocyte migration in the small intestine, is known to regulate intestinal inflammation [21]. Monocyte chemoattractant protein 1 (MCP-1) that attracts monocytes among other cells plays a critical role in colitis and is increased in IBD patients [22,23]. It has also been shown that IL-16 activates expression and production of

proinflammatory cytokines such as IL-1β and tumor necrosis factor alpha (TNF-α) in human monocytes and is significantly increased in IBD patients as compared to healthy controls [24].

The current evidence clearly suggests a link between increased levels of chemokines, cytokines and IBD pathogenesis. However, the available human studies are limited by the patient sample size and the number of chemokines/cytokines measured. Therefore, the purpose of this study was to perform an extensive examination of circulating chemokine and cytokine concentrations in IBD patients. Given the role of T helper cells, macrophage and neutrophils in IBD development and progression, in this study we focused specifically on T helper cell, macrophage, and neutrophil mediated chemokines and cytokines.

2. Materials and methods

2.1. IBD patients

A total of 42 age-matched serum samples were collected at Palmetto Health Hospital at Richland Medical Park by surgical collaborator team of Dr. Raja Fayad from a cohort of 24 patients with chronic CD (18 females and 6 males with a mean age of 41.6 years and an age range between 31 and 76), 18 patients with UC (12 females and 6 males – with a mean age of 39.2 years and age range between 30 and 76) and 10 serum samples from healthy donors (6 females and 4 males with a mean age of 47 years and an age range between 38 and 62) over a period of two years. The average body mass index of IBD patients was 21.4 kg/m^2. The diagnosis of IBD was based on standard clinical, endoscopic and histological criteria. All patients had symptomatic active IBD or strictures that required surgical treatment. The serum samples were collected from the IBD patients before any treatment of antibiotics or steroids. Normal, healthy donors had no active gut or intestinal disease or symptoms at the time of blood collection. Formal consent was obtained from the patients, who were informed about the future use of the serum. The study was approved by the institutional review board for the study of human subjects at University of South Carolina and Palmetto Health systems (PH IRB # 2012-094; PRO-00021486).

2.2. Chemokine and cytokine analysis by multiplex™ ELISA

Levels of macrophage, neutrophil, and T helper cell-derived chemokines and cytokines (IL-16, TNF-α, I-309, CXCL6, IFN-γ, IL-1β, MIF, CCL25, CCL23, CXCL5, CXCL13, CXCL10, CXCL11, MCP1 and CCL21) were determined in the serum using a luminex Elisa assay kit (Bio Rad, Hercules, CA, USA). In brief, IL-16, TNF-α, I-309, CXCL6, IFN-γ, IL1β, MIF, CCL25, CCL23, CXCL5, CXCL13, CXCL10, CXCL11, MCP1 and CCL21 analyte beads contained in an assay buffer were added to pre-wet vacuum wells followed by 50 μl of assay buffer with beads. The buffer was then removed from the wells underwent a wash cycle. Next, 50 μl of standard or serum sample was added to each well and the plate was incubated for 1 h and subjected to continuous shaking (at setting #3) using a Lab-Line™ Instrument Titer Plate Shaker (Melrose, IL). The filter bottom plates were then washed and vortexed at 300x*g* for 30 s. Subsequently, 25 μl of anti-mouse detection Ab was added to each well and incubated for 30 min at room temperature (RT). Next, 50 μl of streptavidin–phycoerythrin solution was added and the plate was again incubated with continuous

shaking for 10 min at RT. Finally, after washing 125 μl of assay buffer was added and BioRad™ readings were measured using a Luminex™ System (Austin, TX) and calculated using BioRad software. The Ab BioRad™ MAP assays are capable of detecting >10 pg/ml for each analyte.

2.3. Power and statistical analysis

Power calculations were performed in order to determine the probability ($1 - \beta$) of detecting a significant difference (δ) between systemic levels of the chemokines and cytokines in IBD patients as compared with normal healthy donors. Based on preliminary investigations, we calculated that at least 40 IBD subjects and 10 healthy donors were needed to show significant differences between the groups, with a power of 95%, and consideration of Type 1 Error 0.001. Data are expressed as the mean ± SEM and compared using a two-tailed paired student's t-test or an unpaired Mann Whitney *U*-test. The results were analyzed using Microsoft Excel (Microsoft, Seattle, WA) for Macintosh computers. The results were considered statistically significant if p values were < 0.05.

3. Results

3.1. Chemokines increases in inflammatory bowl disease patients

It has been shown that systemic CC chemokines levels are increased in many autoimmune diseases. Therefore, we sought to determine whether any systemic changes in CC chemokine concentrations are characteristic of disease in IBD patients. As hypothesized, we found that serum levels of macrophage migration factor (MIF), thymus-expressed chemokine (TECK: CCL25), macrophage inflammatory protein-3 (MIP3: CCL23), monocyte chemoattractant protein-1 (MCP-1: CCL2), and macrophage inflammatory protein-3 beta (MIP3β/Exodus-2: CCL21) are increased in IBD patients as compared to healthy donors (Fig. 1A) ($P < 0.05$). Surprisingly, however, we noticed a slight increase in serum levels of I-309 (CCL1) in IBD patients as compared to healthy controls (Fig. 1A) ($P < 0.05$). These data indicate that systemic CC chemokines are significantly increased in IBD patients.

3.2. Increased levels of CXC chemokines in inflammatory bowl disease patients

Among CXC chemokines, the role of CXCR3 in chronic inflammation has been intensively investigated in the past. CXCL10, a CXCR3 ligands level is increased during inflammation as reported in UC patients. However, the relationship between other CXC chemokines and IBD has been less well characterized in the literature. Therefore, in the present study we report that serum levels of IFN-γ-Inducible Protein-10 (IP-10: CXCL10), interferon-inducible T-cell alpha chemoattractant (I-TAC: CXCL11), lipopolysaccharide-induced chemokine (LIX: CXCL5), B cell attracting chemokine-1 (BCA-1: CXCL13), and granulocyte chemotactic protein-2 (GCP-2: CXCL6) are increased in IBD patients as compared to healthy donors (Fig. 2) ($P < 0.05$). Taken together, these data indicate an increase in level of CXC chemokine in IBD patients.

3.3. Pro-inflammatory cytokine, interleukin-16 and interferon-γ levels in inflammatory bowl disease patients

Tumor necrosis factor alpha (TNF-α) and interleukin-1 beta (IL-1β) are often over-produced during a variety of inflammatory diseases, including IBD. Further, IL-1β is a potent pro-inflammatory cytokine that is produced and activates by many immune and inflammatory cells and activated macrophages are known to be an important source for increased IL-1β expression in IBD patients. Moreover, it is well known that Th1 differentiation leads to subsequent IFN-γ production, which is a major factor for IBD progression. The cytokine IL-16 selectively regulates migration of all $CD4^+$ expressing T cells regardless of their activation state, a key factor for IBD pathogenesis. Here we report a significant increase in circulating levels of IL-16, TNF-α, IL-1β, and IFN-γ in IBD patients as compared to healthy donors (Fig. 3).

4. Discussion

Accumulating evidence suggest that chemokines are important not only for overall systemic inflammation, but also for homeostasis and proper balance of immune function [25]. Chemokines are produced by a wide variety of cells including T cells, macrophages, and neutrophils, all of which are present in IBD lesions and are known to play a prominent role in enhancing the severity of intestinal as well as systemic inflammation. In the present study we found that systemic levels of few individual chemokines were significantly increased in IBD patients as compared to healthy donors. Further, we also report an increase in circulating levels of cytokines including TNF-α, IL-1β, IL-16, and IFN-γ in IBD patients as compared with normal healthy donors.

IBD is mediated, in large-part, by the infiltration of T cells that produce Th1 cytokines in the mucosa [26,27]. In support of this, it has been reported that $CXCR3^+$ cells and $CD4^+$CXCR3 T cells are increased in the intestinal LP of IBD patients as compared with healthy LP [28]. In fact, we have shown that systemic levels of CXCR3 ligands CXCL9, CXCL10, and CXCL11 are significantly increased in both experimental colitis and IBD patients when compared to normal healthy donors [29,30]. Moreover, CXCL10 expression is also increased in the mucosa of IBD patients [18]. Further, mechanistic studies performed in mice by our group also support the role of CXCR3 ligands in the pathogenesis of colitis [29,30]. In the present investigation we report a significant increase in circulating levels of CXCL6, CXCL10, CXCL11 in IBD patients as compared to normal healthy donors supporting the above view on the importance of these chemokines in the progression and maintaining the symptoms of IBD.

In UC patients, circulating frequency of neutrophils has been shown to increase, when compared to healthy donors [31]. Further, it has been shown that neutrophil activation, migration, and degranulation are important effector mechanisms for the gut damage in IBD [32]. In the present study we found a significant increase in circulating levels of chemokines CXCL5 and CCL23 that are primarily responsible for neutrophil activation and systemic level increase in IBD patients. This finding corroborates the previous investigations that have implicated a potential role of neutrophils on the regulation of leukocyte migration, inflammation in general, and subsequent advancement of disease severity in IBD patients.

In IBD patients, an increase in turnover and activation of monocytes occurs, which are a source of intestinal macrophages [33]. The chemokine MCP-1 (CCL2) is a potent chemotactic and activator of monocytes and macrophages [34]. Several studies have documented elevated CCL2 mRNA and protein expression in the mucosa of IBD patients [23,35]. Further, experimental studies performed in rodents support a role for CCL2 in experimental colitis [36–38]. For example, mice deficient in CCL2 show a significant reduction in the severity of colitis both macroscopically and histologically along with a decrease in mortality compared with wild-type control mice [39]. This was associated with a downregulation of myeloperoxidase activity, IL-1β, IL-12p40, and IFN-γ production, as well as infiltration of $CD3^+$ T cells and macrophages in the colonic mucosa [39]. These investigations clearly support a notion that CCL2 is crucial for mediating systemic and in particular intestinal inflamma-tion. The available literature on the impact of CCL23 in IBD is less clear. In fact, to our knowledge, there are no investigations that have reported a relationship between CCL23 and IBD. However, it has been implicated the role of CCL23 in atherosclerosis among other diseases [40]. Consistent with previous investigations, we report that circulating CCL2 is increased in IBD patients. However, for the first time we also report an increase in CCL23 in patients with IBD as compared to healthy donors.

CCL25 is another chemokine that is specifically expressed in the small intestine and is important for mucosal homeostasis in the gut [41]. A known CCL25 receptor CCR9 expression is limited to inflamed small intestine in CD patients as compared to colon [42]. Further, rodent studies also support a role for CCL25/CCR9 interactions in experimental colitis [21]. In the present study we report an increase in levels of CCL25 in IBD patients as compared to healthy donors supporting the previous findings on the association between CCL25 and progression of IBD.

The elevated levels of inflammatory mediators have been documented in the literature suggest that pro-inflammatory cytokines play a large role in driving IBD pathogenesis and many other autoimmune diseases. It has been shown that IL-16 expression increase in IBD patients as compared to healthy donors [24]. Further, IL-16 exerts a strong chemoattractant activity on $CD4^+$ cells and induces intestinal inflammation through PepT1 upregulation [43]. TNF-α is increased in the colon tissue of IBD patients [44]. Specifically, TNF-α levels have been reported to be elevated in tissue, LP cells, and fluids [45] of IBD patients. Similarly, IL-1β expression has been reported to be increase in activated macrophages isolated from IBD patients [46]. Likewise, IFN-γ a known inducer of Th1 responses has been reported to play a critical role in the induction and progression of colitis [47]. It has been shown that chemokine anti-CXCL10 treatment abrogate spontaneous colitis, which also coincided with decrease levels of TNF-α and IFN-γ [48]. In the present study, we demonstrated that systemic levels of TNF-α, IL-1β, IL-16 and IFN-γ are increased in IBD patients as compared to healthy donors. These results strongly support the previous finding on regulation of IBD and experimental colitis by these pro-inflammatory and cell-mediated cytokines.

In summary, it is sensible to assume that IBD is not just an inflammatory disease, but is likely comprised of intricate interactions of many signaling pathways and immune cell types mediated by chemokines and cytokines in the intestine as well as other part of the immune

and non-immune organs. The interplay between effector immune cells with the intestinal environment initiates inflammation, due to constant exposure to the foreign antigen that mediates immune cells to secrete pro-inflammatory mediators and induce chronic intestinal inflammation as well as systemic inflammation and colon cancer. In the present study, analysis of IBD patients suggests a potential role of chemokines in immune mediating signaling pathways is in central to systemic inflammation, but in particular to intestinal inflammation. A therapeutic approach can thus be hypothesized to include blocking of chemokines to receptors on inflammatory cells, which may be very advantageous for combatting IBD symptoms.

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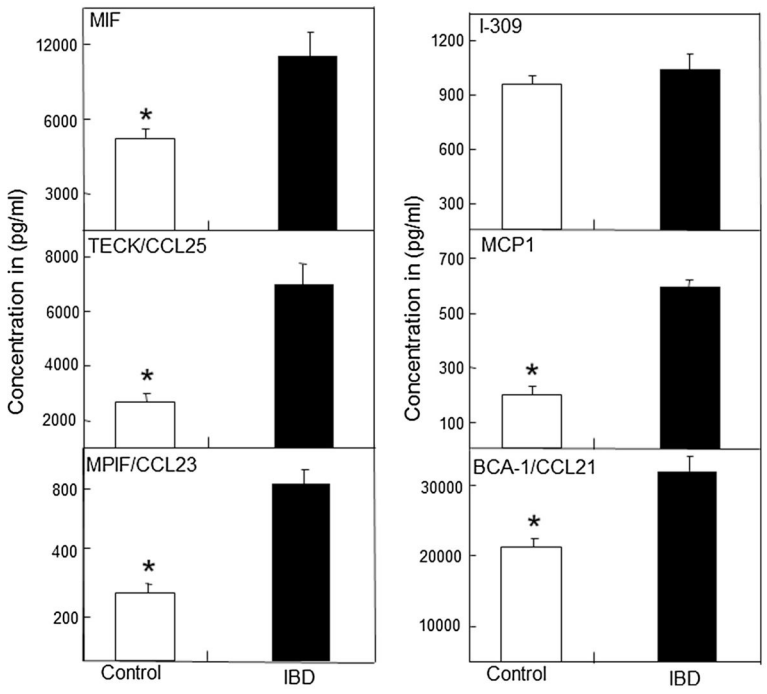

Fig. 1.

Serum level of MIF, CCL21, CCL23, CCL25, I-309, and MCP-1 during IBD. Sera from IBD patients ($n = 42$, ■) and normal healthy donors ($n = 10$ □) were isolated and evaluated for the presence of CC chemokines (MIF, CCL21, CCL23, CCL25, I-309 and MCP-1) by multiplex ELISA that was capable of detecting >10 pg/ml of each chemokines. The data presented are the mean MIF, CCL21, CCL23, CCL25, I-309, and MCP-1 concentrations ± SEM in IBD patients and healthy donors. Asterisk(s) indicate statistically significant differences, i.e., $p < 0.05$ (*), between the two groups.

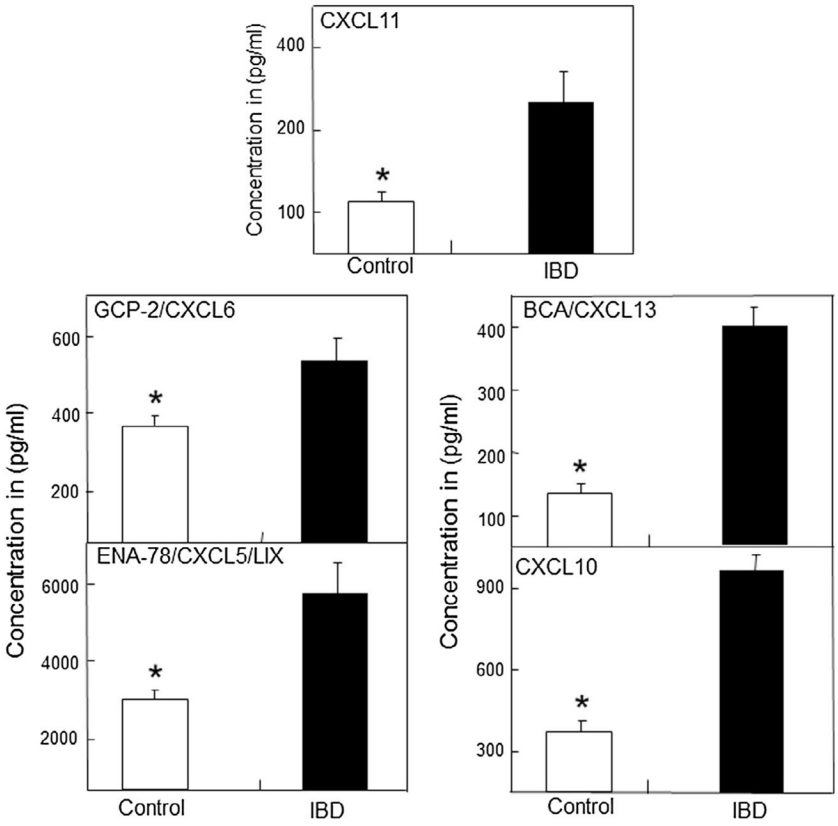

Fig. 2.

Systemic levels of CXCL5, CXCL6, CXCL10, CXCL11, and CXCL13 during IBD. Sera from IBD patients (n = 42, ■) and normal healthy donors (n = 10 □) were isolated and evaluated for the presence of CXC chemokines (CXCL5, CXCL6, CXCL10, CXCL11, and CXCL13) by multiplex ELISA that was capable of detecting >10 pg/ml of each chemokines. The data presented are the mean CXCL5, CXCL6, CXCL10, CXCL11, and CXCL13 concentrations ± SEM in IBD patients and healthy donors. Asterisk(s) indicate statistically significant differences, i.e., $p < 0.05$ (*), between the two groups.

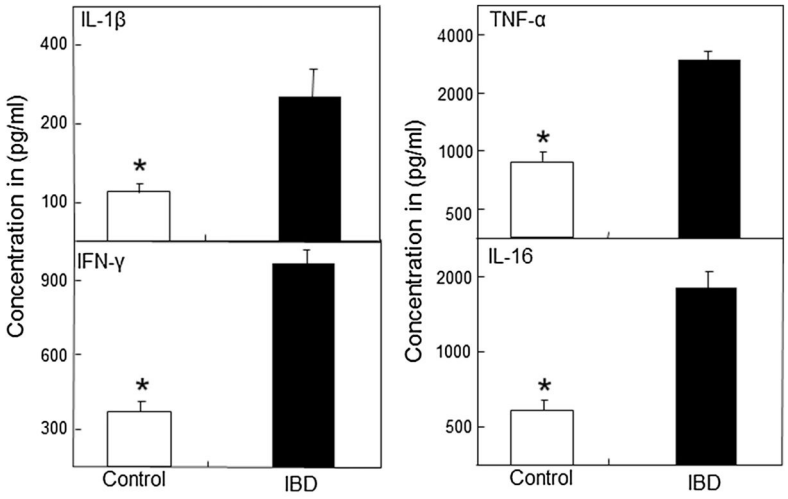

Fig. 3.

Changes in systemic TNF-α, IL-1β, IFN-γ and IL-16 cytokines level during IBD. Sera from IBD patients (n = 42, ■) and normal healthy donors (n = 10 □) were isolated and evaluated for the presence of cytokines (TNF-α, IL-1β, IFN-γ and IL-16) by multiplex ELISA that was capable of detecting >10 pg/ml of each chemokines. The data presented are the mean TNF-α, IL-1β, IFN-γ and IL-16 concentrations ± SEM in IBD patients and healthy donors. Asterisk(s) indicate statistically significant differences, i.e., $p < 0.05$ (*), between the two groups.