

Expression of CXC Chemokine Receptors in Acute Ulcerative Colitis: Initial Study from an Animal Model

Abstract

Background: Ulcerative colitis (UC) is an inflammatory disease which is characterized by infiltration of inflammatory cells, crypt abscesses, distortion of the mucosal glands, and goblet cell depletion. The existence of neutrophil-rich inflammation in colon tissues of patients with UC is one of the most significant histological features of this disease. Nonetheless, the expression of CXC chemokine receptors which appear as the main chemical mediators governing the migration of neutrophils into the mucosal tissue of patients with UC has not been well clarified. **Materials and Methods:** In this experimental study, the UC model was induced in Wistar rats by administration of 2 ml 4% acetic acid into the large colon through the rectum. Animals were anesthetized after 48 h; their colon tissue samples were isolated for macroscopic and histopathological examination. The expression of receptor_{1,7} of CXC chemokine was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) technique. **Results:** Heavy infiltration of neutrophils, coagulative necrosis, and ulcers were observed in H and E staining, which pathologically proved the UC model. qRT-PCR results indicated that CXCR2 as one of the important ELR⁺ chemokine family receptors bears the highest expression in the UC group (32 fold) than the control group ($P \leq 0.05$). In addition, other CXCRs of this group including CXCR1 did not possess any change ($P > 0.05$). In contrast, RLR negative chemokine family receptors did not show any changes with the normal group. **Conclusion:** The results showed that CXCR2 is the only receptor for CXCL family which was remarkably upregulated in experimental UC and that CXCR2 might play a significant role in the pathogenesis of UC.

Keywords: CXCR, chemokines, inflammation, ulcerative colitis

Introduction

Ulcerative colitis (UC) is implied as one of the important causes of gastrointestinal disease.^[1] UC is just a pathological condition in which the inflammatory reaction and morphologic modifications are limited to the colon. The rectum seems involved in most of the patients. The typical histologic findings appear to be acute and chronic inflammation of the mucosa by polymorphonuclear cells (PMNs) and mononuclear cells, crypt abscesses, distortion of the mucosal glands, and goblet cell depletion.^[2-6] The endless existence and trafficking of immunocytes into the mucosal section are called physiologic inflammation and mirror the production of chemokines by cells inside the gastrointestinal mucosa. Trafficking of immunocytes to the site of the inflammation or injury occurred through the interaction of small molecules, called chemokines and chemokine receptors.^[7-9]

The chemokines are a superfamily of low molecular weight which serves as effective chemoattractants for inflammatory cells. Chemokines had been ordered into four subfamilies, based on the existence of cysteines at the amino-terminal: CXC, CC, CX3C, and C.^[10-12] Through CXC, chemokine can be subclassified based on either the presence or absence of a tripeptide motif ELR at the NH₂ terminus into Glu-Leu-Arg (ELR)⁺ and ELR⁻ CXC family.^[13,14] The characteristic of ELR⁺ chemokines is their ability to recruit PMNs, specifically neutrophils into the inflamed tissues; this subclass contains CXCR2 and CXCR1 that are shown to be the main receptors of ELR⁺ CXC subfamily which are strong chemoattractants for neutrophils.^[15,16] ELR⁻ CXC subfamily includes chemokines such as CXCL9, CXCL10, and CXCL11, and their main receptor is CXCR3 which primarily attracts

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activated Th1 cells and natural killer cells. CXCR4, CXCR5, CXCR6, and CXCR7 are other members of ELR⁻ subfamily. Although CXCR4 and CXCR7 through binding to their ligand CXCL12 play an important role in trafficking of many cells such as T and B lymphocytes, and hematogenic stem cells, CXCR5 is a chemoattract for B-lymphocytes.^[17-19] On the other hand, several studies have shown the importance of CXC chemokines and their receptors in UC, for instance, Farooq *et al.* showed that blocking of CXCR2 receptor resulted in attenuation of inflammation in an animal model of UC.^[20] Moreover, other studies showed that CXCR2- and CXCR3-knockout mice were protected against colitis.^[21,22] Taken together, accumulating evidence indicates that chemokines and their receptors are key players in the inflammatory status of colitis. However, not only the etiology and pathobiology of colitis are unclear but also the expression of all inflammatory mediators, particularly chemokine receptors, in this disease has not well studied. Herein, we examined the expression levels of 1–7 members of CXCR chemokine receptors at mRNA levels in the experimental model of UC and found that CXCR2 appeared the only receptor for ELR⁺ chemokine which was significantly being upregulated within the UC group. In contrast, the expression of entire members of ELR⁻ CXCRs did not significantly change.

Materials and Methods

Animals

Male Wistar rats (300–400 g) were got from Kurdistan University of Medical Sciences. The animals were fed a regular chow pellet regimen, had free admission to water, and were kept on a 12-h light/dark cycle. The experimental protocol was approved (Approval ID: IR.MUK.REC.1397.319) by Kurdistan University of Medical Sciences. In this study, 12 adults male Wistar rats were randomly divided into two groups of control and acetic acid (AA)-induced colitis groups. Experimental colitis was induced in 6 rats by administration of AA and designed as AA-induced colitis group. The other 6 rats which were treated with normal saline designated as the control group. It should be noted that all the rats should be kept for 2–3 months to reach an ideal weight of 300–400 g to reduce their mortality during the test.

Induction of ulcerative colitis and sample preparation

Induced colitis was carried out as described previously.^[23] rats were lightly anesthetized with ether, and then, AA 4% (pH = 2.3) was administered into the distal colon through a cannula (2-mm internal diameter and 6-cm long into the colon from the rectum). In order to induce colitis, rats were sacrificed by chloroform after 48 h, then 6 cm of the distal part of colon was removed and used as sample for the molecular and microscopic evaluations. About 2 cm from the mucosal part of the colon tissue was placed in an RNase-free tube and immediately

stored at –70°C freezer for mRNA extraction, and the rest of removed colons were fixed in 10% formalin for later histopathological evaluations.

Evaluation of edema

In order to evaluate the amount of edema, 6 cm of the distal part of colon was separated and weighed, then weight/length ratio of wet colon in each group was calculated.^[24]

Histological analysis

Colonic tissues were fixed overnight in 10% formalin, dehydrated in ethanol, then embedded in paraffin, and sectioned at 5-µm thick. Histological sections were stained with hematoxylin/eosin (H and E) according to the standard protocols and used for the detection of inflammation and verification of the model. To confirm UC, microscopic analysis was performed to study white blood cell (WBC) infiltration, ulcers, and other characters by our pathologist (BN) who was blind to each group.

RNA extraction and quantitative real-time polymerase chain reaction

Total RNA was extracted from the colonic mucosa tissue using RNA isolation kit according to the manufacturer's instructions (#740955.50; NucleoSpin RNA, Germany), and cDNA synthesis was performed for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) using PrimeScript™ RT Reagent Kit (#RR037A; Takara, Japan). Real-time qRT-PCR was performed by Corbett Rotor-Gene 6000 real-time PCR system (Corbett Research, Australia) and carried out using SYBR Green dye detection protocol. The primers were designed by Gene Runner and are listed in Table 1. The expression of target genes was quantified using the comparative Cycle Threshold (CT) method.^[25] Amplification condition was as follows: initial denaturation at 94°C for 30 s, followed by 40 cycles of denaturation at 95°C for 5s, annealing temperature at 55°C for 30 s,

Table 1: The list of primers

Numb	Name	Direction	Oligo sequences 5'→3'
1	CXCR1	Forward	TTCATCTGGATTGCTCCTGAG
		Reverse	AGCTAAGAAGGAACACCAGTG
2	CXCR2	Forward	TTGTACAATCGAAGCACCTG
		Reverse	AACGAGAATACCTTGCACAG
3	CXCR3	Forward	TGCTGAGCCGTGAAATCTTC
		Reverse	ACTTGACGTTCACTGACCTC
4	CXCR4	Forward	TATCCCTGACATCATCTTCG
		Reverse	TGATGATGCAGTAACAGGAC
5	CXCR5	Forward	TCACCTACAACATGGACGATC
		Reverse	AGGATTACCAGTACGAGGATG
6	CXCR6	Forward	AGAAAGATCTGGACGATGAC
		Reverse	ACACTACCAGATACATGCAG
7	CXCR7	Forward	ACTACTCGGACATCAACTGG
		Reverse	TGGCAATCATACCGATCACG
8	CXCR8	Forward	AGTTCAACGGCACAGTCAAG
		Reverse	ATACTCAGCACCAGCATCAC

extension temperature at 72°C for 30 s, and final extension temperature at 72°C for 5 min. GAPDH was used as a housekeeping gene, and of course, the expression of CXCR and GAPDH genes was quantified in AA-induced colitis and control groups. We calculated the mean \pm standard deviation for each group using $2^{-\Delta CT} [2^{-(CT_{CXCR} - CT_{GAPDH})}]$. Then, the fold change was calculated using this formula: $2^{-\Delta CT} \text{ AA-induced colitis group} / 2^{-\Delta CT} \text{ control group}$.^[25]

Statistical analysis

In this study, SPSS version 20.0, IBM, Chicago and *t*-test were used to compare the results of gene expressions and colon/body weight ratio of rats in the UC and control groups ($P \leq 0.05$).

Results

Macroscopic evaluation of colon tissue

Macroscopic analysis showed: thickening of the colon, severe hemorrhagic ulcers, and bloody mucosa in AA-induced colitis group. In the other hand, there were no signs of inflammation or tissue damaging in the control group [Figure 1a and b]. Finally, the whole body weight was measured before sacrificed, and the ratio of the mean wet colon weight to the mean whole body weight was calculated through both the groups and their results, as shown in Figure 1c; this ratio was much higher in the AA-induced colitis group than the control group.

Histological observation

Histopathological evaluations represented epithelial destruction, goblet cells depletion, ulcerated mucosa and infiltration of WBC mostly PMNs in AA-induced colitis [Figure 2]. In the other hand, we can see healthy and intact architecture of the colon tissue without any inflammation through the control group [Figure 3].

ELR⁺ receptor expression in mucosa of experimental colitis model

Our investigations from quantitative real-time PCR showed the overexpression of CXCR2 in inflamed mucosa of AA-induced colitis, but not in healthy ones ($P < 0.05$);

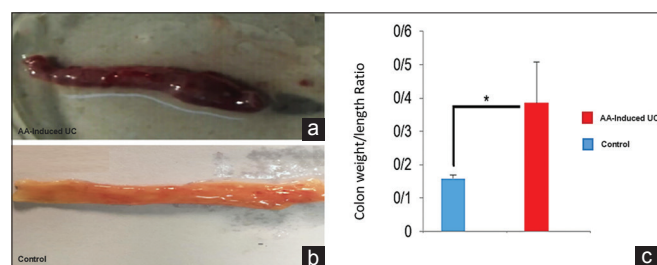


Figure 1: Macroscopic evaluations of the colon tissue from the acetic acid-induced colitis and control groups. (a and b) Gross macroscopic architecture of the colon in the acetic acid-induced colitis and control groups. (c) Colon weight/length ratio through the acetic acid-induced colitis and control groups. This ratio considers as edema, and each column represents fold change + standard error of the mean (SEM) for six male Wistar rats. *($P < 0.05$)

in fact, results of fold change showed approximately 33 times higher through the AA-induced colitis group rather than the control group. However, unlike CXCR2 expression, we did not find any change in the expression of CXCR1 between the AA-induced group and the control group ($P > 0.05$) [Figure 4].

ELR⁺ receptor expression through mucosa of experimental colitis model

Our investigations of quantitative real-time PCR showed that CXCR5 relative expression had lightly elevated in inflamed mucosa than healthy ones. Although the levels of CXCR5 expression at mRNA were slightly unregulated (1.4 times) but it was not statistically significant. Other CXCRs such as CXCR3, CXCR4, CXCR6, and CXCR7 mRNA expression in the mucosa of the AA-induced colitis group remained unchanged in comparison with the control group ($P > 0.05$) [Figure 5].

Discussion

Due to the incomplete and unknown etiology of UC and importance of the disease as one of the most important causes of gastrointestinal disorders on the one hand, and on the other hand, the importance of the immune system in etiology of UC, we were about to evaluate mRNA of CXCRs family as an important part of the immune system. Growing of evidence indicates that neutrophils are the most important players in acute inflammation. ELR⁺ C-X-C motif chemokine family is involved in trafficking of neutrophils through the binding of their cognate receptors. CXCL1 and CXCL8 are two important ELR⁺ CXCL family chemokines, and their interaction with CXCR2 leads in the migration of neutrophils.^[26,27] In line with previous studies, we have reported here that CXCR2 is remarkably overexpressed in

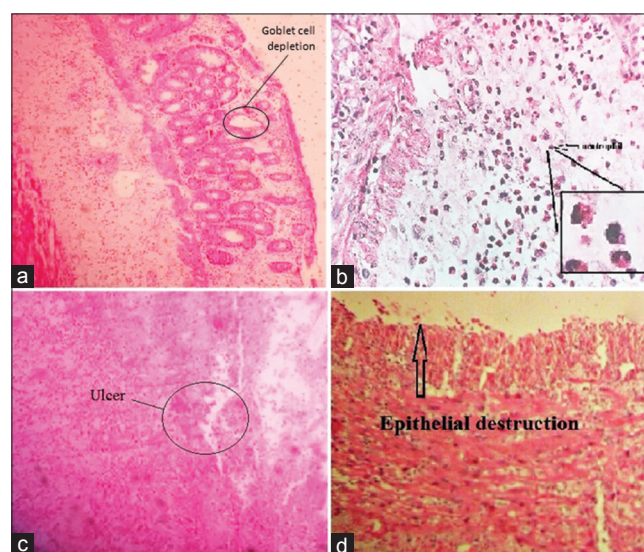


Figure 2: Histological observations from colon biopsies of acetic acid-induced colitis. (a) Goblet cell depletion ($\times 10$). (b) Dominant infiltration of neutrophils ($\times 40$). (c) Ulcerated tissue and neutrophil infiltration ($\times 10$). (d) Epithelial destruction ($\times 10$)

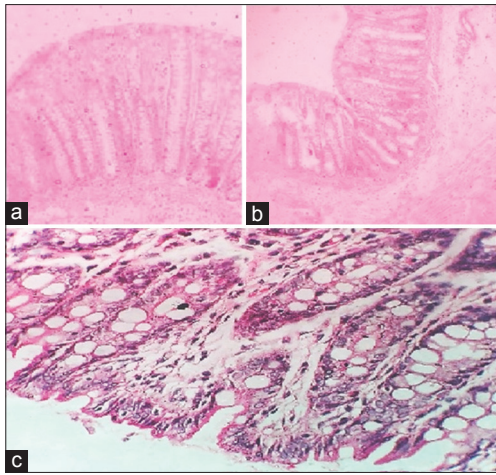


Figure 3: Histological observation from colon biopsies of the control group. As we can see in this figure, architecture of the colon tissues is solely normal, and there are no ulcer or infiltration of neutrophils (a and b, $\times 4$, c $\times 40$)

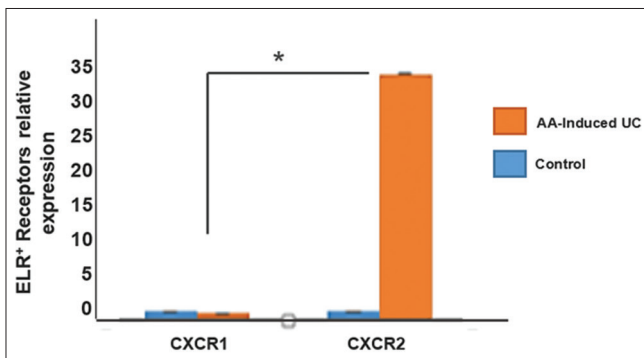


Figure 4: ELR⁺ CXCRs mRNA expression of the mucosa of the colon tissues from biopsies of the acetic acid-induced colitis group and control group. CXCR2 ELR⁺ CXCR chemokine receptor is highly upregulated in the acetic acid-induced colitis group than the control group. Each column represents a fold change \pm SEM for six male Wistar rats. The statistical results for CXCR2 in the acetic acid-induced colitis group show a statistically significant difference compared to the control group ($P \leq 0.05$). However, in contrast, CXCR1 remained unchanged in compared through both the groups ($P > 0.05$)

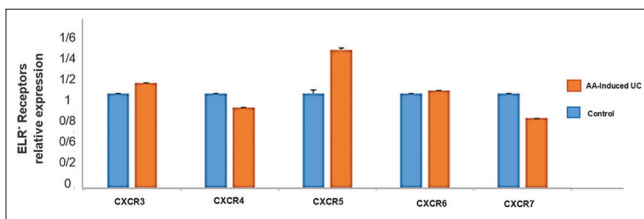


Figure 5: ELR⁻ CXCR mRNA expression of the mucosa of the colon tissues from biopsies of the acetic acid-induced colitis group and control group. Each column represents a fold change \pm SEM for six male Wistar rats. The statistical results for ELR⁻ CXCRs in the acetic acid-induced colitis group show no significant difference in compared with the control group ($P > 0.05$)

experimental UC than the control. Based on our observation, we have considered that this model seems an acute induced UC and the main infiltrated cells appear to be neutrophils. In contrast, the expression levels of CXCR1, another receptor for ELR⁺ chemokines, remain unchanged. This

discrepancy could be explained by the following reasons: first of all, maybe CXCL8 which is main and high-affinity ligand for CXCR1 does not exist in the rats.^[26,28] Second, previous studies have shown that CXCR1, unlike CXCR2, needs more concentration of ligands to respond. Moreover, CXCR2 can alone activate PMNs, but CXCR1 does not have this ability.^[29] Since the sensitivity of CXCR2 is higher than CXCR1 in responding to its ligands, we postulate that this could be another reason explaining the remarkable upregulation of CXCR2 in our model. Finally, from the biological point of view, there are some structural differences between rat CXCRs and human CXCRs, for instance, in terms of homology, there are about 58% of differences between rat CXCR1 and human CXCR1. Based on these facts, the results from human studies and experimental models can vary from each other.^[30]

We envision that in our model, some CXCR2-expressing neutrophils may react with CXCR2 ligands, CXCL6 and CXCL3, which have been remarkably upregulated in the colonic tissue (Boshagh *et al.* in press) and infiltrate into the inflamed colon. Other studies established that anti-CXCR2 mAb decreases neutrophil infiltration as well as the severity of colitis. Accordingly, CXCR2 knockout rats showed that more mild symptoms of UC, such as ulcer and bleeding, will be attenuated.^[20,31,32] Some clinical studies have clearly shown that the levels of CXCL8 increased in the serum of patients with active UC. Consistently, it has been demonstrated that CXCR1 and CXCR3 were upregulated on the cell surface of colonic macrophages and T-lymphocytes in patients with active UC.^[33] However, in our hands, the expression levels of both CXCR1 and CXCR3 were similar to the control group. Our observations are consistent with the fact that CXCR3 is a receptor of CXCL10 and chemoattracts T-cells.^[33]

Herein, we demonstrated the expression of other CXCRs such as CXCR3, CXCR4, CXCR5, CXCR6, and CXCR7 are almost similar in the control group and the UC group. However, Werner *et al.* reported that the expression levels of CXCR4 in peripheral blood T-cells and colon epithelial cells were higher in patients with UC than the healthy controls.^[34] In contrast with the present study which is observed that the expression levels of CXCR6 are similar in both control and experimental UC, a previous study exhibited that the expression of CXCR6 and its ligand CXCL16 was significantly higher in mucosa tissue of patients with Crohn's disease than the healthy controls.^[35] This discrepancy could be due to the difference in nature of UC which occurs in human and the experimental models which are induced in the animal models.

Conclusion

The results of the current study showed that the CXCR2 seems more likely to be the only chemokine receptor which remarkably upregulated in experimental UC and that CXCR2 could be a suitable candidate target for the

treatment of UC. However, further studies, especially human studies, are required.

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Nil.

Conflicts of interest

There are no conflicts of interest.

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