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Chemokine Receptor CXCR3 Expression in Inflammatory Bowel Disease

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Summary: CD4+ T lymphocytes in the lamina propria (LP) of the gut play a central role in the immune response in inflammatory bowel disease (IBD). CXCR3 is a chemokine receptor expressed on activated T lymphocytes, and a key component for the recruitment of T helper (Th1) effector cells to the site of inflammation. To determine if CXCR3 is involved in localization of T cells to the gut in IBD patients, we investigated the expression of CXCR3 on CD4+ T lymphocytes in the LP and in the submucosa of resection specimens from 51 IBD patients and 15 control patients. Positive cells were microscopically scored using a semiquantitative analysis on a five-point scale. We found that CD4+ T cells, CXCR3+ cells, and CD4+CXCR3+ T cells in the LP were slightly increased in both IBD groups compared with control non-IBD specimens. In addition, CD4+ and CXCR3+ cells in the submucosa were significantly increased in the CD group compared with the control

group. CD4+ and CXCR3+ expression was not statistically different between CD and UC. Flow cytometry was used to analyze the percentage of CXCR3+ cells within the CD4+ T-cell population isolated from biopsy specimens and peripheral blood from IBD patients and control patients. There was no difference in the percentage of CD4+CXCR3+ cells between the different groups in the gut as well as in the circulation. These results suggest that CD4+CXCR3+ T cells migrate to the normal and inflamed intestinal mucosa, indicating a role in maintaining normal gut homeostasis. The selective expression of CXCR3+ cells in the submucosa of CD patients might also indicate that these cells play a role in inflammation. **Key Words:** Inflammatory bowel disease—Crohn's disease—Ulcerative colitis—Chemokine receptors—CXCR3—CD4+ T cell—Immunohistochemistry.

INTRODUCTION

Crohn's disease (CD) and ulcerative colitis (UC) collectively termed inflammatory bowel disease (IBD) are chronic inflammatory conditions of the gastrointestinal tract. In the normal gut, there is a balance between the tolerogenic (regulatory/suppressor cell response) and the immunogenic potential damaging T helper (Th1) response. In IBD, this homeostasis seems to be disturbed, resulting in mucosal inflammation (1).

In the mucosa, a large resident population of CD4+ T lymphocytes is found diffusely spread throughout the lamina propria. Adhesion molecules have been impli-

cated in the selective recruitment of these lymphocytes within the intestine. Specifically, the $\alpha 4\beta 7$ integrin, which interacts with the mucosal addressin MAdCAM-1 on endothelial cells, is critical for normal gut homing of lymphocytes (2).

In addition, chemokines are involved in the recruitment of leukocytes. Chemokines are small peptides defined by the presence of four characteristic cysteines, and depending on the motif displayed by the first two cysteines, they have been classified into CXC or alpha, CC or beta, C or gamma, and CX3C or delta chemokines (3). Chemokine receptors are G-protein coupled, seven-span transmembrane receptors named based on the chemokine class they bind. CD4+ effector T cells, generated in vitro, express a broad range of chemokine receptors, some of which are preferentially expressed on Th1 (CXCR3, CCR5) or Th2 cells (CCR3, CCR4, CCR8) [see review by D'Ambrosio et al. (4)]. In particular, expression of CXCR3, a 40–41kD protein, is highly restricted to acti-

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vated T cells and natural killer (NK) cells, and is not expressed on other leukocytes (5). CXCR3 functions as a chemotactic receptor for the interferon-gamma (IFN γ)-induced chemokines interferon [IFN]- γ -inducible protein 10 (IP-10), Mig (monokine-induced IFN- γ), and interferon-inducible T cell alpha chemoattractant (I-TAC) (6), and is thought to be a key component for the recruitment of Th1 effector cells into sites of inflammation, as the CXCR3 ligands are important in the trafficking and recruitment of Th1 cells. Increased expression of CXCR3 was found in active Th1 lymphocytes in multiple sclerosis(7), rheumatoid arthritis(8) and hepatitis(9). Differential expression of this chemokine receptor on T cells in the mucosa of IBD patients could direct us to a specific activated inflammatory T cell subset involved in the pathogenesis of IBD.

Therefore, we analyzed the expression of CD4+CXCR3+ on cells present in the intestinal mucosa, and peripheral blood from IBD patients and controls. We report that the expression of CXCR3 on CD4+ T cells in the lamina propria (LP) and on peripheral blood CD4+ T cells in CD and UC patients is not significantly different compared with controls. Only in the submucosa of the gut of patients with CD was a significant increase in CXCR3+ cells detected. We conclude that CXCR3 may play a role in homeostatic lymphocyte migration into the lamina propria, and that inflammation results in CXCR3-dependent migration in the submucosa.

MATERIALS AND METHODS

Patients

Resection specimens were obtained from 66 patients undergoing a resection of their bowel. Twenty-two patients had UC and 29 patients had CD. Thirteen UC patients and 11 CD patients were on corticosteroid treatment. The control group consisted of 15 patients who had a bowel resection for non-IBD related disease (adenoma, adenocarcinoma, or diverticulitis). The patients' clinical data are shown in Table 1.

Specimens were snap-frozen in liquid nitrogen and stored in -70°C until analysis. Four μm serial section were cut in a cryostat and mounted on glass slides (Star Frost adhesive slides, Knittelgläser, Braunschweig, Germany), the slides were fixed in acetone at room temperature for 10 minutes and dried overnight, then stored at -70°C until immunohistochemical analysis was performed. Sections were cut and stained with hematoxylin and eosin for histologic analysis.

Lymphocytes were isolated from gut biopsy samples from six patients with CD, six with UC, and seven control patients. Patients committed to a trial were excluded.

For the isolation of peripheral blood mononuclear cells (PBMC), heparinized blood was drawn from 10 healthy subjects, 10 patients with CD, and 5 patients with UC—different from the patients from which the biopsies were taken.

Immunohistochemistry

Before use, the slides were warmed to room temperature and air-dried. The sections were washed between all steps with phosphate-buffered saline (PBS). After a wash in PBS for at least 5 minutes, sections were incubated for 10 minutes in 10% normal goat serum (Dako A/S, Glostrup, Denmark) in PBS and then incubated with CXCR3 purified mouse antihuman monoclonal antibody (Pharmingen, San Diego, CA, U.S.A.), which was diluted to a concentration of 1 $\mu\text{g}/\text{ml}$ in PBS/1% (w/v) bovine serum albumin (Sigma, St. Louis, MO, U.S.A.), for 1 hour at room temperature. Subsequently, endogenous peroxidase activity was quenched by placing the slides in a solution of 0.1% Sodium-azide in PBS and 0.3% hydrogen peroxide (H_2O_2) for 10 minutes. Sections were then incubated with biotinylated goat antimouse antibody (Dako) diluted 1:200 in PBS and 10% human normal serum (CLB, Amsterdam, The Netherlands) for 30 minutes. Sections were then incubated with Streptavidine/alkaline phosphatase (AP) (Dako) diluted 1:100 in PBS/1% bo-

TABLE 1. Clinical data of IBD patients and controls tested for the expression of CXCR3 on CD4+ T lymphocytes in surgical specimens

		Ulcerative colitis n = 22	Crohn's disease n = 29	Control n = 15
Age (years)	Median (range)	40 (23–63)	36 (20–79)	53 (25–79)
Sex ratio	M:F	7:15	9:20	5:10
Disease duration (years)	Mean $\pm$ SD	7.6 $\pm$ 1.6	7.7 $\pm$ 1.4	
Localization of the bowel	Ileum	—	20	2
Specimen resected	Colon	22	8	13
	Stomach	—	1	—
Steroid treatment		13	11	—

vine serum albumin (BSA) together with normal mouse serum diluted 1:10 in PBS/1% BSA for 30 minutes. After that, sections were incubated with CD4/FITC (fluorescein isothiocyanate) (Leu 3a/FITC; Becton Dickinson, San Diego, CA, U.S.A.) diluted 1:20 in PBS/1% BSA for 1 hour, and were then incubated with rabbit anti-FITC (Dako) diluted 1:1000 in PBS/1% BSA for 15 minutes, and incubated with horseradish peroxidase (HRP) conjugated goat antirabbit antibody (Dako) 1:50 in PBS together with 10% normal human serum for 30 minutes. After washing with PBS and Tris-HCl buffer (pH 8.5), AP activity (blue staining) was detected by Naphtol-AS-MX-phosphate (Sigma) as substrate and Fast blue BB (Sigma) as dye for 20 minutes. Then HRP activity (red staining) was detected using H_2O_2 as substrate and 3,3'-amino-9-ethylcarbazole (AEC)/Perhydrol (Merck, Whitehouse Station, NJ, U.S.A.) as dye for 10 minutes. Sections were then dipped in Formol/Macrodex for 5 minutes for the fixation. After washing with tap water and distilled water in turn, slides were warmed and mounted in Kaiser's glycerol gelatine (Merck). For control sections the primary antibodies were omitted.

Gut Lymphocyte Analysis

Biopsies were collected in RPMI + 5% human serum albumin (HSA) on ice. To get a cell suspension, samples were mashed through a 35 μ m Medicon filter with the Medimachine (Dako). This is a nonenzymatic desegregation method. We used this method to avoid enzyme-induced bias. Medicon filters were washed with PBS supplemented with 0.5% BSA and 0.02% NaN_3 (FACS [fluorescence activated cell sorter] buffer) to minimize loss of cells. Then the isolated lymphocytes were filtered through a 35 μ m strainer cap (Falcon; Becton Dickinson). Cell pellets were incubated with phycoerythrin (PE)-labeled antihuman CXCR3 monoclonal antibody (Pharmingen) and PE-Cy5-labeled antihuman CD4 antibody (Immunotech; Beckman Coulter, Brea, CA, U.S.A.) for 20 minutes on ice. The live gates and compensation of the FACSscan (Becton Dickinson) were set using cells from peripheral blood; IgG1 PE, IgG FITC, and IgG1 PC5 (all from Immunotech) were used as controls. After incubation with monoclonal antibodies, cells were washed and resuspended in ice-cold FACS buffer. Flow cytometry (FACSscan; Becton Dickinson) was used to determine the percentage of CXCR3+ cells within the CD4+ T cell population.

Peripheral Blood Analysis

Ficoll-Hypaque (Sigma) density gradient centrifugation (2,800 rpm, 4°C, 15 min) was used for PBMC iso-

lation. PBMC were washed once in PBS, and then suspended in 10 ml RPMI+ glutamine (glu) + antibiotics (ab)/antimycotics (am) + 10% FCS. Cells were stained and analyzed by FACSscan as described above.

Microscopic Analysis

Two independent observers blinded for clinical data randomly analyzed the sections. Cells positive for CD4+, CXCR3+ or CD4+CXCR3+ double-stained cells were scored according to the percentage of these cells in all lymphocytes within LP of the intestine. A score of 0 presented minimal infiltration, while 4 presented infiltration by numerous positive cells in the LP. Percentage of positive cells from 0 to 10% was scored by 0; 10 to 25% was scored by 1; 25 to 50% was scored by 2; 50 to 75% was scored by 3; and 75 to 100% was scored by 4. Individual readings were identical or differed by only one point; minor differences between the observers were resolved by conjunct agreement.

Statistical Analysis

The mean scores of three different positive cells between three patients groups were compared with non-parametric test (Mann-Whitney *U* test) using SPSS statistics for Windows (SPSS, Inc., Chicago, IL, U.S.A.) A two-tailed $p < 0.05$ was considered significant.

RESULTS

CXCR3 Expression in the Gut Mucosa

The two IBD groups were well matched for age, sex ratio, and disease duration, but the location of the UC and CD was significant different. As expected, in UC, most preparations were obtained from the colon, while CD mostly involved the ileum ($p < 0.05$). As compared with the control group, the age of the control group members is much higher than the CD group, and they all suffered from the disease in the colon ($p > 0.05$), while there was no difference between the UC group and control group in age (Table 1).

CXCR3 expression was analyzed in resections from CD and UC patients and controls. The number of CD4+ T cells, CXCR3+ cells, and CD4+CXCR3+ T cells were determined. The number of CD4+, CXCR3+, and CD4+CXCR3+ T cells in the lamina propria did not significantly differ between IBD patients and controls (Table 2) (Fig. 1). The observed differences between UC and CD in terms of numbers of CD4+, CXCR3+, and CD4+CXCR3+ cells in the lamina propria also were not

FIG. 1. Detection of CD4+, CXCR3+, and CD4+CXCR3+ T cells in the gut lamina propria (LP) and submucosa (SM). **A:** Ulcerative colitis patient ($\times 250$); **B:** Crohn's disease (CD) patient ($\times 250$); **C:** LP and submucosa staining of a CD patient ($\times 250$); **D:** Control ($\times 250$).

statistically significant (Table 2). Only CD4+ T cells and CXCR3+ cells in the submucosa were significantly increased in the CD group as compared with the control group ($p < 0.05$), but no difference was found of the CD4+CXCR3+ cells between the three groups. Because in the CD group, specimen derived from the colon and ileum were analyzed, we also compared the colon specimen from the CD group ($n = 8$) with the UC colon ($n = 22$) and control colon ($n = 13$). This analysis is similar to the analysis involving the ileum in that only the number of CD4+ T cells and CXCR3+ cells are significantly enhanced in the submucosa. The mean scores of the positive CD4+, CXCR3+, and CD4+CXCR3+ cells in the LP and in the submucosa in IBD were not related to clinical parameters including sex, disease duration, and location ($p > 0.05$). Finally we studied the relationship between histologic activity of inflammation in the sections and CXCR3 staining. The sections were labeled active or nonactive and compared with the level of CXCR3 stain-

ing. Statistical analyses indicate no relationship between these parameters.

FACScan Analysis

The percentage of CXCR3+ cells in isolated gut lymphocytes and peripheral blood lymphocytes from different patient groups was analyzed with flow cytometry. In the lymphocytes isolated from the gut, the mean percentage of CD4+CXCR3+ double-positive cells for UC, CD, and the control group was $39.5\% \pm 6.7\%$, $44.2\% \pm 7.2\%$, and $50.5\% \pm 7.6\%$, respectively. On statistical analysis, this was not significant (Fig. 2). In peripheral blood lymphocytes (PBL), the mean percentage CD4+CXCR3+ cells for UC, CD, and control group were $26.2\% \pm 7.8\%$, $29.9\% \pm 4.8\%$, and $31.2\% \pm 3.0\%$, respectively (Fig. 2) (not statistically significant). In Figure 3, a representative sample of the expression of CD4+CXCR3+ on PBL of a CD patient is illustrated. The percentage of CD4+CXCR3+ isolated from the gut was always higher when compared with PBLs within the same patient group, but the difference was not significant (Fig. 2).

DISCUSSION

Mucosal CD4+ T cells play an important role in the pathogenesis of IBD. Because of the presumed role of activated CD4+ Th1 cells in IBD, we studied the presence of a Th1-related marker, CXCR3, on these lymphocytes. In this study we demonstrate that there is no quantitative difference in the number of CD4+ T cells and

TABLE 2. Microscopic score (1–5) of T-cell infiltration in intestinal lamina propria and submucosa

		Crohn's disease		Ulcerative colitis		Control	
		Mean	SEM	Mean	SEM	Mean	SEM
CD4+	LP	2.83	± 0.18	3.09	± 0.27	2.20	± 0.39
	SM	2.45*	± 0.23	2.24	± 0.34	1.36	± 0.43
CXCR3+	LP	1.55	± 0.15	1.68	± 0.24	1.27	± 0.23
	SM	1.21*	± 0.16	1.43	± 0.27	0.64	± 0.25
CD4+CXCR3+	LP	1.10	± 0.13	1.05	± 0.15	0.93	± 0.18
	SM	1.03	± 0.17	1.14	± 0.21	0.57	± 0.20

* $p < 0.05$ compared with control group; SEM, standard error of the mean; LP, lamina propria; SM, submucosa.

FIG. 2. No significant differences in the percentages of positive CD4+CXCR3+ T cells in gut lymphocytes (GL) derived from biopsies and peripheral blood lymphocytes (PBL) from control, ulcerative colitis, and Crohn's disease patients. The GL and PBL were isolated from different patients.

CXCR3+ lymphocytes in the LP in IBD patients compared with control patients. Only in the submucosa of CD patients were more CD4+ and CXCR3+ cells found. CD4+ and CXCR3+ expression was not statistically different between CD and UC. The observed enhancement of the number of CD4+ T cells in the submucosa is in agreement with the findings of Fell et al. (10) and Camoglio et al. (11) who also described a transmural increase in CD3+ cells in CD. Because of the role ascribed to the CD4+ T cells in the pathogenesis of IBD, one could expect a difference in the number of CD4+ T cells in the LP. One explanation for the finding that we do not observe a difference could be that there is not a quantitative difference but rather a qualitative difference in CD4+ T cells, meaning that different subsets of activated CD4+ T

cells exist. It is likely that the specificity of the CD4+ T cells in the pathogenesis of IBD depends on different subsets of CD4+ T cells. Already a number of CD4+ T-cell activation markers that play critical roles in pathogenesis of IBD have been described. For example, OX40 (12) and CD40L (13), as markers of activated CD4+ T cells, are over-expressed in the LP of patients with IBD. In addition, CD40L expressed on LP T cells is able to induce proinflammatory cytokine production in monocytes (13).

It has been suggested that the chemokine receptor CXCR3 is important in the Th1 inflammatory response. In our study, there was no difference in the number of CXCR3+ T cells or CD4+CXCR3+ cells in the LP between IBD patients and controls; only the number of CXCR3-expressing cells in the submucosa was significantly enhanced in CD compared with control and UC patients. Other reports dealing with CXCR3 expression in the gut have been incomplete in regard to the expression in normal compared with pathologic tissue. Qin et al. (8) reported that in colonic biopsy specimen from patients with UC, >70% of the lymphocytes in an LP infiltrate were positive for CXCR3, particularly below the area of epithelial ulceration. However, no CD or control tissues were compared. Furthermore, in a recent report of Agace et al. (14) in which the expression of several chemokine receptors on normal human intestine were analyzed by flow cytometry, virtually all lamina propria lymphocytes and intraepithelial lymphocytes expressed CXCR3 and CCR5. Interestingly, chemokine receptors associated with Th2 (Tc2) lymphocytes, CCR7, CXCR1, and CXCR2 were not expressed. In another study, the ligand of CXCR3, IP-10, also was markedly expressed in the mucosa of control biopsies and up-regulated in UC patients (15). Combined, these studies confirm our observation that CXCR3 is expressed in the

FIG. 3. FACS analysis of CD4+CXCR3+ T cells in peripheral blood lymphocytes isolated from a Crohn's disease patient.

normal gut LP. This might indicate that CXCR3 plays a role in activated T-cell recruitment in the healthy mucosa as well as in the pathogenesis of CD. The role of CXCR3 in the pathogenesis of CD is not as absolute as has been described in rheumatoid arthritis synovial fluid (8), where CXCR3 is a general marker for T cells associated with inflammation. The reason for this discrepancy could be that the microenvironment of the normal gut is different from that of the normal synovium. In healthy joints, no foreign antigens are present, but the gut is constantly exposed to a large amount of different antigens. In healthy gut, a balance between both tolerance and immune reactivity maintains homeostasis through the action of regulatory cells and cytokines (16).

CONCLUSION

In conclusion, CXCR3 is involved in the trafficking of CD4+ T lymphocytes into the healthy mucosa of the gut, and also plays a role in the recruitment of activated lymphocytes to specific sites of inflammation.

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