CXC Chemokine Receptor 1 (CXCR1) is Expressed Mainly by Neutrophils in Inflamed Gut and Stomach Tissues

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Ohtani, N., Ohtani, H., Oki, M., Naganuma, H. and Nagura, H. CXC Chemokine Receptor 1 (CXCR1) is Expressed Mainly by Neutrophils in Inflamed Gut and Stomach Tissues. Tohoku J. Exp. Med., 2002, 196 (3), 179–184 —— CXC chemokine receptor 1 (CXCR1) is one of the important receptors for CXC chemokines with ELR motif, of which interleukin 8 (IL-8; CXCL8) is representative. To identify the cell type(s) of CXCR1-expressing cells in inflamed stomach and gut tissues, we performed immunoperoxidase method using pre-fixed frozen sections. In chronic gastritis associated with Helicobacter pylori infection (7 cases), CXCR1 was positive in neutrophils (polymorphonuclear leucocytes) in the lamina propria near the neck region and those in pit abscess. In ulcerative colitis (6 cases) and Crohn’s disease (5 cases), CXCR1 was sporadically expressed by neutrophils in the mucosa, and particularly CXCR1+ neutrophils were abundantly distributed in inflammatory granulation tissue in ulcer base. Double staining confirmed co-localization of CXCR1 and neutrophil elastase. Neither CD3+ T lymphocytes nor CD68+ macrophages were positive for CXCR1. Immunoelectron microscopy confirmed the cell surface localization of CXCR1. Neutrophils protect the host from microbial pathogens. However, they also cause damages to host tissues in chronic inflammation. Therefore, our study underscores the importance of CXCR1 expression in inflammatory processes. —— CXCR1; inflammation; gastrointestinal tract; neutrophils
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Neutrophils (polymorphonuclear leucocytes) play important roles in the inflammatory processes particularly in acute and/or active phases as one of the important mediators of innate immunity. Interleukin 8 (IL8; CXCL8), which belongs to CXC chemokines

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with ELR motif, is known to be a representative chemoattractant for neutrophils, with CXC chemokine receptors 1 and 2 (CXCRs 1 and 2) as its corresponding receptors (Zlotnik and Yoshie 2000; Godaly et al. 2001; Thelen 2001). CXCL6 (GCP-2) and CXCL1 (GROα) also bind to CXCR1. In chronic active gastritis and inflammatory bowel disease, IL-8 and GROα are abundantly expressed mainly by inflammatory cells including macrophages and neutrophils themselves and partly by epithelial cells (Arai et al. 1998; Uguccioni et al. 1999; Eck et al. 2000). Therefore, we could expect abundant CXCR1 expression in acute and/or active inflammatory lesions, presumably in neutrophils. In gut tissue, Williams et al. (2000) reported that CXCR1 is expressed by non-neutrophilic cells (macrophages and lymphocytes) in actively inflamed mucosa of ulcerative colitis (UC), stressing the importance of IL-8 signals to induce immune responses. They also described the expression of CXCR1 in epithelial cells. In the present report we adopted prefixed frozen sections for the immunohistochemical analyses of chemokine receptors. To detect chemokine receptors we need to be cautious for the fixation condition, because we have already confirmed that pre-fixed frozen sections are optimal for the immunohistochemical detection of chemokine receptor, CCR4, on the cell surface (Katou et al. 2001). We show here a clear membrane localization of CXCR1, and that positive cells were mainly neutrophils, not lymphocytes, in actively inflamed stomach and gut tissues.

**MATERIALS and METHODS**

We examined seven patients with chronic gastritis associated with *Helicobacter pylori* (*H. pylori*) infection, six with UC, and five with Crohn’s disease (CD). For the control, normal colonic tissues were used. These samples were obtained at surgical resection, and all patients with gastritis were operated for gastric cancer. Immediately after resection, fresh samples, 10×10 mm in size, were fixed in periodate-lysine-4% formaldehyde (4% PLP) for 4–6 hours. Tissue samples were washed in phosphate-buffered saline with sucrose, embedded in O.C. T. compound, and rapidly frozen. Frozen sections were used for the immunohistochemistry. The primary antibodies are listed in Table 1. Peroxidase-labeled Envision plus kit (Dako Japan, Kyoto) was used as the secondary antibody. The coloring solution was 0.006% H2O2/0.05% diaminobenzidine tetrahydrochloride (DAB; Dojin, Kumamoto) in 0.05 M Tris HCl buffer or Cobalt-DAB (DAB+0.02% CoCl2). The endogenous peroxidase activity was blocked by immersing sections in 100% methanol/0.3% H2O2 for 15 minutes after the incubation with the primary antibodies, and by adding 0.05% NaN3 in the coloring solution. In two cases, Vector red (Vector Lab., Burlingame, CA, USA) was also used as chromogen. For the negative control, the primary antibodies were replaced with isotype-matched control antibodies (DAKO Japan).

**Table 1 List of primary antibodies used**

<table>
<thead>
<tr>
<th>Antibodies to</th>
<th>Monoclonal Clone</th>
<th>Isotype</th>
<th>Final concentration</th>
<th>Sources</th>
</tr>
</thead>
<tbody>
<tr>
<td>CXCR1*</td>
<td>42705.111</td>
<td>IgG2a</td>
<td>1:1000 (1 µg/ml)</td>
<td>R &amp; D Systems, Minneapolis, MN, USA</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>NP57</td>
<td>IgG1</td>
<td>1:60 (1 µg/ml)**</td>
<td>DAKO Japan, Kyoto</td>
</tr>
<tr>
<td>CD8</td>
<td>SK-7</td>
<td>IgG1</td>
<td>1:10*</td>
<td>BD, San Jose, CA, USA</td>
</tr>
<tr>
<td>CD68</td>
<td>EBMI11</td>
<td>IgG1</td>
<td>1:200 (1.5 µg/ml)*</td>
<td>DAKO Japan</td>
</tr>
</tbody>
</table>

*The immunogen was human CXCR1-transfected NSO mouse myeloma cells. This antibody reacts with hCXCR1 transfecants but not with parental cells. This antibody does not crossreact with hCXCR2, and it blocks IL-8-induced release of myeloperoxidase from human granulocytes (R & D systems).**

**Concentration for the second step immunohistochemistry in double immunohistochemistry.
To identify neutrophils, we used neutrophil elastase, since the enzymatic activity can reflect the degree of activation of neutrophils in Crohn's disease (Gouni-Berthold et al. 1999; Dhote et al. 2000), and it causes tissue damage (Kawahata et al. 2000). The combination of double labeling immunohistochemistry was as follows: CXCR1/neutrophil elastase, CXCR1/CD3, and CXCR1/CD68. In brief, the first-step immunohistochemistry was done by the same method as in single immunohistochemistry with Cobalt-DAB as chromogen. Specimens were washed in 0.1 M glycine buffer, pH 2.2 for 15 minutes. The second-step immunohistochemistry was done with alkaline phosphatase-conjugated Envision (DAKO Japan) as the secondary antibody and with Vector red as chromogen. For the immunoelectron microscopy, we used pre-embedding immunoperoxidase method as described previously (Katou et al. 2001). The present study was approved by the Ethical Committees of Tohoku University Graduate School of Medicine and of Sendai City Hospital.

RESULTS

Our preliminary study revealed that washing in hypertonic buffer (300 mM NaCl/0.01 M phosphate buffer), three times, each for 5 minutes after the incubation with anti-CXCR1 antibody was preferable to obtain a better signal to noise ratio. In all cases of gastritis, neutrophils infiltrated mainly the neck region, and immunoreactivity for CXCR1 was mainly observed in the lamina propria around this neck region in all cases (Fig. 1a). Isotype-matched control antibody (IgG2a) revealed no reactivity (Fig. 1b). Higher magnification revealed that CXCR1 was localized along the cell membrane, and positive cells had lobulated nuclei, indicating that they were neutrophils (polymorphonuclear leucocytes) including band neutrophils (Fig. 1c). Fig. 1c also shows that not all neutrophils express CXCR1. In one case, neutrophils in pit abscesses were positive for CXCR1 (Fig. 1d). For UC and CD, we selected cases with active mucosal inflammation. In UC, CXCR1+ cells were sporadically detected in inflamed mucosa with most of neutrophils negative for CXCR1. In CD mucosa, CXCR1 reactivity was scarcely observed. Occasionally neutrophils in crypt abscess were positive for CXCR1 in UC (the same as in pit abscess in Fig. 1d). The most abundant reactivity for CXCR1 was observed in neutrophils in the areas of ulcer base in UC and CD (Fig. 1e). Epithelial cells did not show membrane-type staining for CXCR1 in any cases. In the normal control colonic mucosa, the expression of CXCR1 was sparse or absent (data not shown).

Double staining performed in 2 patients with gastritis, 3 with UC and 2 with CD confirmed that CXCR1 (membrane localization) was co-localized with neutrophil elastase (cytoplasmic localization) (Fig. 1g). No clear relationship was observed between the intensity of neutrophil elastase and reactivity of CXCR1 in the inflamed mucosa. However, neutrophils that permeated to the surface of ulcer base in UC and CD lacked the reactivity for CXCR1, although they expressed neutrophil elastase abundantly (Fig. 1f). Neither T-lymphocytes nor CD68 macrophages were positive for CXCR1 (Figs. 1d and 1h). Immunoelectron microscopy (performed in 1 patient with gastritis and 1 with UC) revealed a clear membrane localization of CXCR1 along the cell surface, and positive cells were judged to be neutrophils because of lobulated nucleus and abundant cytoplasmic granules and vacuoles (Fig. 2a). Eosinophils identified by electron dense eosinophil-specific granules were negative for CXCR1 (Fig. 2b). No clear localization of CXCR1 was observed in lymphocytes or macrophages (Fig. 2a).

DISCUSSION

This is the first study to demonstrate that CXCR1 is localized along the cell membrane of neutrophils in human inflammatory lesions.
Fig. 1.  

a: Immunohistochemistry for CXCR1 (red) in chronic active gastritis. b: Negative control with the primary antibody replaced by isotype-matched control antibody. c: Higher magnification of CXCR1 staining (black) with hematoxylin counterstaining. Note that positive cells (arrows) have lobulated nucleus. d: CXCR1 (black) is also expressed by neutrophils in pit abscess (center), and red color represents CD68 (macrophages). e-g: Double staining of CXCR1 (black)/neutrophil elastase (red) in the ulcer base of Crohn’s disease. Hematoxylin counterstaining. e: Low magnification. The surface area of ulcer base (S) and granulation tissue (Gr). f: Neutrophils permeated to the surface of ulcer base (S), which lack CXCR1 reactivity. g: The area of inflamed granulation tissue (Gr) in ulcer base, where neutrophils elastase (red)-expressing neutrophils are positive for CXCR1 (black) on the cell surface. h: Double staining of CXCR1 (dark brown)/CD3 (red) in inflamed mucosa in ulcerative colitis. CXCR1+ cells are different from CD3+ T-cells. Scale bar, 200 μm in Fig. 1a, 1b, 25 μm in Fig. 1c, d, f-h, and 250 μm in Fig. 1e.
Our previous study on CCR4 (Katou et al. 2001) and the present results confirmed the methodological reliability to use PLP-fixed frozen sections for the immunohistochemical detection of chemokine receptors. IL-8 (CXCL8) and GROα (CXCL1) are upregulated in UC, CD and chronic gastritis (Arai et al. 1998; Uguccioni et al. 1999; Eck et al. 2000). Therefore, our data suggest that neutrophils are the main cell sources that could be chemoattracted to actively inflamed tissue by the signals of IL-8 and other CXC chemokines through CXCR1. Neutrophils are not only important for the defense against microbial pathogens but they also cause epithelial damages in chronic inflammation (Yabuki et al. 1997). Therefore, CXCR1 may be crucial for the control of inflammatory process. As shown in the present study, not all neutrophils express CXCR1. This may be related to the activation status of neutrophils, and more detailed study will be required to elucidate the activation status of neutrophil in chronic inflammatory diseases.

Monocytes/macrophages, lymphocytes and vascular endothelial cells are other candidates for CXCR1 expression (Rossi and Zlotnik 2000). Furthermore, epithelial cells may express CXCRs-1 and -2 during the transepithelial migration of neutrophils (Godaly et al. 2000). Our present observation did not detect CXCR1 on these non-neutrophil cells. The differences from the previous study (Williams et al. 2000) may be due to the differences of antibody, fixation and tissue processing methods. As already stated in the introduction section, a precaution is required for the detection of membrane receptor proteins. Due to a possible limitation of the sensitivity of our present method, our study does not completely exclude the CXCR1 expression in these cells at a lower expression level.

We observed a phenotypical change of neutrophils in inflamed tissues; neutrophils in inflammatory granulation tissue of UC and CD abundantly express CXCR1 while those permeated outside the surface of ulcer base lacked CXCR1 although they expressed neutrophil elastase extensively. This suggested that neutrophils that permeated on the surface of
ulcer base do not require stimulatory signals through corresponding chemokines any more, with an abundant expression of neutrophil elastase. This observation may correspond to an in vitro observation that the cell surface neutrophils-elastase activity increases after stimulated by IFN-γ and IL-8 (Owen 1997), or this may be related to the cell senescence. In conclusion, CXCR1, mainly expressed by neutrophils, is one of the important chemokine receptors to modulate inflammatory process.

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