Expression of Interleukin-8 Receptors (CXCR1 and CXCR2) in Premenopausal Women with Recurrent Urinary Tract Infections

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The migration of neutrophils through infected tissues is mediated by the CXC chemokines and its receptors (CXCR1 and CXCR2). It has been proposed that a CXCR1 deficiency could confer susceptibility to acute pyelonephritis in children. The objective of the study is to assess the surface expression of CXCR1 and CXCR2 and the existence of polymorphisms in the CXCR1 gene in premenopausal women with recurrent urinary tract infections. The study included 20 premenopausal women with recurrent urinary infections, with normal urinary tracts, and without diseases potentially associated with relapsing urinary infections and 30 controls without previous urinary infections. The levels of CXCR1 and CXCR2 expression on neutrophils were measured and analyzed by flow cytometry by measuring the mean fluorescence intensity (MFI) channel. The promoter and coding regions of the CXCR1 gene were analyzed for the presence of polymorphisms by a sequence-based typing method. Patients with recurrent urinary tract infections exhibited median levels of CXCR1 expression, determined from MFI values, similar to those of the controls. The analysis of CXCR2 showed that patients with recurrent urinary infections had lower median levels of expression, determined from the MFI values, than the controls (P = 0.002, Mann-Whitney U test). No polymorphisms were detected at the promoter or at the exon 1 region of the CXCR1 gene either in the patients or in the controls. Polymorphisms were detected at the exon 2 of CXCR1, but their frequencies did not differ between patients and controls. We have found a low level of CXCR2 expression in patients with recurrent urinary tract infections. These results suggest that a low level of CXCR2 expression may increase the susceptibilities of premenopausal women to urinary tract infections.

Recurrent urinary tract infections (RUTIs) are frequent among healthy young women who generally have anatomically and physiologically normal urinary tracts (23). It has been estimated that each year in the United States about 6 million to 8 million young women have acute cystitis (20). Moreover, about 25% of these women who have had an initial infection will experience RUTIs, resulting in considerable morbidity and the associated health care costs (25). There is no satisfactory theory to explain the predisposition of some young healthy women with normal urinary tracts to RUTIs, but it seems to be the result of the combination of various factors inherited by the host and several behavioral conditions (5).

In response to infection with uropathogenic Escherichia coli, the most common pathogen involved in community-acquired urinary tract infections (UTIs) (28), human uroepithelial cells secrete interleukin-8 (IL-8) as well as other members of the CXC cytokine family, which are potent neutrophil chemotactici and activating peptides (15, 10, 16). Neutrophils respond to the CXC cytokine gradient by leaving the general circulation and accumulating in the subepithelial tissue, and eventually, end-stage renal disease (9, 29). Additional evidence of the importance of the IL-8 receptors in the neutrophil migration process comes from the observation of the absence of neutrophil recruitment to the urinary tract after the administration of a blocking IL-8 receptor homologue antibody in mice (14).
It has been proposed that the increased susceptibility to UTIs observed in IL-8 receptor-knockout mice could have a human counterpart. Frendeus et al. have demonstrated that neutrophils from children prone to acute pyelonephritis show decreased levels of expression of CXCR1 but not CXCR2 compared with those of age-matched controls, which may explain their susceptibilities to recurrent acute pyelonephritis (6). In addition, preliminary results suggest that the low level of expression of CXCR1 could be related to polymorphisms in the promoter region of the CXCR1 gene (7). Therefore, the confirmation of a deficient expression or function of the CXC receptors in humans could provide new genetic clues to explain individual susceptibilities to UTIs.

To our knowledge the expression of CXC receptors in adults with RUTIs has not been previously assessed. The objective of the present study was to investigate the surface expression of CXCR1 and CXCR2 on human neutrophils as well as the presence of single-nucleotide polymorphisms in the CXCR1 gene from premenopausal women with RUTIs and from a healthy control group.

### MATERIALS AND METHODS

#### Study population

The study group included 20 premenopausal women with a history of RUTIs and normal urinary tracts identified from the Infectious Diseases Outpatient Unit between January 2002 and January 2003. For further comparison, 30 volunteer premenopausal females who were not matched with the patients and who did not have a history of UTIs were also studied. The present study was conducted with the approval of the hospital Ethics Committee and the informed consent of all participants. The human experimentation guidelines of the U.S. Department of Health and Human Services and those of the authors’ institution were followed in the conduct of the clinical research.

Women were considered to meet the case definition for RUTIs if they had experienced either three or more symptomatic UTIs in the past year or two such episodes in the past 6 months. A UTI was defined by bacterial growth ≥10⁵ CFU/ml in a culture of midstream urine from a women experiencing two or more symptoms of cystitis (dysuria, urgency, frequency, suprapubic pain, or hematuria) or, in the absence of culture, the demonstration of pyuria on urine analysis and two or more urinary tract symptoms, as well as the complete and rapid resolution of symptoms after the start of antibiotic therapy. Acute pyelonephritis was clinically defined as an armpit temperature of >38°C, pyuria, and hematuria.

Premenopausal women with a history of urological abnormalities or urological manipulation or in which the imaging evaluation (echography and pyelography) had demonstrated urological abnormalities potentially related to RUTIs were excluded. Patients with diseases associated with RUTIs (diabetes mellitus, cirrhosis, immunosuppressive treatments, transplant recipients) were also excluded.

**Patient samples.** Samples were obtained during an infection-free interval. Blood samples were collected by venipuncture into Vacutainers (Becton Dickinson, Mountain View, CA) equipped with an argon laser (excitation wavelength, 488 nm). The mixture was left for an additional 15 to 30 min in the dark at room temperature before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Fluorescence-activated cell sorter lysing solution was added by gentle vortexing, and the cytometry was performed on ice to the laboratory and analyzed by flow cytometry within 1 to 6 ho f collection.

**Reagents.** Phycoerythrin (PE)-labeled mouse immunoglobulin G2a (IgG2a) monoclonal antibodies to human CXCR1 (clone 42705.111) and CXCR2 (clone 48311.211) were from R&D Systems Inc. (Minneapolis, MN). Fluorescein isothiocyanate (FITC)-labeled mouse IgG1 monoclonal antibody to human CD45 (clone 2D1) was from BD Biosciences (San Jose, CA). FITC-labeled mouse IgG2a (clone X39) and PE-labeled mouse IgG1 were from BD Biosciences.

**Immunofluorescence and flow cytometry analysis.** Direct immunofluorescence staining of whole blood was performed by using a lysis, no-wash procedure, according to the manufacturer’s instructions (BD Biosciences). Briefly, 50 µl of EDTA-anticoagulated whole blood was mixed with 20 to 50 µl fluorochrome-conjugated monoclonal antibodies (PE-labeled anti-CXCR1 and anti-CXCR2 or FITC-labeled anti-CD45) and incubated for 15 min in the dark at room temperature. As negative controls, additional samples were incubated with PE-labeled mouse IgG2a or FITC-labeled mouse IgG1. The staining with FITC-labeled anti-CD45 monoclonal antibody, which recognizes the common leukocyte antigen present on neutrophils, monocytes, and lymphocytes, was used as an internal control to verify the integrity of the target cell population among different individuals and to exclude fragments of red blood cells. Then, 450 µl of fluorescence-activated cell sorter lysing solution was added by gentle vortexing, and the mixture was left for an additional 15 to 30 min in the dark at room temperature before analysis on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA) equipped with an argon laser (excitation wavelength, 488 nm). The data were analyzed with 1.0 CellQuest software (Becton Dickinson). Forward and side light-scatter parameters were used for the gating of the neutrophil population. A histogram of fluorescence distribution was constructed, and the relative mean fluorescence intensity (MFI) was obtained from the histogram and expressed as an index of membrane surface expression.

**Sequence-based typing.** Genomic DNA was extracted from peripheral blood by using a commercially available kit (QiAamp blood DNA isolation kit; QIAGEN, Hilden, Germany), following the manufacturer’s instructions. Specific primers for the detection of SNPs were PCR amplified and analyzed using a fluorescent dideoxy sequencing method.
were designed for PCR amplification of the promoter and the coding regions of the CXCR1 gene, according to the published genomic DNA sequences (GenBank accession number L19592) (24). A 519-bp fragment encompassing the promoter region and exon 1 was generated by using primers IL8RAPro.Fw (5'-GAGGTCTCTGCTGTAAGTCA-3') and IL8RAPro.Rv (5'-CTCAACCTCC CACGAAATG-3'). The whole coding region of exon 2 (a 1,203-bp fragment), from the end of intron 1 to beyond the natural stop codon, was generated by using primers IL8RAInt1.Fw (5'-GCCTTGAATCCGAGCTACTAAAT-3') and IL8RAEx2.Rv (5'-CCTCAGGTTGTGTTATCTCT-3'). The PCR was performed in a final volume of 50 µl containing 100 to 500 ng of genomic DNA, 2 pmol/ml of specific primers, 0.5 to 2 U Taq Expand DNA polymerase (Boehringer Mannheim), 2 mM MgCl2, 5 mM deoxynucleoside triphosphates, and 10 mM Tris-HCl (pH 9.5). The cycling conditions were 8 min at 98°C; 35 cycles of 30 s at 94°C, 30 s at 58°C, and 90 s at 72°C; and a final extension step of 10 min at 72°C. Samples (1 to 2 µl) of the resulting PCR products were subjected to direct sequencing by the dye-terminator method (ABI PRISM dRhodamine Terminator cycle sequencing ready reaction kit) and analyzed with an automated ABI 3100 DNA sequencer (Applied Biosystems). The sequencing primers for the promoter region and exon 1 was generated by using primers IL8RAPro.Fw (see above) and IL8RA6072.Fw (5'-GAGGTCCTGGAAATGACAC-3'). A 519-bp fragment encompassing the promoter-exon 1 and exon 2 fragments were IL8RAPro.Fw (see above) and IL8RAPro.Rv (5'-GAAGTCTCTGCTGTAAGTCA-3'), respectively.

Statistical analysis. The levels of expression of CXCR1 and CXCR2 on neutrophils from women with RUTIs and from healthy controls were compared by the Mann-Whitney U test. Values are expressed as medians (horizontal bar), 25th and 75th percentiles (boxes), and 10th and 90th percentiles (bars). Significant differences between groups are indicated (Mann-Whitney U test).

RESULTS

Clinical data. The study group was composed of 20 premenopausal women with RUTIs (median age, 26 years; age range, 21 to 39 years) and 30 premenopausal female volunteers who had never experienced an UTI (median age, 27 years; age range, 18 to 46 years). The main clinical findings for the premenopausal patients with RUTIs are shown in Table 1. Nine of the 20 patients with RUTIs had experienced their first UTI before the age of 15 years. The lifetime number of UTIs was ≥10 in 65% of the women with RUTIs. By consideration of the total number of acute pyelonephritis episodes of each patient during their lives, 11 patients (55%) had experienced cystitis with or without one episode of acute pyelonephritis and 9 patients (45%) had experienced cystitis with more than one episode of acute pyelonephritis. The nine patients with an early onset of UTI had experienced a total of 32 episodes of acute pyelonephritis (74% of the total episodes of acute pyelonephritis of the study group), while the 11 patients with a late onset of UTI had experienced a total of 11 episodes of acute pyelonephritis.

Chemokine receptor expression. The surface expression of CXCR1 and CXCR2 was measured on neutrophils and expressed as arbitrary units of MFI. The values obtained for each premenopausal patient with RUTIs included in the study are represented in Table 1. The median MFI values (25th and 75th percentiles) of CXCR1 and CXCR2 in the healthy control group were 116.22 (103.67 and 139.46) and 18.94 (16.2 and 23.62), respectively. The median MFI values of CD45 were comparable between the patients and the healthy controls (18.38 [15.89 and 23.71]) for patients with RUTIs and 18.36 [16.62 and 23.41] for healthy controls for samples simultaneously stained for CXCR1; (19.22 [16.78 and 30.1] for patients with RUTIs and 18.84 [17.21 and 22.7] for the healthy controls for samples simultaneously stained for CXCR2). The median MFI value of CXCR1 observed in the premenopausal patients with RUTIs was 118.9 (102.66 and 158.89), similar to the median MFI values found for the healthy controls (Fig. 1). However, three patients with the onset of UTIs during childhood had CXCR1 MFI values below the 5th percentile of the CXCR1 MFI values for the healthy control group. The analysis of CXCR2 revealed that premenopausal women with RUTIs showed significantly lower median CXCR2 MFI values (14.96 [12.82 and 17.23]; P = 0.002) than the controls (Fig. 1). A deeper analysis in which the patients were classified by the age of onset of UTIs showed that although the median MFI values of CXCR1 (112.14 [63.58 and 169.01]) for those with the onset of UTIs before the age of 15 years and 122.31 [103.29 and
pyelonephritis show low levels of CXCR1 neutrophil surface expression compared to those for age-matched controls. This deficient CXCR1 expression could be the result of genetic polymorphisms in the promoter region of the CXCR1 gene (6, 7). In our study, patients with RUTIs had a median CXCR1 MFI value comparable to that for the healthy controls, although the small number of patients included could have influenced these results. However, a closer analysis of the data revealed that three patients (patients 1, 2, and 3) with RUTIs had a median CXCR1 MFI value below the 5th percentile of the median MFI value for the healthy controls. These three patients began having UTIs before the age of 15 years, experienced their first sexual intercourse after that age, and have had more than one episode of acute pyelonephritis. The onset of UTIs before the age of 15 years, particularly before the first sexual intercourse, has been reported to be a risk factor for RUTIs, supporting the idea that inherited factors may be important in some women with relapsing UTIs (21). Therefore, our observation suggests that a deficient expression of CXCR1 could be implicated in the increased susceptibility to RUTIs in the subset of premenopausal women with an early onset of urinary tract infections through deficient neutrophil chemotaxis to the urinary tract.

An unexpected observation of our study is the low median level of MFI CXCR2 surface expression on neutrophils from premenopausal women with RUTIs compared to that on neutrophils from healthy controls. This lower level of surface expression was even more marked in the subgroup of premenopausal women in whom the onset of UTIs occurred before the age of 15 years, which again strengthens the idea that individual factors could be particularly important in the pathogenesis of RUTIs in women with the onset of urinary tract infections during childhood. It is interesting that the two patients (patients 1 and 2) with the lowest levels of CXCR2 surface expression also had very low levels of CXCR1 surface expression. Different studies have suggested that CXCR1 and CXCR2 are regulated by agonist-dependent mechanisms. Binding of IL-8 and ENA-78 rapidly downmodulates CXCR1 and CXCR2 due to internalization of the ligand-receptor complex (4). It is probable that IL-8, ENA-78, as well as other chemotactic cytokines regulate the expression of CXCR1 and CXCR2 through similar or identical pathways. Therefore, the expression of both receptors may be regulated in a similar way.

Neutrophil recruitment is driven not only by IL-8 but also by other chemotactic factors, such as ENA-78 and GRO; and thus, a low level of surface expression of a multiligand-specific receptor such as CXCR2 could cause a deficient neutrophil chemotaxis. Our data demonstrating the low level of expression of CXCR2 in premenopausal women with RUTIs suggests a handicapped capacity of GRO-α and ENA-78, and maybe of other CXCR2 binding chemokines, to exert their chemotactic activities. These data differ from those from previous in vitro studies that used kidney epithelial cell layers infected with E. coli and in which the addition of an anti-CXCR1 antibody but not an anti-CXCR2 antibody reduced E. coli transepithelial neutrophil migration (8). However, other studies have demonstrated a partial inhibition of neutrophil chemotaxis by using specific neutralizing antibodies directed against ENA-78 and GRO, which are CXCR2-dependent chemokines (14). Further studies are needed to address whether the CXCR2-deficient surface expression in the patients with RUTIs is caused by...
specific gene defects or, alternatively, by upstream regulatory mechanisms.

In the second part of the study we screened the promoter and the entire coding region of the CXCR1 gene in 20 premenopausal patients with RUTIs and in 30 healthy controls and detected three missense exchanges. Two of these have already been reported (11, 27). Although previous studies have suggested the existence of polymorphisms in the promoter region and exon 1 of the CXCR1 gene in children prone to acute pyelonephritis, we were not able to detect such polymorphisms in either patients or healthy controls (7). The analysis of exon 2 has demonstrated that two patients were heterozygous for the two previously reported nonsynonymous polymorphisms, S276T and R335C of the mature protein. These two patients (patients 2 and 3) also had low levels of MFI CXCR1 surface expression (Table 1). Another patient (patient 20), who presented with the novel nonsynonymous S276R polymorphism, showed an average level of MFI CXCR1 surface expression in the flow cytometry analysis. In conclusion, no significant differences in the presence of single-nucleotide polymorphisms in the promoter and the coding regions (exons 1 and 2) of the CXCR1 gene were detected between patients and controls. It is worth mentioning that the R335C polymorphism was observed in the only healthy control individual showing reduced surface expression of CXCR1. The same polymorphism was also observed in one of the three patients showing reduced surface expression of CXCR1.

The CXCR1 receptor is a member of the superfamily of G-protein-coupled receptors that consists of seven transmembrane domains, three intracellular loops, and three extracellular loops. The S276T and the S276R polymorphisms are located in the third extracellular loop, while the R335C polymorphism is located in the C-terminal tail of the CXCR1 gene. The C-terminal tail of CXCR1 is necessary for receptor phosphorylation and desensitization (17), and the replacement of arginine by cysteine at position 335 may confer the potential of homo- or heterodimerization through disulfide bonds (2). Therefore, the R335C polymorphism has the potential to influence receptor functions by changing the secondary or tertiary structure of the receptor. The S276T and S276R variations are not expected to lead to major differences in receptor structure or function. Further studies are needed to evaluate the relevance of the R335C polymorphism on CXCR1 functions.

In conclusion, we have found no differences regarding the average level of neutrophil CXCR1 expression between premenopausal patients with RUTIs and healthy controls. However, three patients that began having UTIs during childhood presented low, below-average CXCR1 levels on the neutrophil surface. These low levels of CXCR1 on the neutrophil surface were not related to specific polymorphisms in the CXCR1 gene. We have also found an average lower level of expression of CXCR2 in premenopausal women with RUTIs, particularly in those with an early onset of urinary tract infections, compared with that for the healthy controls. These results suggest that a low level of expression of CXCR2 could be associated with increased susceptibilities to RUTIs in certain patients, particularly in those premenopausal women with the onset of UTIs during childhood. The identification of these women at high risk of developing serious and repetitive urinary tract infections could justify the implementation of preventive anti-biotic therapeutic strategies.

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