Interleukin-8 is essential for normal urothelial cell survival

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Tseng-Rogenski S, Liebert M. Interleukin-8 is essential for normal urothelial cell survival. Am J Physiol Renal Physiol 297: F816–F821, 2009. First published June 17, 2009; doi:10.1152/ajprenal.90733.2008.—Interleukin-8 (IL-8; CXCL8) has been shown to play a role in multiple cellular processes. Here, we report an additional role of IL-8 as a growth and survival factor for normal urothelial cells. Supplementation exogenous recombinant human IL-8 to normal urothelial cells promoted cell growth through the Akt pathway. Inhibition of IL-8 expression by small inhibitory RNA (siRNA) caused normal urothelial cells to die. Additions of recombinant human IL-8 rescued the normal urothelial cells treated with IL-8 siRNA. This rescue effect could be blocked by antibodies to the IL-8 receptor CXCR1 but not by CXCR2, suggesting that normal urothelial cells normally have IL-8 autocrine and paracrine activity for survival and growth mediated by CXCR1. IL-8 mRNA levels were lower in samples from patients with interstitial cystitis, a urinary bladder disorder associated with urothelial cell dysfunction and/or loss. Taken together, these results suggest that IL-8 is an important normal urothelial growth factor and is necessary for normal urothelial cell survival in vitro and in vivo. Lower IL-8 expression levels in the urinary bladder may contribute to pathophysiology of interstitial cystitis.

Interleukin-8 (IL-8; CXCL8) was originally identified as a chemotactic factor secreted by activated leukocytes that promotes directional migration of neutrophils and T lymphocytes (2, 36, 37). In addition to participating in acute and chronic inflammation, IL-8 promotes angiogenesis by stimulating growth and survival of endothelial cells (24). IL-8 also directly supports cell growth for several types of cancer cells, normal fetal colon and hematopoetic progenitors (1, 7, 27, 39, 42, 43). More recently, studies showed that IL-8 protects against cell death (1, 27, 42). Because of strong IL-8 biologic activity, IL-8 expression is tightly controlled, and normal levels are usually low (14).

IL-8 is the prototype of the CXC chemokine family (2, 36, 37). CXC chemokines mediate their biological functions by interacting with specific G protein-coupled CXC chemokine receptors (CXCRs) (2, 35–37, 42). CXCR1 and CXCR2 bind to IL-8 with high affinity. CXCR1 binds more specifically to IL-8, whereas CXCR2 binds to IL-8 as well as other members of CXC chemokine protein family with similar high affinity (2, 36, 37). Activation of CXCRs results in signal transduction (27, 35, 42). Urothelial cells, which line the urinary tract from the renal pelvis through the urinary bladder, constitutively express but normally do not secrete IL-8 (3, 13, 38). IL-8 release by urothelial cells is increased in response to urinary tract infection (20, 38). IL-8 is well-known to play an important role protecting the urinary tract by recruitment of leukocytes to resolve infection (12, 40). Additionally, bladder urothelial cells express both CXCR1 and CXCR2 (12, 40). Other functions of IL-8 in the normal urinary tract have not been studied.

Because of the emerging understanding of additional functions of IL-8, we explored functions of IL-8 in urothelial cells. In this report, we show that IL-8 is a growth factor essential for normal urothelial cell survival. The expression of IL-8 in the nonmalignant urothelial cell disorder, interstitial cystitis, was also evaluated. Additional roles of IL-8 in urothelial biology are also explored.

MATERIALS AND METHODS

Cells and tissues. Urothelial cells were cultured from ureteral tissues as previously described (25) in a low-calcium, serum-free growth medium [Keratinocyte-SFM (KGSF) supplemented with 0.2 ng/ml of epidermal growth factor, 30 µg/ml of bovine pituitary extract, and 1% penicillin-streptomycin, Invitrogen]. Normal bladder and ureteral tissues as well as interstitial cystitis bladder tissues were obtained under University of Michigan Institutional Review Board-approved protocols. Patients with interstitial cystitis met the National Institute of Diabetes and Digestive and Kidney Diseases-modified criteria (21). Normal specimens were obtained from normal volunteers and from tissues removed surgically for conditions other than interstitial cystitis.

Growth evaluation. Cells per well were seeded at 7.5 × 10^4/well into a 24-well plate with 500 ng/ml of recombinant human IL-8 (rIL-8; PeproTech) and incubated at 37°C/5% CO2. Samples were added to normal urothelial cells for growth studies. On the first day of the treatment, groups treated with the AKT inhibitor X were pretreated with the inhibitor for 1 h before the addition of rIL-8 and/or vehicle control. Old media were replaced with fresh media containing rIL-8, AKT inhibitor X, and/or vehicles daily. On day 4, cells were trypsinized, collected, and resuspended in Trypan blue for cell counting using a hemocytometer. The experiment was conducted in triplicates and each well was counted twice.

Small inhibitory RNA. To optimize transfection reactions using the transfection agent (Lipofectamine 2000, Invitrogen), normal human urothelial cells were seeded onto a 24-well plate to reach different cell densities the next day. Transfections were carried out using 0.8 µg per well of FITC-conjugated synthetic nucleotides (Invitrogen). Cells were observed 24 h after transfection and evaluated using a fluorescence microscope and documented by photography. Fully confluent normal urothelial cell cultures are more tolerant of treatment with transfection reagent compared with cell cultures at lower confluencies.
(data not shown) and therefore transfections were performed on confluent cultures.

Diced small inhibitory RNA (siRNA) pool was synthesized using a commercially available kit (BLOCK-iT complete Dicer RNAi kit, Invitrogen). Briefly, total RNA of normal human urothelial cells was used to synthesize an 864-bp-long IL-8 cDNA fragment using oligo(dT) primers for first-strand cDNA and then IL-8–1 (5′-TTCA-GAGACAGCAGACACACA-3′) and IL-8–5 (5′-GCTAGCAGC- TAGGGTTGCCAG-3′) primers for the subsequent PCR. The cDNA was ligated to T7 promoter sequences at both ends and subjected to second PCR amplification using T7-specific primers. The resulting T7-IL-8 DNA fragment served as the template in the following T7 in vitro transcription to yield IL-8 RNA fragment, which was then subjected to a dicer reaction to produce IL-8 siRNA pools. The siRNA was purified before use. The HM-LacZ was used in the same way, except for using lacZ gene. The HM-lacZ was used in the transfection reactions as a control to demonstrate the specificity of the IL-8 siRNA. Commercially available siRNAs were also purchased (Ambion). The sequences for these IL-8 siRNAs (siRNA ID 2261) are 5′-GUGCAUUUGCCAAGATT-3′ (sense) and 5′-UCCUGGCAGAAUCCTT-3′ (anti-sense).

Normal human urothelial cells were transfected with IL-8 and/or control siRNA using Lipofectamine 2000 (Invitrogen). A day before transfection, 1.5 × 10⁵ cells per well were seeded onto 24-well plates and incubated at 37°C/5% CO₂ overnight to reach 100% confluence. The next day, 0.8 μg of siRNA in 50 μl media and 3 μl of Lipofectamine 2000 in 50 μl media were mixed, incubated at room temperature for 20 min, and added into each well. H₂O₂ was used in the mock transfection as a control for the transfection reagent. Transfection was performed in KSGF without antibiotics. Media were changed 24 h posttransfection and cells and the media were harvested 48 h after the transfection to assess IL-8 expression.

Addition of rIL-8 (500 ng/ml; PeproTech) after siRNA treatment was performed to evaluate IL-8 “rescue” of the normal urothelial cells. To block the rescue effect of rIL-8, 10 μg/ml of anti-CXCR1 and/or anti-CXCR2 antibodies (R&D Systems) were added in addition to rIL-8.

IL-8 RT-PCR. Total RNA (2 μg) was incubated with 0.5 μg of oligo(dT) primers (Invitrogen) at 65°C for 15 min to denature RNA and the primers and then chilled on ice. Pre-RT-PCR mix [8 μl for each reaction composed of 4 μl of 5× transcription buffer, 2 μl of 0.1 M DTT, 1 μl of 10 mM dNTPs, and 1 μl (40 U) of MTLV-reverse transcriptase (Invitrogen)] was added into each reaction and incubated at 37°C for 30 min, followed by the addition of another 0.5 μl of reverse transcriptase, and then another 30 min of incubation at 37°C. The reaction was then incubated at 65°C for 15 min to stop the reverse transcription. The cDNA product (2 μl of diluted cDNA) was used to set up the subsequent PCR with IL-8–1 and IL-8–5 primers (30 cycles; see above for primers’ sequences) using PCR Platinum Supermix (Invitrogen). PCR product was separated on a 1% agarose gel to check the expression levels of IL-8 after siRNA transfection. For interstitial cystitis tissues obtained by bladder biopsies and normal control tissues, RNA was isolated from the tissues containing urothelium and muscle layers using RNazol (Tel-Test) and evaluated using a cytokine RT-PCR amplifier kit (Clontech), following the manufacturer’s directions.

IL-8 quantitation. IL-8 levels were quantified by a commercial enzyme immunoassay (BD OptEIA Set for human IL-8, BD Biosciences) following manufacturer’s instructions. Briefly, an immunoassay flat-bottomed 96-well plate was coated with capture antibody at 4°C overnight. The next day, the plate was washed and blocked with assay diluent at room temperature for 1 h. Samples and standards were added into the wells and incubated at room temperature for 2 h. After being washed, detection antibody and avidin-horseradish peroxidase (HRP) were added into the wells and incubated at room temperature for 1 h. The plate was washed. After addition of substrate, the plate was incubated at room temperature for 30 min. Stop solution (2 N HCl) was added to each well, and the plate was read at 450 nm. Results were determined from a standard curve.

CXCR1 and CXCR2 staining. After cells were transfected with siRNA in the presence of rIL-8 for 24 h, cells were collected and seeded onto eight-chamber slides and incubated at 37°C/5% CO₂ overnight. To only stain the cell surface receptors, unfixed cells were rinsed in PBS and blocked with 5% FBS in PBS (FBS/PBS) for 20 min. Cells were then stained at 4°C with anti-CXCR1 or CXCR2 (R&D Systems) at 10 μg/ml in FBS/PBS for 2 h, followed by three washes with PBS. Cells were then stained at 4°C with FITC-conjugated anti-mouse antibody (Zymed) for 1 h. After three washes in PBS, cells were fixed in ice-cold 3% paraformaldehyde in PBS for 20 min. Slides were briefly rinsed in PBS, mounted, and evaluated on a fluorescence microscope.

Western blotting. Normal urothelial cells were seeded onto six-well plates (120,000 cells per well) and incubated at 37°C/5% CO₂ overnight. The next day, media were replaced with fresh media supplemented with 500 ng/ml of rIL-8 (1 ml per well; PeproTech), except for the zero time point, and incubated at 37°C/5% CO₂ for 5, 10, 20, 30, and/or 60 min. At each time point, cells were washed twice with ice-cold PBS and homogenized in cell lysis buffer (10 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% sodium deoxycholate, 5 mM EDTA, 0.1% NP-40, 1 μg/ml aprotinin, 1 μg/ml leupeptin and HALT

Fig. 1. IL-8 acts as a growth factor for normal urothelial (NU) cells through Akt pathway. A: different amounts of recombinant human IL-8 (rIL-8) were added to normal human urothelial cell cultures. The growth rate of NU cells was monitored by toluidine blue O staining. B: levels of phosphorylated Akt increased on the addition of rIL-8. C: growth-promoting effect of IL-8 was blocked by the addition of an Akt-specific inhibitor in a dosage-dependent manner. The difference between IL-8 alone and IL-8 with Akt inhibitor is statistically significant. Differences significant from controls are indicated by * (P < 0.05).
phosphatase inhibitor cocktail, Thermo Scientific). Cell lysates were separated on nanography SDS-PAGE (10 μg total protein per lane) and transferred onto nitrocellulose membrane. Western blotting was performed using phospho-Akt pathway sampler kit as recommended by the manufacturer (Cell Signaling Technology). The membrane was blocked in 5% skim milk/25 mM Tris-HCl-buffered saline containing 0.1% Tween-20 (TBST) at room temperature for 1 h and then incubated with primary antibody at 1:1,000 dilutions in 5% BSA/TBST at 4°C with gentle shaking overnight. The membrane was then washed three times with TBST and incubated with secondary antibody (anti-rabbit-HRP; Cell Signaling Technology) at 1:2,000 dilutions at room temperature for 1 h, followed by three washes, development using chemiluminescent HRP substrate (Immobilon Western; Millipore), and exposure to X-ray film (Kodak Biomax XAR film).

**Statistical analysis.** Each experiment was performed at least twice. Statistical analysis was performed using a commercially available computer statistics package (Sigma Stat v3.5, Systat Software). ANOVA was used to compare treatments, and pairwise comparisons were performed (Holm-Sidak method). Differences were considered significant for P values <0.05.

**RESULTS**

**IL-8 promotes the growth of normal urothelial cells in vitro through Akt signaling.** Because normal urothelial cells constitutively express but normally do not secrete IL-8 (2, 13, 38), and IL-8 has been shown to stimulate growth in a variety of cell types (1, 24, 27, 39, 43), growth of normal urothelial cells treated with rIL-8 was monitored. The results indicated that rIL-8 promoted the growth of normal urothelial cells in culture in a dose-dependent manner (Fig. 1A), suggesting IL-8 serves as a growth factor for human normal urothelial cells. Both 77 amino acid and 72 amino acid rIL-8 were able to promote normal urothelial cells to a similar degree in our studies (data not shown). This result was also confirmed using MTT assay (data not shown). The addition of rIL-8 to the normal urothelial cells led to changes on the levels of phosphorylated Akt, suggesting the involvement of Akt pathway as shown in Fig. 1B. Furthermore, the addition of an Akt-specific inhibitor could block the growth-promoting effect of rIL-8 in a dosage-dependent fashion (Fig. 1C). These data clearly indicate that Akt pathway plays a pivotal role on the growth-promoting effect of IL-8 in normal urothelial cells.

**IL-8 siRNA causes cell death in normal human urothelial cells in vitro.** To investigate whether IL-8 has additional functional roles in normal urothelial cells, IL-8 levels were modulated using siRNA. We noticed a reduction of IL-8 levels in the mock-transfected cells, indicating existing toxicity from the transfection reagent. Both IL-8 siRNAs were able to further lower the amount of IL-8 significantly (Fig. 2). In addition to the IL-8 RNA Dicer pool (HM-IL-8), we also used commercially available IL-8-specific siRNA (Ambion).

Cells transfected with control siRNA remained attached and living (Fig. 3A), while cells transfected with IL-8 siRNA
rounded up, detached from the culture plate, and eventually died (Fig. 3B). This result indicated that IL-8 expression is essential for the survival of normal human urothelial cells in culture. To determine whether IL-8 depletion caused the normal urothelial cell death after IL-8 siRNA treatment, rIL-8 was added to IL-8 siRNA-treated normal urothelial cells (“IL-8 rescue”). IL-8 supplementation supported normal urothelial survival following the IL-8 siRNA treatment (Fig. 3C). Both 77 and 72 amino acid rIL-8 rescued normal urothelial cells in siRNA assays (data not shown). To determine whether this response was receptor mediated, anti-CXCR1 and/or anti-CXCR2 antibodies were also included in the experiments. Addition of anti-CXCR1, not anti-CXCR2, antibody abolished the rescue effect of rIL-8 (Fig. 3C), indicating the involvement of CXCR1. In the process of optimizing the IL-8 siRNA treatment, addition of anti-CXCR1 into the normal urothelial cell culture also reduced the expression of IL-8 (data not shown). All these results strongly suggest that an autocrine or paracrine activation of CXCR1 plays a pivotal role in the survival of normal urothelial cells.

Since CXCR1 was shown to mediate IL-8 function as a survival factor for normal urothelial cells, we then examined the expression of CXCR1 on normal urothelial cells after siRNA transfection. To stain only the cell surface CXCR1 and 2, cells were stained with antibodies before fixation. No CXCR1 was detected on untreated and/or control siRNA-transfected normal urothelial cell surface (Fig. 4A). However, the surface expression CXCR1 was readily detected on IL-8 siRNA transfection, indicating that reduction of IL-8 expression led to higher CXCR1 surface expression (Fig. 4B). Interestingly, RT-PCR results did not show more steady-state CXCR1 mRNA expression on siRNA transfection (data not shown). Expression of CXCR2 was detected in a subpopulation of normal urothelial cells, but no change, either in the number of positive cells or in the strength of staining, was observed after siRNA transfection with either control or IL-8 siRNA (data not shown).

**IL-8 mRNA expression in bladder samples from interstitial cystitis patients.** Interstitial cystitis is a poorly understood disorder of the urinary bladder characterized by pain, urinary frequency, and urgency without evidence of infection (21, 41). Studies have shown that many patients with interstitial cystitis have alterations in the urothelial layer, including partial or complete loss of urothelial cells and thinning or “cracks” in the urothelial lining (17, 23, 41). We examined the IL-8 expression in the bladder biopsies from interstitial cystitis patients. The cytokine RT-PCR result showed that nearly all of normal samples (5 of 6 normal bladder and ureter tissues; 83%) expressed IL-8 mRNA. In sharp contrast, the majority of interstitial cystitis samples (9 of 13; 69%) had undetectable IL-8 mRNA (Table 1; P < 0.05), while the expression levels of other three mRNAs (Cyclophilin, a housekeeping control, DRø, and DRβ) remained unchanged (data not shown).

**DISCUSSION**

In our studies of cultured urothelial cells, we observed that IL-8 was abundantly expressed. We therefore initiated research to delineate the role(s) of IL-8 in urothelial biology. Although cultured normal human urothelial cells express high IL-8 levels, addition of exogenous IL-8 increased cell growth. Unexpectedly, inhibition of IL-8 expression by siRNA showed that loss of IL-8 resulted in cell death. Therefore, IL-8 is critical for the survival of normal urothelial cells in culture, mediated through the CXCR1 receptor.

While we demonstrated that CXCR1 was essential to urothelial survival through IL-8, we were not able to detect CXCR1 mRNA expression by RT-PCR in normal urothelial cells with or without IL-8 siRNA transfection. CXCR1 has been shown to be stored in vesicles in human T cells and then transported to the cell surface for functional activity when needed (10). Similarly, normal urothelial cells may express low levels of CXCR1 stored in the vesicles. Upon IL-8 siRNA transfection, CXCR1 molecules could then be rapidly transported to the cell surface without higher transcription level of mRNA. Since both CXCR1 and CXCR2 are expressed in the human urothelium on bladder tissue sections (40), this working model may very well hold true in vivo.

Similar to our observations, IL-8 has been shown to support endothelial cell survival as well as promoting endothelial cell growth (24). Additionally, IL-8 protects fetal intestinal mucosa from tumor necrosis factor-induced cell death (27). Recent studies in prostate cancer demonstrated the importance of IL-8.

Table 1. **IL-8 expression levels in normal and IC tissues**

<table>
<thead>
<tr>
<th>IL-8 Status</th>
<th>Positive</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>IC</td>
<td>4</td>
<td>9</td>
</tr>
</tbody>
</table>

*RNA was prepared from normal bladder/ureter tissues or bladder biopsies of interstitial cystitis (IC) patients and then subjected to RT-PCR to examine the expression levels of IL-8. X² P < 0.05.*
in supporting prostate cancer cell survival after chemotherapy (28, 42), under hypoxia (28), or following androgen withdrawal (1). IL-8 enhancement of normal urothelial cell growth in our studies involved the Akt pathway, which is consistent with the study reporting a role of Akt pathway in cell proliferation in normal cells (6). In addition, the growth-promoting effect of rIL-8 we observed could be due to fewer cells were undergoing cell death in culture, resulting from the activation of Akt pathway by the addition of rIL-8. In this scenario, more cells would survive culture conditions, resulting in higher cell numbers in the group treated with rIL-8. This is consistent with our siRNA experiments indicating that IL-8 plays a critical role in normal urothelial cell survival. The role of Akt pathway in cell survival has been well established (5, 33, 42). Interestingly, we observed similar percentages of dead cells in the growth studies involving rIL-8 and/or Akt inhibitor by Trypan blue staining (data not shown). This observation clearly supports the concept that IL-8 promotes cell growth, rather than inhibiting cell death in normal urothelial cell culture. Our data suggest that IL-8 plays a dual role in normal urothelial cells: first, to promote cell growth and second, to maintain survival. The exact mechanisms and balance of IL-8-mediated urothelial cell growth and survival remain to be determined.

IL-8 has also previously been shown to promote growth of various cells. IL-8 has been shown directly to promote the growth of melanoma (39), prostate (1, 28), and several other types of cancer cells (42, 43), as well as normal fetal colon (27) and hematopoietic progenitor cells (7). IL-8 signaling can lead to activation of epithelial growth factor receptor by metalloproteinase activation (15, 16), which may contribute to IL-8-mediated cell growth. In our studies, the surface level of CXCR1 was very low before IL-8 siRNA treatment. Addition of IL-8 did not increase surface CXCR1 expression and urothelial surface CXCR2 was present under all conditions. Therefore, the urothelial IL-8 growth response we demonstrated could be due either to a direct effect through CXCR2 or to an indirect effect via EGFR activation.

What other roles does IL-8 play in urothelial biology? Our research strongly suggests that in addition to its well-established role in immune defense and angiogenesis, IL-8 also contributes significantly to the survival and growth promotion of normal urothelial cells. These observations may explain why patients with interstitial cystitis show histologic evidence of ulcers, loss of urothelial cells, urothelial thinning, and “breaks” in the urothelial layer (17, 23, 41) because their normal urothelial cells are not producing enough IL-8, resulting in unhealthy urothelium. Previous studies measuring urinary IL-8 in interstitial cystitis patients have been inconsistent (8, 9, 34). Interestingly, two reports indicate that urinary IL-8 levels in interstitial cystitis patients’ urine are slightly lower than normal, although not statistically significant. Our data, which directly measured the IL-8 mRNA level in the biopsy samples, clearly indicated the lower production of IL-8 mRNA in interstitial cystitis patients’ bladder samples (Table 1). Based on our data, IL-8 and/or agents that stimulate IL-8 production may be potential therapeutic agent(s) for this disorder.

IL-8 release has long been recognized as an important early response essential for immunologic defense against urinary tract bacterial infections (11, 12, 20, 31, 38). During bladder infection with uropathogenic Escherichia coli, the luminal, most differentiated urothelial cells (umbrella cells) provide a number of defensive functions (31). First, these cells initially provide a physical barrier to pathogenic bacteria and prevent deeper access through tight junctions (22). Second, umbrella cells absorb the initial bacterial invaders and begin to release IL-8 (3, 38). Binding of pathogenic E. coli causes apoptotic cell death (18, 30, 31). This apoptosis is associated with suppression of NF-kB (19), a transcription factor which regulates IL-8 transcription (14, 43). It is possible that lower levels of IL-8 in the umbrella cells contribute to apoptosis in this process. In dying, the umbrella cells undoubtedly release more IL-8, resulting in mobilization of the immune system, and as we showed, increased growth and survival of the exposed subluminal urothelial cells. Gene expression changes during urothelial regeneration in the mouse model of E. coli bladder infection have been described (32), and members of the CXC family are increased during this process. Additionally, lower levels of CXCR1 have been found on leukocytes from women with recurrent urinary tract infections and children with pylonephritis (11, 26). These data suggest that alterations in IL-8-CXCR1/2 pathways could contribute to susceptibility to infection. The levels of urothelial expression of CXCR1 were not explored in those studies, and contribution of urothelial CXCR1 to those pathologic processes remains to be studied. Our unexpected findings that IL-8, besides its host defense functions, also serves as a urothelial growth factor essential for survival contribute to the concept that the urothelial cells, through multiple mechanisms, serve a “sentinel” function, guarding the urinary tract against infection.

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GRANTS

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IL-8 and Urothelial Cells


