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Tumor Necrosis Factor- α induces expression and release of interleukin-6 by human urothelial cells

Zun-Yi Wang* and Dale E. Bjorling

Department of Surgical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, WI.

Abstract

Objective—We examined the effects of tumor necrosis factor- α (TNF- α) on expression and release of interleukin-6 (IL-6) by human urothelial cells (HUCs) and investigated whether the effects of TNF- α are mediated by mitogen-activated protein kinase (MAPK) pathways.

Materials and Methods—HUCs were treated with TNF- α at 1–10 ng/ml for 2–24 hours. Expression of IL-6 and TNF- α receptor 1 (TNFR1) mRNAs were examined by real-time PCR. Release of IL-6 into culture medium was determined by ELISA. Presence of TNFR1 protein and TNF- α -induced activation of MAPK pathways was examined by immunoblotting analysis. The effects of selective blockers of MAPK pathway on TNF- α -induced IL-6 expression and release were determined.

Results—TNF- α increased IL-6 mRNA expression and stimulated release of IL-6 in a concentration- and time-dependent manner. The effects of TNF- α were mediated by TNFR1. TNF- α induced phosphorylation of the ERK1/2 and JNK, and TNF- α -induced IL-6 expression and release were inhibited by selective ERK1/2 and JNK blockers.

Conclusions—These results demonstrate that TNF- α increases expression and release of IL-6 by HUCs and the effects of TNF- α are mediated by TNFR1. Also, the ERK1/2 and JNK pathways are involved in TNF- α -induced expression and release of IL-6 in HUCs and may represent therapeutic targets in inflammatory urinary tract diseases.

Keywords

Cytokines; Inflammatory mediators; signal transduction; TNF; tumor necrosis factor- α ; interleukin-6; urothelial cells; mitogen-activated protein kinase

Introduction

Tumor necrosis factor- α (TNF- α) is a pro-inflammatory cytokine that is primarily produced by activated macrophages, T lymphocytes, and mast cells [1, 2]. TNF- α is the most rapidly produced pro-inflammatory cytokine during activation of innate host defenses [3] and epithelial and endothelial cells are primary targets of TNF- α [4]. TNF- α exerts multiple effects on targeted cells, including stimulating production and release of inflammatory cytokines by these cells [5]. To date, two sub-types of TNF- α receptors, 1 and 2, have been identified, and most effects of TNF- α appear to be mediated by the TNF- α receptor 1 (TNFR1) [1, 2, 6]. Binding of TNF- α to TNFR1 triggers a series of intracellular events, including activation of mitogen-activated protein kinase (MAPK) pathways [7].

Address correspondence to: Zun-Yi Wang, Ph.D, Department of Surgical Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, 2015 Linden Drive, Madison, WI 53719; Tel: 608 263 8374; FAX 608 263 7930; wangz@svm.vetmed.wisc.edu.

Interleukin-6 (IL-6) is a multifunctional cytokine involved in innate host defense and inflammatory responses, and IL-6 production is increased during various inflammatory conditions [8]. IL-6 cannot be detected in urine from healthy persons, but urine IL-6 concentrations are increased in children and adults with urinary tract infection [9]. IL-6 is also significantly increased in urine from patients with inflammatory bladder diseases [10], suggesting that IL-6 may participate in pathology underlying inflammatory urinary tract diseases [10].

Besides acting as a barrier separating the environment and submucosal tissues, urothelial cells play an important role in regulating urinary tract functions by producing and releasing a number of signaling molecules including cytokines [18, 19]. In the present study, we examined the effects of TNF- α on expression and release of IL-6 from cultured human urothelial cells (HUCs) and investigated whether the effects of TNF- α are mediated by MAPK pathways.

Materials and Methods

Culture of HUCs

HUCs were derived from segments of ureter obtained from a healthy donor as a byproduct of kidney transplant surgery in the absence of cancer, active infection, or other bladder disorders and immortalized by human papillomavirus E6 as described previously [11, 12]. Use of HUCs were reviewed and approved by University of Wisconsin-Madison Health Sciences-Institutional Review Boards. Cells were cultured in Ham's F12 medium (Invitrogen, Carlsbad, CA), supplemented with 0.1 μ g/ml hydrocortisone, 5 μ g/ml transferin, 10 μ g/ml insulin, 0.1 mM nonessential amino acid, 27 mg/ml dextrose, 2.0 mM L-glutamine, 100 u/ml penicillin, 100 μ g/ml streptomycin, and 2 % fetal bovine serum [12].

Measurement of IL-6 by ELISA

HUCs were seeded in 12-well plates (~ 50,000 cells/well), 2 ml medium per well. Cells were allowed to grow to 70–80% confluence, treated with test drugs for various times, and control cells were treated with vehicles used to prepare drugs. Conditioned medium was then collected, and IL-6 was measured using paired IL-6 antibodies from R& D Systems (human IL-6 DuoSet, Minneapolis, MN) following the manufacture's instructions. Samples were diluted 1:1 in sample buffer and run in duplicate.

Immunoblotting

Cells were grown in 25 cm² flasks (~ 200,000 cells/flask). Cell lysates were prepared using a commercial mammalian cell extraction reagent (Pierce, Rockford, IL) with a protease inhibitor cocktail (Roche Diagnostics, Indianapolis, IN) added immediately prior to protein isolation. Cell lysates were collected by centrifuging at 10,000g for 30 minutes at 4° C. Protein content was determined by the Bradford assay (Pierce, Rockford, IL). 20 μ g of protein sample per lane was loaded and resolved on 10% SDS gel by electrophoresis and transferred to nitrocellulose membranes. Membranes were blocked in 10% fat-free dry milk in TBST (20 mM Tris-HCl, 137 mM NaCl, 0.05% Tween-20; pH 7.5) for 1 hour and incubated with primary antibody overnight at 4° C. Membranes were then washed free of primary antibody and incubated at room temperature for 1 hour with secondary antibody conjugated with horseradish peroxidase. Signals were revealed with ECL detection reagent (Pierce). Membranes were stripped and re-blotted with α -tubulin antibody as a loading control.

Real-time RT-PCR analysis

HUCs were seeded in 6-well plates (~ 100,000 cells/well). Total RNA was isolated using RNeasy® Mini Kits (QIAGEN, Valenlia, CA) and treated with DNase I (Invitrogen) to remove genomic DNA. First strand cDNA was generated using a cDNA synthesis kit according to the manufacturer's instructions (Invitrogen). Primers were designed using Primer Express® software (Applied Biosystems, Foster City, CA) from gene sequences obtained through GenBank. PCR was done using an ABI 7300 instrument (Applied Biosystems), and SYBR Green was used to reveal real-time PCR product accumulation. Samples were amplified in duplicate using the following thermal cycling conditions: 94° C for 10 min, followed by 40 cycles of amplification at 94 °C for 30 seconds and then 60° C for 1 minute to allow for denaturing and annealing-extension., and expression level of IL-6 was normalized to expression of human S26, a constitutively-expressed ribosomal protein in the same sample. Primer sequences used were: IL-6 (forward: 5'-CCA GGA GCC CAG CTA TGA AC-3', reverse: 5'-CCC AGG GAG AAG GCA ACT G-3'); TNFR1 (forward: 5'-GAA ATG GGT CAG GTG GAG ATC T-3', reverse: 5'-GTT CTT CCT GCA GCC ACA CA-3'); S26 (forward: 5'- AGT CAG GAA TCG ATC TCG TGA AG -3', reverse: 5'-CAG CAC CCG CAG GTC TAA AT-3').

Reagents

Polyclonal rabbit anti-TNFR1 (1:1000), monoclonal anti- α -tubulin (1:10,000), monoclonal anti-JNK-2 (1:500) antibodies, and secondary goat anti-rabbit IgG and goat anti-mouse IgG (both conjugated to horseradish peroxidase and used at 1:20,000 dilution) were obtained from Santa Cruz (Santa Cruz, CA). Polyclonal rabbit anti-phospho-ERK1/2 (1:1000), phospho-p38 (1:1000), pan-ERK1/2 (1:1000), and p38 (1:1000) antibodies were obtained from Cell Signaling (Danvers, MA). Polyclonal rabbit anti-phospho-JNK2 (1:3000) antibody was from Promega (Madison, WI). U0125, U0126 and SP600125 (dissolved with DMSO at 10 mM as the stock solution and diluted in PBS to desired concentration) were obtained from EMD Chemicals (Gibbstown, NJ). SB202474 DiHCl and SB203580 HCl (EMD Chemicals) were dissolved in PBS. U0126 is a selective ERK1/2 inhibitor, and U0125 is structurally similar to U0126 and is used as the inactive control congener of U0126. SB203580 HCl is a selective p38 kinase inhibitor, and SB202474 DiHCl is an inactive control congener. SP600125 is a selective JNK kinase inhibitor, but no inactive control congener is available [13]. Recombinant human TNF- α , goat polyclonal TNFR1 agonistic antibody and monoclonal TNFR1 neutralizing antibody were from R&D Systems (Minneapolis, MN), and these antibodies selectively recognize different epitopes of TNFR1. TNFR1 antibodies that activate TNFR1 exert an agonistic effect [15], and those that prevent binding of TNF- α to TNFR1 neutralize the effects of TNF- α [16, 17].

Statistical analysis

Data are presented as arithmetic means $\pm$ SEM. Data were analyzed using one-way ANOVA followed by Tukey's post hoc multiple comparison test (GraphPad Prism, San Diego, CA). A p value < 0.05 was considered indicative of significant differences.

Results

TNF- α increased IL-6 expression and release by HUCs

HUCs were treated with TNF- α at various concentrations (1–10 ng/ml) for 6 hours. TNF- α stimulated IL-6 release in a concentration-dependent manner, and the effect of TNF- α appeared to reach maximum at 10 ng/ml (Figure 1 A). HUCs were therefore treated with TNF- α at a submaximal concentration of 3 ng/ml for various periods of time, and medium was collected to measure IL-6 release. TNF- α (3 ng/ml) increased IL-6 release in a time-

dependent manner (Figure 1B). TNF- α (3 ng/ml) also enhanced IL-6 mRNA expression, and the effect of TNF- α on IL-6 mRNA expression peaked 1 hour after exposure to TNF- α . IL-6 mRNA content remained increased 4, 8, and 12 hours after treatment with TNF- α compared to controls at each time point (Figure 1C).

Effects of TNF- α were mediated by TNFR1

TNFR1 protein (Figure 2A) and mRNA (Figure 2B) were present in HUCs, and treatment with TNF- α (3 ng/ml) did not alter expression of TNFR1 (Figure 2A, B). Treatment of HUCs with a selective TNFR1 agonistic antibody (5 μ g/ml) [15] stimulated IL-6 release by about 5-fold compared to concentrations of IL-6 in control samples treated with the same amount of normal goat IgG (Figure 2C, n=6, $p < 0.01$ vs control). IL-6 release induced by TNF- α (3 ng/ml, for 6 hours) was prevented by a selective TNFR1 neutralizing antibody (2.5 μ g/ml) [16, 17] applied 15 minutes before TNF- α (Figure 2D, n=6, $p < 0.01$ vs control), and control samples were treated with the same amount of normal mouse IgG.

Effects of TNF- α were mediated by ERK1/2 and JNK MAPK pathways

TNF- α (3 μ g/ml) induced phosphorylation of ERK1/2 15 minutes after application of TNF- α (Figure 3A, n=4). Incubation of TNF- α (3 μ g/ml) for 1 hour increased expression of IL-6 mRNA (Figures 3B), and IL-6 release (Figure 3C) was enhanced by TNF- α (3 μ g/ml) for 6 hours. These effects of TNF- α were inhibited ($p < 0.01$ vs TNF- α -treated group, n=6) by pretreatment with U0126 (10 μ M), an inhibitor of ERK1/2 phosphorylation, but not by its inactive congener U0125 ($p > 0.05$ vs TNF- α -treated group, n=6), applied 15 minutes before TNF- α (Figures 3D and E).

TNF- α (3 μ g/ml) also induced phosphorylation of JNK-2 15 minutes after application of TNF- α (Figure 4A, n=4). Incubation of TNF- α (3 μ g/ml) for 1 hour increased expression of IL-6 mRNA (Figures 4B), and IL-6 release (Figure 4C) was enhanced by TNF- α (3 μ g/ml) for 6 hours. These effects of TNF- α were inhibited ($p < 0.01$ vs TNF- α -treated group, n=6) by pretreatment with SP600125 (10 μ M), an inhibitor of JNK, applied 15 minutes before TNF- α (Figure 4B).

Treatment of HUC with TNF- α (3 μ g/ml) failed to induce phosphorylation of p38 (Figure 5A, n=4). Lysates of PC12 cells treated with NGF (provided by Cell Signaling) as a positive control were subjected to electrophoresis on the same gel as lysates of HUCs, demonstrating that the antibody was able to detect p38 MAPK phosphorylation in PC12 cells using this immunoblotting protocol (data not shown), and absence of specific signal in urothelial cells (Figure 5A) indicated that TNF- α indeed did not induce p38 MAPK phosphorylation in urothelial cells. Although TNF- α (3 μ g/ml, for 1 hour)-induced expression of IL-6 mRNA (Figures 5B) and IL-6 release (TNF- α , 3 μ g/ml, for 6 hours, Figure 5C) were inhibited ($p < 0.01$ vs TNF- α -treated group, n=6) by pretreatment with SB 203580 (10 μ M; an inhibitor of p38 applied 15 minutes before TNF- α), SB202474 (the inactive congener of SB203580) also induced a similar inhibition on the expression of IL-6 mRNA and IL-6 release ($p < 0.01$ vs TNF- α -treated group, n=6) (Figure 5B). These results indicate that inhibition of IL-6 expression and release by SB203580 is not specific to the p38 pathway.

Discussion

In the present study, we found that: 1) treatment with TNF- α increased expression and release of IL-6 by HUCs; 2) the effects of TNF- α were mediated by TNFR1; and 3) TNF- α -induced IL-6 expression and release were at least partly mediated by the MAPK ERK1/2 and JNK pathways.

Previous studies have shown an increase in mast cell numbers and activation in inflammatory diseases of the urinary tract [14]. TNF- α is a major cytokine secreted by activated mast cells. Mast cell-derived TNF- α has been shown to induce IL-8 release from urothelial cells, and the effects of TNF- α on urothelial cells are mediated by TNFR1 [5]. The present study demonstrates that TNF- α stimulates expression and release of IL-6 by urothelial cells, and the effect of TNF- α on IL-6 release was concentration- and time-dependent. IL-6 was significantly increased in urine from patients with inflammatory bladder diseases [10]. Our data suggest that urothelial cells are responsive to inflammatory stimuli by synthesizing and releasing IL-6 and may participate in pathology of inflammatory urinary tract diseases.

TNFR1 is a 55 kD protein expressed in various cell types [12] and mediate effects of TNF- α primarily [1, 2, 6]. Our data demonstrate that TNFR1 is present in urothelial cells and that expression of TNFR1 was not affected by treatment with TNF- α . Recently, selective antibodies against different epitopes of TNFR1 have been developed. Some of the antibodies bind to TNFR1 and induce activation of TNFR1, exerting an agonistic effect [15], and others prevent binding of TNF- α to TNFR1, neutralizing the effects of TNF- α [16, 17]. In the present study, the selective TNFR1 agonistic antibody induced IL-6 release, and the selective TNFR1 neutralizing antibody abolished TNF- α -induced IL-6 release from HUCs. Therefore, our data suggest that the effects of TNF- α on IL-6 release by HUC are mediated by TNFR1.

As an inflammatory mediator, IL-6 has been shown to increase sensitivity of afferent nerve fibers during inflammation [18]. IL-6 receptors are expressed in afferent neurons and their nerve fibers, suggesting that IL-6 may directly affect afferent function, and administration of IL-6 into rat paws induced hyperalgesia in a dose-dependent manner [20]. In combination with its soluble receptors, IL-6 sensitized skin afferent nociceptors and increased release of calcitonin-gene related peptide [21]. Pretreatment with IL-6 neutralizing antiserum inhibited carrageenin-induced hyperalgesia in rats [20]. Afferent nerve fibers in the urinary tract are located in close proximity to urothelial cells, suggesting that chemical mediators derived from urothelial cells can significantly influence function of afferent nerve fibers [18]. Interestingly, IL-6 concentrations in urine correlated positively with pain scores in patients with inflammatory bladder diseases [10]. Thus, increased expression and release of IL-6 during inflammation may contribute to increased afferent functions in patients with inflammatory urinary tract disorders.

The MAPK are a group of protein serine/threonine kinases that mediate signal transduction from the cell surface to the nucleus in response to a variety of extracellular stimuli [22, 23]. The MAPK consist of three major families of protein kinases, including p38, ERK and JNK [22, 23], and activity of MAPK depends on their phosphorylation status [22, 23]. In human neutrophils, TNF- α suppresses apoptosis through activation of both ERK1/2 and p38 pathways [17]. TNF- α -induced IL-6 release was mediated by p38 pathways in rheumatoid synovial fibroblasts and airway smooth muscle cells [15]. In human pulmonary microvascular endothelial cells [24] and murine embryo fibroblasts [25], TNF- α induced activation of JNK pathways. Thus, TNF- α is capable of activating all three major MAPK pathways, although activation of ERK1/2, p38, or JNK by TNF- α may vary among cell types. We found that TNF- α induced phosphorylation of ERK1/2 and JNK in HUCs. Moreover, IL-6 mRNA expression and release induced by TNF- α were inhibited by selective antagonists of ERK1/2 and JNK. These results suggest that both ERK1/2 and JNK pathways mediate TNF- α -induced expression and release of IL-6 in HUCs. TNF- α did not induce phosphorylation of p38 in HUCs. Although TNF- α -induced IL-6 mRNA expression and release were moderately inhibited by a selective p38 blocker (SB 203580), the effects of TNF- α were also reduced by the inactive p38 blocker congener (SB 202474), suggesting

that inhibition of the effects of TNF- α by these chemicals may not be related to specifically blocking the p38 pathway. Similarly, Bellei et al. reported that SB 203580 and SB202474 inhibited melanin synthesis in B16 melanoma cells and the inhibition by these compounds did not involve the p38 pathway [26]. It is possible that SB 203580 and SB202474 may affect other unknown cellular targets in certain cell types [26]. Cumulatively, our results indicate that the p38 pathway does not participate in TNF- α -induced release of IL-6 by HUCs.

In conclusion, our results demonstrate that TNF- α increases expression and release of IL-6 by HUCs via activation of TNFR1 and the effects of TNF- α are at least partly mediated by the ERK1/2 and JNK pathways. Interestingly, bladder inflammation created by treatment of rats with cyclophosphamide is associated with phosphorylation of ERK1/2 in bladder urothelial cells [27] and increased IL-6 expression in bladder [28], and blocking activation of ERK1/2 inhibits bladder hyperreactivity-induced by cyclophosphamide [29], suggesting that ERK1/2 kinase is involved in regulating bladder response to inflammation. Our observation that the ERK1/2 and JNK pathways are involved in release of pro-inflammatory mediators, such as IL-6 from urothelial cells, together with previous findings, indicate that MAPK ERK1/2 and JNK are potential therapeutic targets for treatment of patients with inflammatory urinary tract diseases. It should be noted that TNF- α -induced IL-6 expression is a complicated process and various signaling pathways are likely to contribute to regulating IL-6 expression and the observations may vary between different cell lines. Future studies using primarily cultured human urothelial cells will provide a more complete understanding of regulating IL-6 expression in urothelial cells.

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Figure 1.

A: Treatment of human urothelial cells (HUCs) with TNF- α for 6 hours increased IL-6 release in a concentration-dependent manner. Data are presented as mean $\pm$ SEM of 6 replicates. B: Treatment of HUCs with TNF- α at a submaximal concentration of 3 ng/ml increased IL-6 release in a time-dependent manner. Data are presented as mean $\pm$ SEM of 6 replicates. C: Treatment of HUCs with TNF- α at a submaximal concentration of 3 ng/ml stimulated IL-6 mRNA expression. The relative IL-6 expression level was normalized to expression of human S26, a constitutively-expressed ribosomal protein in the same sample. Data are presented as mean $\pm$ SEM of 6 replicates. ** $p < 0.01$ vs control group.

Figure 2.

A: TNFR1 protein and mRNA (B) were present in the HUCs, and treatment with TNF- α (3 ng/ml) did not alter expression of TNFR1. The relative TNFR1 mRNA expression level was normalized to expression of human S26 mRNA, a constitutively-expressed ribosomal protein in the same sample. Data are presented as mean $\pm$ SEM of 6 replicates. C: Treatment of HUCs with a selective TNFR1 agonistic antibody (5 μ g/ml) stimulated IL-6 release. Data are presented as mean $\pm$ SEM of 6 replicates. D: IL-6 release induced by TNF- α (3 ng/ml, for 6 hours) was prevented by a selective TNFR1 neutralizing antibody (2.5 μ g/ml) applied 15 minutes before TNF- α . Data are presented as mean $\pm$ SEM of 6 replicates. ** $p < 0.01$ vs control group. ## $p < 0.01$ vs TNF- α -treated group.

Figure 3.

A: TNF- α (3 ng/ml) induced phosphorylation of ERK1/2. Expression of IL-6 mRNA induced by TNF- α (3 μ g/ml, for 1 hour, B) and IL-6 release induced by TNF- α (3 μ g/ml, for 6 hours, C) were inhibited by pretreatment with U0126 (10 μ M), an inhibitor of ERK1/2 phosphorylation, but not by its inactive congener U0125 (D, E), applied 15 minutes before TNF- α . Data are presented as mean $\pm$ SEM of 6 replicates. ** $p < 0.01$ vs control group. ## $p < 0.01$ vs TNF- α -treated group.

Figure 4.

A: TNF- α (3 ng/ml) induced phosphorylation of JNK2. Expression of IL-6 mRNA induced by TNF- α (3 μ g/ml, for 1 hour, B) and IL-6 release induced by TNF- α (3 μ g/ml, for 6 hours, C) were inhibited by pretreatment with SP600125 (10 μ M), an inhibitor of JNK, applied 15 minutes before TNF- α . Data are presented as mean $\pm$ SEM of 6 replicates. ** $p < 0.01$ vs control group. ## $p < 0.01$ vs TNF- α -treated group.

Figure 5.

A: TNF- α (3 ng/ml) failed to induce phosphorylation of p38. Expression of IL-6 mRNA induced by TNF- α (3 μ g/ml, for 1 hour, B) and IL-6 release induced by TNF- α (3 μ g/ml, for 6 hours, C) were inhibited by pretreatment with SB203580 (10 μ M), an inhibitor of p38 applied 15 minutes before TNF- α . However, SB202474 (the inactive congener of SB203580) also induced a similar inhibition (D, E). Data are presented as mean $\pm$ SEM of 6 replicates. ** $p < 0.01$ vs control group. ## $p < 0.01$ vs TNF- α -treated group.