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ORIGINAL PAPER

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NF-κB-dependent gene expression of proinflammatory cytokines in T24 cells: possible role in interstitial cystitis

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Abstract Our previous report of predominant activation of nuclear transcription factor NF-κB in the bladder urothelium of interstitial cystitis (IC) patients suggests a potential role for this nuclear factor in the pathogenesis of the disease. Although NF-κB has been implicated in the pathogenesis of several inflammatory diseases, the downstream mechanism(s) by which it can mediate its effects are still fragmentary. In this study, we examined the role of this nuclear factor on the induction of proinflammatory cytokine gene expression in human bladder carcinoma T24 cells and further examined their corresponding protein levels in the urine of IC patients. T24 cells transduced with a dominant-negative super-repressor IκB mutant (pAxCAmIκB-M) or wild-type adenoviral vectors in the presence or absence of rhTNF-α. Transduction efficiency and ability of pAxCAmIκB-M to inhibit NF-κB activation were monitored by in situ reporter β-galactosidase and gel mobility shift assays, respectively. Expression profile analysis of proinflammatory cytokines was measured in cells and urine of IC patients using RT-PCR and ELISA, respectively. The activation of NF-κB by rhTNF-α was associated with 27, eight, ten and sevenfold increases in the TNF-α, IL-1β, IL-6 and IL-8 transcripts, respectively. In contrast, abrogation of the TNF-α-induced cytokine gene expression by an adenovirus super-repressor IκB mutant vector demonstrate that these effects were NF-κB-dependent. Interestingly, the NF-κB-induced expression of these transcripts correlates with increased protein levels of NF-κB-regulated proinflammatory factors in the urine of IC patients in comparison to controls. That these factors are capable of activating NF-κB in urothelial cells suggests a pivotal role for this nuclear transcription factor in the pathophysiology of the disease, possibly by inducing aberrant immune and inflammatory responses within the bladder of IC patients.

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Introduction

Interstitial cystitis (IC) is an indolent bladder disorder that has continued to be a challenging concern in urology. The disease presents with a varying symptom complex, including urinary frequency, nocturia, urinary urgency, pain on bladder filling, and suprapubic pain, which may ultimately limit bladder capacity. Despite assertive investigation in the past two decades, the cause and pathophysiology of the disease remain elusive. Several theories of its pathogenesis have been proposed, but none fully account for the manifestation of the disease. We previously reported the activation of the nuclear factor NF-κB in bladder biopsies of IC patients, predominantly in the cells of the urothelium and submucosal layer [3]. The transcription factor nuclear factor kappa B is a heterodimeric, sequence-specific transcription factor. It was first described as a regulator of the expression of the kappa light-chain gene in murine B lymphocytes [24], but has subsequently been found in many cell types. Several forms of NF-κB proteins have been characterized [5]. All members are distinguished by the presence of the Rel homology domain (RHD), which essentially determines DNA binding to the κB element. The activated form of NF-κB is a heterodimer, which usually consists of two proteins, a p65 (also called RelA) subunit and a p50 subunit. In unstimulated cells, NF-κB is found in the cytoplasm and is bound to IκB-α and IκB-β, which prevent it from entering nuclei [6]. In stimulated cells, however, specific kinases phosphorylate IκB leading to rapid degradation by proteasomes and subsequent nuclear translocation and binding of NF-κB to specific sequences in the promoter regions of target genes [10].

Recently, NF-κB has been suggested to play a pivotal role in chronic inflammatory diseases such as RA and

CBD[6]. NF-κB is activated in macrophages and epithelial cells in bronchial-biopsy specimens from patients with asthma [7], as well as in synoviocytes and endothelial cells in the joints of patients with active rheumatoid arthritis [20]. The targeted disruption of IκB-α in mice prolonged the activation of NF-κB in response to inflammatory stimuli, and the animals died of widespread inflammation [16]. A number of factors have been shown to activate NF-κB, and several signal transduction pathways may be involved; all of these stimuli act by means of protein kinases that phosphorylate IκB. These include pathogens, cytokines, growth factors, activators of protein kinase C, viruses, oxidants, and stress [5, 17].

Of the less investigated potential etiological factors in the pathogenesis of IC are ischemia (hypoxia) and stress. By using an animal model [18], the bladder mucosa was found to be more sensitive to ischemia than smooth muscle as evidenced by the greater rate of high energy phosphate degradation. This increased mucosal sensitivity to ischemia may thus be related to the etiology of IC, especially in light of recent evidence that there is reduced blood flow related to IC [14]. These data clearly suggest an etiological role for anoxia/ischemia in IC and support our hypothesis that NF-κB activation may be a key player in the pathogenesis of IC. Furthermore, it should be emphasized that the nuclear factor NF-κB can be specifically inhibited by antioxidants [8] and that the beneficial use of locally administered antioxidant (SOD) in IC patients two decades ago [19], which received little attention, further supports our hypothesis.

In conjunction with activator protein 1 (AP-1) and the nuclear factor of IL-6 (C/EBP-β), NF-κB regulates the expression of many genes involved in immune and inflammatory responses [11, 25]. Among the induced proinflammatory genes in chronic inflammatory diseases are cytokines, growth factors, enzymes, chemokines, adhesion molecules that play a key part in the recruitment of inflammatory cells, and biological messengers that control and coordinate the functions of many cells [7]. This can be exemplified by IL-1β, TNF-α, IL-6, INF-α, and various chemokines that summon cells to sites of inflammation, such as IL-8, Gro and MCP-1. Cyclooxygenase-2, another inducible enzyme regulated by NF-κB [26], is responsible for the increased production of prostaglandin and thromboxane in inflammatory diseases [9]. Although there are many similarities among the inflammatory responses in patients with chronic inflammatory diseases, there are also some differences in the type of inflammatory cells involved and in the mediators of inflammation.

In this study, we examined whether NF-κB can amplify and perpetuate mechanism(s) that can exaggerate the disease-specific inflammatory process via the cell-specific coordinated activation of several proinflammatory genes in human bladder epithelial carcinoma cells. We further examined whether NF-κB-induced gene expression correlates with cytokine/chemokine protein levels in the urine of IC patients.

Materials and methods

Cell culture

Bladder carcinoma T24 cells were purchased from the American Type Culture Collection (Rockville, Md.). Cell culture medium and reagents were obtained from Mediatech (Herndon, Va.). The super-repressor IκB (pAxCAmIκB-M) and wild-type (pAxCA) adenoviral constructs were a generous gift from Dr. I. Verma (Salk Institute, Calif.). The pAxCAmIκB-M construct expresses a mutant IκB form capable of binding NF-κB in an irreversible fashion. Primers for proinflammatory cytokines were obtained from Clontech Laboratories (Palo Alto, Calif.). Enzyme-linked immunosorbent assay kits were purchased from Chemicom International (Temecula, Calif.).

Adenovirus transduction

T24 cells were cultured in McCoy's medium supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin at 37°C in an air/5% CO_2 incubator. Subconfluent cultures were transduced using 20 MOI (multiplicity of infection) for 24 h with an adenovirus (pAxCAmIκB-M) or the wild-type (pAxCA) vectors prior to the addition of rhTNF-α (0 or 10 ng/ml) for an additional 24 h period. The super-repressor construct expresses a mutant IκB (pAxCAmIκB-M) protein capable of inhibiting NF-κB activation in an irreversible fashion. Transduction efficiency of T24 cells was determined using a reporter Adv-βgal construct, carrying a β-galactosidase gene down-stream of a CMV promoter, followed by β-galactosidase activity in situ staining as described previously [2].

Mobility gel shift assays

To evaluate the efficacy of pAxCAmIκB-M in inhibiting NF-κB activation, nuclear extracts were prepared from wild-type- or pAxCAmIκB-M-transduced T24 cells stimulated with rhTNF-α (10 ng/ml) as described previously [2, 4]. Briefly, cells were harvested on ice, washed in phosphate buffered saline, and suspended in hypotonic buffer and subsequently subjected to Dounce homogenization. After centrifugation, the lysis of nuclei was achieved by suspension in low-salt buffer, followed by adjustment of the KCl concentration to 300 mM using high-salt buffer. Nuclear extracts were obtained by centrifugation (25,000 *g*, 30 min at 4°C), and optimized by dialysis in a buffer containing 20 mM HEPES, pH 7.9, 20% glycerol, 100 mM M KCl, 0.2 mM PMSF and 0.5 mM DDT. Total proteins were determined (BCA, Pierce) in the supernatants and samples were stored at −80°C. Crude nuclear extracts (10 g) were preincubated with 4 g of poly(dI-dC) in a reaction mixture containing 12% glycerol, 12 mM HEPES, 4 mM Tris-HCl, 60 mM KCl, 1 mM EDTA and 1 mM DTT at 30°C for 15 min. ^{32}P-dATP-labeled oligonucleotides containing consensus binding sites for NF-κB or AP-1 (Promega), were added and incubated for an additional 30 min. DNA probes were prepared by end-labeling the positive and negative-strand oligonucleotides using ^{32}P-ATP and T4 polynucleotide kinase. Competition assays were performed by incubating extracts with 50–200-fold molar excess with unlabeled nucleotides. Samples were then resolved onto a 6% nondenaturing polyacrylamide gel, dried, and exposed to Kodak XAR film for 12–48 h at −70°C.

RT-PCR

Total cellular RNA was isolated from the cells after experimental manipulation using the RNAzol Kit (Tel-Test, Friendswood, Texas) and prepared for RT-PCR. Total RNA (200 ng) was

primed with 12–18-mer oligo(dT) (Promega, Madison, Wis.) and reverse transcribed with molony murine leukemia virus reverse transcriptase (GIBCO, BRL) in the presence of RNasin (20 units, Promega) in a total reaction volume of 20:1 as described previously [1]. PCR amplification of various cytokines was performed using 1 μl of the synthesized cDNA in the presence of specific amplimer sets (Clontech Laboratories) with a 3-temperature, 35 cycle program, including 94°C (denaturing) for 15 s, 64°C (annealing) for 15 s, and 7°C (extension) for 1.5 min using Taq DNA polymerase (Promega) in a Perkin-Elmer GeneAmp 2400 thermal cycler. For quantitative analysis, amplification of the housekeeping gene G3PDH was performed using the amplimer set 5′GAAGGTGAAGGTCGGAGTCAACG for the sense and 5′TGCCATGGGTGGAATCATATTGG for the antisense (Clontech Laboratories). The PCR products were subsequently resolved by electrophoresis on a 2% agarose gel containing ethidium bromide, visualized under UV and analyzed by densitometric scanning (Model GA-700, BioRad, Hercules, Calif.).

ELISA assay

Urine samples were collected from IC patients ($n = 25$) and healthy controls ($n = 10$) and appropriately stored for subsequent analysis. All patients were clinically diagnosed as having interstitial cystitis and were selected according to NIDDK criteria. All patients were females who underwent thorough physical examination and completed a 7-day voiding diary. Patients had symptoms of urgency and frequency in the absence of other detectable causes. Other specific conditions, such as cancer and infections, were ruled out. Commercially available (Chemicom International) screening kits, based on enzyme-linked immunosorbent assay, were used for the detection of cytokines in the urine of IC patients and healthy controls. Each kit included appropriate positive and negative controls for quantitative immunodetection of each urine sample. Detection of inflammatory mediators included IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, TNF-α and IFN. Data were analyzed by ANOVA and expressed as mean ± SEM with significance set at $P < 05$.

Results

Transduction efficiency

As anticipated, the transduction efficiency of T24 cells by the reporter Adv-β-Gal construct had reached 100% as evidenced by in situ staining for β-galactosidase activity in comparison to the endogenous enzymatic activity of control parental cells (data not shown). Consistent with previous studies, activation of NF-κB in T24 cells occurred as early as 30 min following the addition of rhTNF-α at a concentration of 10 ng/ml.

Inhibition of NF-κB activation by the IκB adenoviral mutant construct

Activation and inhibition of NF-κB in T24 cells was confirmed by gel mobility shift assays (Fig. 1). Nuclear extracts and a [^{32}P]ATP-labeled double-stranded oligonucleotide containing the consensus κB binding site, were used to evaluate the activation (rhTNF-α at 10 ng/ml final concentration) or inhibition of NF-κB by the IκB super-repressor adenoviral mutant vector

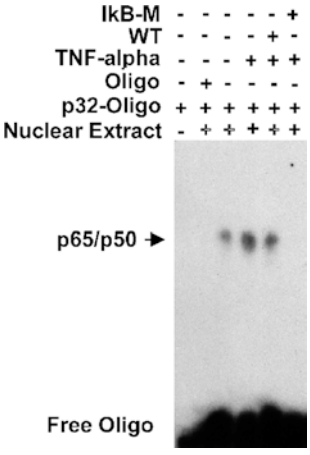

Fig. 1 Inhibition of rhTNF-α-induced activation of NF-κB by the IκB super-repressor adenovirus construct. T24 cells were stimulated with rhTNF-α in the presence of the IκB super-repressor or the wild-type adenovirus vectors. Nuclear protein extracts (10 g) were added to a reaction mixture containing [^{32}P]-labeled, double-stranded DNA oligonucleotide (5′-AGTTGAGGGGACTTTCCCAGGC-3′) encompassing the κB binding site, and poly(dI-dC) and BSA in binding buffer and allowed to incubate at 30°C for 15 min. Competitive assays were performed by the addition of a molar excess of unlabeled olgonucleotides. The reaction mixtures were then fractionated onto 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to Kodak x-ray film for 48 h at −70°C. Autoradiograms were analyzed by densitometric scanning. The *arrow* indicates the p65/p50 complex

(pAxCAmIκB-M) in T-24 cells. The results revealed that TNF-α had stimulated NF-κB activation in parental cells or cells transfected with wild-type vector (pAxCA), as indicated by the p65/050 complex DNA binding in the nuclear extract. This activation was inhibited in cells transduced with the IκB-M super-repressor construct or when a molar excess of unlabeled oligo was added to the reaction mixture (Fig. 1). The nuclear complete inhibition of NF-κB activation may be attributed to the higher transduction (95–100%) efficiency by the super-repressor vector.

Cytokine gene expression

As determined by RT-PCR analysis, stimulation of T24 cells by rhNF-α resulted in an increase in the levels of TNF-α (Fig. 2a), IL-1β (Fig. 2b), IL-6 (Fig. 2c), and IL-8 (Fig. 2d) transcripts by 27, eight, ten and sevenfold, respectively, when compared to parental untreated cells or cells transduced with the wild-type viral construct. The n-fold increase, expressed as a percent of the control, in the levels of NF-KB-regulated genes is depicted as a bar graph for each gene. The specificity of NF-κB-induction of proinflammatory gene expression was determined by stimulating the cells in the presence or absence of the IκB super-repressor construct in comparison to parental cells or cells transduced with the wild-type virus. Indeed, the levels were decreased to basal expression levels, suggesting that the TNF-α

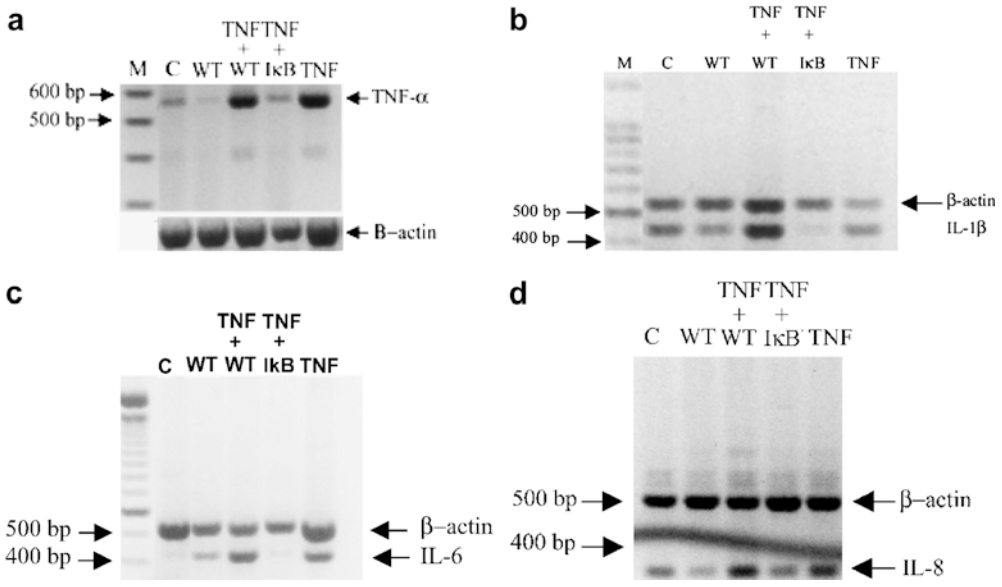

Fig. 2 NF-κB-specific upregulation of proinflammatory cytokines in T24 cells. Total RNA was extracted from stimulated or control cells by RNAzol kit. One microgram of RNA was reverse transcribed using oligo(dT) primers and moloney murine leukemia virus reverse transcriptase (MMLV-RT) in a total volume of 20 l at 42°C for 15 min using a 2400 Perkin-Elmer thermal cycler. One microliter of the synthesized cDNA was amplified with 35 cycles of 3-temperatures including 94°C (denaturing) for 15 s, 64°C (annealing) for 15 s, and 72°C (extension) for 1.5 min. The PCR products were resolved on 2% agarose/EtBr gels, examined under UV and analyzed by densitometric scanning. **a**–**d** represent gene expression profile analysis of TNF-α, IL-1β, IL-6, and IL-8, respectively, in T24 cells stimulated with rhTNF-α (10 ng/ml) in presence of IκB super-repressor or the wild-type adenovirus vectors stimulated with the rhTNF-α (10 ng/ml). Data is depicted as n-fold increase in mRNA expression relative to the housekeeping gene

induction of cytokine gene expression is primarily mediated via an NF-κB-dependent signaling pathway.

Cytokine protein levels in the urine of IC patients

The level of urine cytokines of IC patients was monitored using enzyme-linked immunosorbent assay (ELISA). A summary of urinary cytokines of IC patients is given in Table 1. The differences in the urine levels of these factors in both IC patients and control subjects are depicted in Fig. 3. The levels of interleukin-1β and tumor necrosis factor-α, which can both activate and are amplified by NF-κB, were significantly elevated by approximately threefold in the urine of IC patients in comparison to the levels detected in the control subjects. Another NF-κB-regulated gene product, IL-6, significantly increased by sixfold in IC patients as opposed to controls. Whereas the levels of interleukin 10 were not altered, other important immunoregulatory cytokines such as interleukin 2, interleukin 4 and interferon gamma were significantly increased by 7, 2.4 and 15-fold, respectively, in the urine of IC patients when compared to the urine levels of the healthy subjects.

Discussion

The nuclear transcription factor NF-κB has been shown to play a central role in the gene expression induced in cells by inflammatory and immune mediators [7]. This transcription factor is also known to induce anti-apoptotic effects in cells treated with apoptotic agents such as TNF-α, ionizing radiation or cancer chemotherapeutic agents [15].

In chronic inflammatory diseases, such as asthma, rheumatoid arthritis, inflammatory bowel disease and psoriasis, several cytokines recruit activated immune and inflammatory cells to the site of the lesion, thereby amplifying and perpetuating the inflammatory state [5, 6, 7]. While identification of the causes of these diseases is still fragmentary, one theory suggests they are caused by the interplay between genetic and environmental factors. One ubiquitous transcription factor of particular importance in immune and inflammatory responses is the NF-κB. This nuclear transcription factor is known to modulate the transcription of target genes by binding to specific recognition elements in the upstream 5′ promoter region. Recently, a number of reports have demonstrated that NF-κB regulates a concerted increase in the gene expression of many proinflammatory cytokines, chemokines, inflammatory enzymes and adhesion molecules. These include interleukin-1β, TNF-α, IL-6 and IL-8, which may exacerbate inflammatory and immune responses [5, 6, 7].

There have been relatively few direct measurements of the activation of NF-κB in inflammatory and in inflamed tissues. To this end, we have demonstrated for the first time that bladder biopsies from IC patients, but not controls, had predominant activation of this nuclear factor in the cells of the urothelium and to a lesser extent in the submucosal layer [3]. To further explore the pathophysiologic role of this nuclear factor in mediating immune and inflammatory responses in the bladders of

Table 1 Detection of proinflammatory cytokines in the urine of IC patients and control subjects. Urine was collected from IC patients ($n = 25$) and control ($n = 10$) subjects and subsequently subjected to quantitation of NF-κ6B-regulated proinflammatory cytokines and chemokines using a commercially available ELISA kit as described in Materials and Methods. Numbers (in parentheses) indicates the number of samples used in each group. The urine levels are measured in pg/ml and are expressed as (Mmean ± SEM) for each factor. The multiple comparison differences in urine detectable levels of various factors in the two groups are expressed as fold increase in IC patients relative to levels of the control subjects. These are depicted in Fig. 3 (*bar graph*)

	IL-1β (pg/ml; M ± SE)	IL-2 (pg/ml; M ± SE)	IL-4 (pg/ml; M ± SE)	IL-6 (pg/ml; M ± SE)	IL-8 (pg/ml; M ± SE)	IL-10 (pg/ml; M ± SE)	TNF-α (pg/ml; M ± SE)	IFN-γ (pg/ml; M ± SE)
IC patients	436 ± 65.3	2.79 ± 0.9	239.6 ± 109	69 ± 23	139 ± 17.2	20.0 ± 3.5	364 ± 63.3	407.1 ± 110.3
% positive	9/25 (36%)	24/25 (96%)	18/25 (72%)	16/25 (64%)	15/25 (60%)	17/25 (68%)	25/25 (100%)	23/25 (92%)
Controls	191.5 ± 49.6	0.40 ± 0.07	93.7 ± 6.8	11.6 ± 1.9	17.9 ± 10.5	23.6 ± 3.4	116.1 ± 66.7	27.3 ± 8.1
% positive	1/10 (10%)	9/10 (90%)	9/10 (90%)	3/10 (90%)	4/10 (40%)	9/10 (90%)	10/10 (100%)	4/10 (40%)

Fig. 3 This figure summarizes the multiple comparison differences in urine detectable levels of various factors in the IC and control groups. The results are expressed as a n-fold increase in IC patients relative to levels of the control subjects

IC patients, we used bladder epithelial T24 cells stimulated with a proinflammatory cytokine TNF-α in the presence or absence of a dominant negative inhibitor (pAxCAmIκB-M). TNF-α not only activates NF-κB, but is also amplified by this nuclear transcription factor via a positive regulatory loop, and may thus mimic the chronic activation in vivo. Our data demonstrate that stimulation of these cells by rhTNF-α enhanced the gene expression of proinflammatory cytokines TNF-α, IL-1β, IL-6 and the chemokine IL-8. Inhibition of NF-κB activation by the super-repressor attenuated the TNF-α-induced response in comparison to wild-type transfected T24 cells, suggesting a pivotal contributory role for this nuclear factor in the expression of proinflammatory cytokines and chemokines in bladder epithelial cells.

Cytokine responses have been detected in many urinary tract disease conditions. While normal urine does not usually contain significant cytokine activity [15], urinary IL-1β, IL-6 and IL-8 concentrations were found to be elevated in cystitis [11, 21], pyelonephritis [23], and gonorrhea [22]. These cytokines were suggested to be locally produced by epithelial cells because there was no correlation between their urine and serum levels. Epithelial cells form a barrier between the environment and the submucosal layer, and are highly responsive to external stimuli [12]. Upon stimulation, these cells are capable of interacting with other cellular elements locally in the mucosa and at distant sites via the production of proinflammatory cytokines [13]. Intriguingly, our ELISA analysis demonstrated that levels of NF-κB-regulated proinflammatory cytokine gene products were significantly elevated in the urine of IC patients when compared to those in control subjects. The fact that some of these factors, such as IL-1β and TNF-α, both activate and are amplified by NF-κB via a positive regulatory loop, indicates that the secretion of these factors in the microenvironment and urine may perpetuate NF-κB activation. The persistent activation of this nuclear factor in bladder urothelium, therefore, can exaggerate the disease-specific inflammatory process through

coordinated activation of these proinflammatory cytokine and chemokine genes. Our data clearly demonstrate the potential contributory role of urothelial cell-mediated inflammatory and immune responses in the bladders of IC patients, possibly by enhancing the activation of inflammatory and immune responses in a paracrine fashion.

Of the less investigated potential etiological factors in the pathogenesis of IC are ischemia (hypoxia) and stress. By using an animal model [18], the bladder mucosa was found to be more sensitive to ischemia than smooth muscle, as evidenced by the greater rate of high energy phosphate degradation. The increased mucosal sensitivity to ischemia may thus be related to the etiology of IC, especially in light of recent evidence that reduced blood flow is related to IC [14]. These data clearly suggest an etiological role for anoxia/ischemia in IC and support our hypothesis that NF-κB activation may be a key player in the pathogenesis of IC. Our preliminary studies demonstrated that hypoxia is capable of inducing the proinflammatory cytokine gene expression B, and the use of antioxidants attenuates this response—further documenting the potential role of hypoxia in exacerbating the disease process.

Taken together, our results demonstrate a correlation between NF-κB-induced proinflammatory/immune gene expression in vitro and corresponding protein levels in the urine IC patients—thus suggesting a possible role for this nuclear factor in the pathogenesis of IC. Persistent activation of this nuclear transcription factor and the release of inflammatory mediators in the microenvironment may exacerbate the disease condition by activating epithelial, inflammatory and mast cells in a autocrine and/or paracrine fashion. The release of certain κB-dependent gene products in the urine or serum may serve as noninvasive biomarkers for IC. Since NF-κB activation can be specifically inhibited by antioxidants [8] or the IκB super-repressor, the beneficial use of locally administered antioxidants or gene delivery in IC patients certainly warrants further investigation.

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