Increased Expression of Hypoxia-inducible Factor-1α and Vascular Endothelial Growth Factor Associated With Glomerulation Formation in Patients With Interstitial Cystitis

Jane-Dar Lee and Ming-Huei Lee

OBJECTIVE To examine whether hypoxia occurs in the bladders of patients with interstitial cystitis (IC) by monitoring the expression of hypoxia-inducible factor-1 (HIF-1) and VEGF. Previous studies have reported that bladder perfusion is decreased in patients with IC. Hypoxia induces overexpression of vascular endothelial growth factor (VEGF), which has been reported to be associated with the formation of glomerulations in patients with IC.

METHODS The study group consisted of 32 patients with IC, and the control group consisted of 8 volunteers. We obtained bladder biopsies from both groups and studied the expression of HIF-1α and VEGF proteins by immunoblotting and immunohistochemical and double immunofluorescent staining. Data were analyzed using the Mann-Whitney *U* test.

RESULTS Immunoblotting and immunostaining revealed that the expression of HIF-1α and VEGF proteins was increased in the study group compared with the control group. The relative intensities of HIF-1α and VEGF proteins were 60.60 $\pm$ 7.81 and 43.60 $\pm$ 5.37 in the study group and 26.20 $\pm$ 4.72 and 20.25 $\pm$ 1.45 in the control group, respectively. The overexpression of VEGF in study group biopsies was particularly evident in umbrella cells examined by confocal microscopy.

CONCLUSION Our findings identified increased expression of HIF-1α in bladder tissue and overexpression of VEGF in umbrella cells from patients with IC. These events may be associated with glomerulation formation during hydrodistention in IC bladders. Thus, these molecular findings could offer the therapeutic mechanism for hyperbaric oxygenation application to patients with IC. UROLOGY 78: 971.e11–971.e15, 2011. © 2011 Elsevier Inc.

Interstitial cystitis (IC) is characterized by disruption of the urothelial barrier and a loss of its normal impermeability to urinary solutes, resulting in bladder pain, urgency, and urinary frequency.[1-3] The molecular mechanisms underlying this disease are not well known.[1] Previous studies have reported that blood perfusion in the bladder is decreased in IC, especially during the filling phase.[4] Therefore, the pathophysiological changes observed in patients with IC may result from ischemic/hypoxic conditions in bladder tissue.

Hypoxia-inducible factor-1 (HIF-1) binds to the vascular endothelial growth factor (VEGF) gene and is a key transcriptional mediator of VEGF-mediated angiogenesis in response to hypoxia.[5-8] The activity of HIF-1 is mainly determined by HIF-1α, which helps cells adapt to hypoxia by enhancing cell survival and proliferation.[8-10] VEGF expression is associated with glomerulations formed during bladder hydrodistention in IC patients.[11]

Although a previous study reported up-regulation of HIF-1α and VEGF expression in the ischemic bladders of rats with outlet obstruction,[12] there are no reports on the association between hypoxic severity and HIF-1α expression in the bladders of patients with IC. This first report may help us discover the possible mechanisms underlying the clinical symptoms of urgency, frequency, and pain in relation to glomerulations in IC patients.

MATERIAL AND METHODS

Patient and Tissue Samples

Bladder specimens were obtained from 32 patients (26 females and 6 males) with IC who were undergoing cystoscopy under

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From the Division of Urology, Department of Surgery, Taichung Armed Forces General Hospital; Central Taiwan University of Science and Technology, and Department of Urology, Taichung Hospital, Department of Health, Taiwan

Reprint requests: Ming-Huei Lee, M.D., Department of Urology, Taichung Hospital, Department of Health, No.199, 1st Sec., San-Ming Road, Taichung 40343, Taiwan. E-mail: taic66006@mail.taic.doh.gov.tw

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anesthesia for diagnosis or therapeutic bladder distention. Patients met the cystoscopic criteria established by the National Institute of Diabetes, Digestive and Kidney Diseases,[13] including moderate to severe disease symptoms of >6 months in duration, and had an average age of 37.2 years (range 20-56). In the study group, a cold-cup biopsy was taken at the site showing glomerulations after bladder hydrodistention. Control specimens were obtained from 8 patients (median age 52.7 years; range 42-58; 6 females, 2 males) undergoing a bladder neck suspension procedure for stress urinary incontinence under bladder biopsy, who showed no evidence of IC or bladder mucosal disease. All tissues were stored at −80°C for immunoblotting or were fixed in formalin for immunostaining. All specimens were removed only after obtaining informed written consent from the patients. This study was approved by the Institutional Review Board of Tri-Service General Hospital.

Antibodies

Five primary antibodies were used in the present study: (1, 2) HIF-1α—2 rabbit polyclonal antibodies for immunoblotting (#3716, Cell Signaling, Beverly, MA; dilution 1:500) and immunostaining (sc-10 790, Santa Cruz, Santa Cruz, CA; dilution 1:50); (3) VEGF—a rabbit monoclonal antibody (#1909-1, Epitomics, Burlingame, CA) for immunoblotting (dilution 1:1000) and immunostaining (dilution 1:50); (4) CK7 (cytokeratin 7)—a mouse monoclonal antibody (NCL-L-CK7-OVTL, NovoCastra, UK; dilution 1:500) for immunohistochemical (IHC) staining; and (5) β-actin—a mouse monoclonal antibody (#8226, Abcam, Cambridge, MA; dilution 1:8000) for immunoblotting.

The secondary antibodies for immunoblotting were horseradish peroxidase (HRP)–conjugated goat anti-mouse IgG (#0031430, Pierce, Hercules, CA) or goat anti-rabbit IgG (#0031460, Pierce). The secondary antibodies for immunostaining were the commercial kit (PicTure; Zymed, South San Francisco, CA) for IHC staining and the Alexa-Fluor 488 conjugated goat anti-rabbit (Molecular Probes, Eugene, OR; dilution 1:50) or the Alexa Fluor 546 conjugated goat anti-mouse antibodies (Molecular Probes; dilution 1:200) for whole-mount immunofluorescent (IF) staining. Preliminary experiments of negative controls (samples stained with only primary or secondary antibodies) demonstrated that neither nonspecific staining nor overstaining of the background was found (data not shown) in whole mount IF staining.

Immunoblotting. The method used was modified from our previous studies.[14,15] Each sample stored at −80°C was homogenized on ice and then centrifuged at 13,000*g* for 20 minutes at 4°C. The supernatants were used for determination of protein concentrations or immunoblotting. Protein concentrations were identified by reagents from BCA Protein Assay Kit (#23 225, Pierce) using bovine serum albumin (BSA) as a standard (#23209, Pierce).

For immunoblotting, the antibodies of HIF-1α, VEGF, and β-actin (loading control) revealed molecular weights of about 120, 22, and 42 kDa, respectively. The blots were cut into upper and lower portions at feasible sites for incubation, incubated overnight at 4°C with the diluted primary antibodies, and then incubated with diluted secondary antibody for 1 hour. Finally, the immunoreactive bands were analyzed using MCID software version 7.0 (Imaging Research, St. Catherine's, Ontario, Canada). The results were converted to numerical values (normalized relative to β-actin expression) to compare the relative protein abundance of the immunoreactive bands.

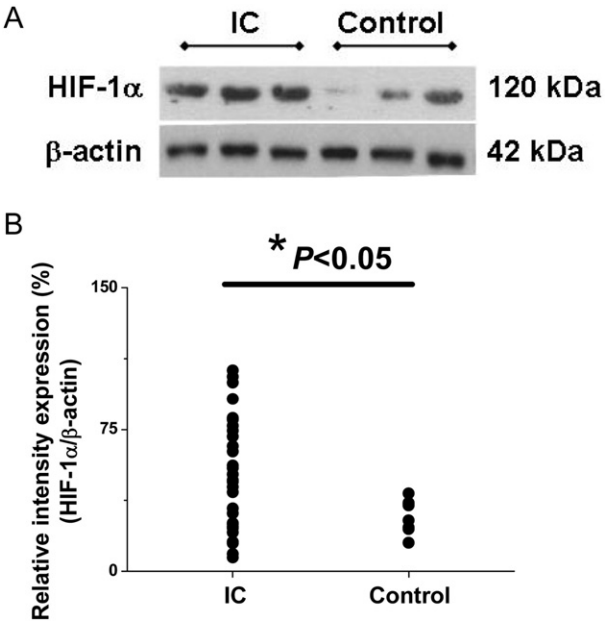

Figure 1. Representative immunoblot **(A)** and relative distribution **(B)** of HIF-1α protein in IC and control groups. β-actin was used as the loading control. Values of HIF-1α expression are normalized relative to β-actin expression. The asterisk indicates a significant difference ($P < .05$).

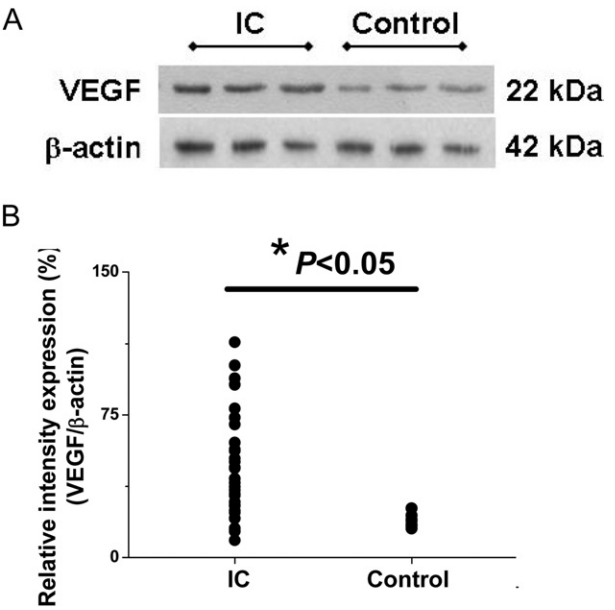

Figure 2. Representative immunoblot **(A)** and relative distribution **(B)** of VEGF protein in IC and control groups. β-actin was used as the loading control. Values of VEGF expression are normalized relative to β-actin expression. The asterisk indicates a significant difference ($P < .05$).

Immunohistochemistry. The method of staining and microscopy were modified from our previous studies.[14,15] The formalin-fixed and paraffin-embedded sections (4 μm) of samples were dewaxed and were then rinsed with phosphate-buffered

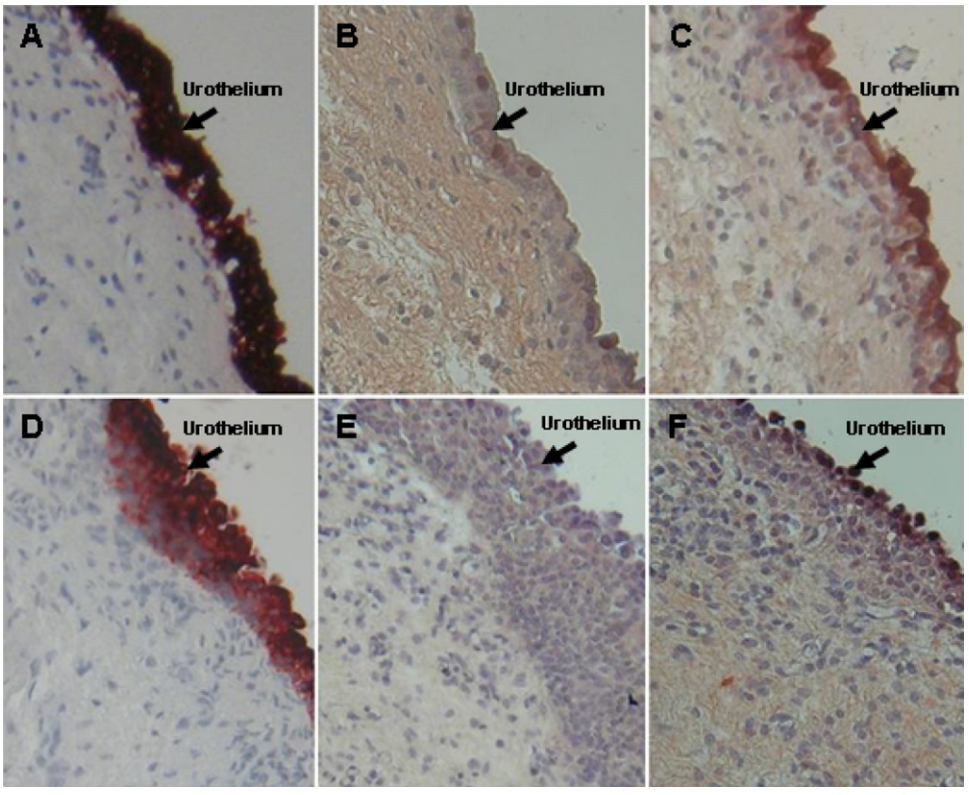

Figure 3. Micrographs of representative sections of IC **(A-C)** and control **(D-F)** groups after CK7 **(A** and **D)**, HIF-1α immunostaining **(B** and **E)**, or VEGF immunostaining **(C** and **F)**. Black arrow = endothelium. Original magnification x400.

saline (PBS). Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide. The sections were stained with primary antibody (CK7, HIF-1α, or VEGF) followed by the commercial kit. Negative control experiments, in which PBS was used instead of the primary antibody, were conducted to confirm the positive results of CK7, HIF-1α, and VEGF. Finally, the sections were counterstained with hematoxylin (Cat. No. 1.05175.0500, Merck, Germany) and rinsed with tap water. Sections were observed using a light microscope (BX50, Olympus, Tokyo, Japan) and the images were photographed.

Whole-Mount Double IF Staining and Confocal Microscopy. The method used for whole-mount IF staining and microscopy was a modified version of that used in our previous studies.[15-17] The whole samples were fixed overnight in formalin and were then washed in PBS. The samples were postfixed and permeabilized with 100% methanol overnight at −20°C. Samples were then washed in PBS twice and incubated in PBS with 0.05% Triton for 10 minutes. After washing in PBS, the samples were incubated in 2 mg/mL proteinase K for 10 minutes at 37°C. The samples were then washed in PBS twice and incubated for 30 minutes in blocking buffer (PBS containing 5% [wt/vol] BSA) to minimize nonspecific binding. The samples were then incubated overnight at 4°C with the diluted primary antibodies (HIF-1α or VEGF). After incubation, the samples were washed in PBS and were then exposed to the respective secondary antibody for 1 hour. Finally, the sections were covered with a slip with mounting solution (Zymed) before being viewed by confocal laser scanning microscopy.

To identify and compare the localization of specific proteins, IF-stained samples were observed with a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). A 488 -nm argon-ion laser and a 543 -nm helium-neon laser were used to give the appropriate excitation wavelengths for observing the fluorescent staining of Alexa Fluor 488 and Alexa Fluor 546, respectively. The micrographs taken from each photomultiplier were subsequently merged so the different-colored labels could be visualized simultaneously.

Statistical Analysis. The difference between groups was analyzed by the Mann-Whitney *U* test (a nonparametric statistical comparison) using SPSS 12.0 software (SPSS, Inc., Chicago, IL), and a significant difference was established if $P < .05$.

RESULTS

The relative intensities and distribution of both HIF-1α and VEGF were approximately 2.3- and 2.1-fold higher in the IC group than in the control group, respectively (60.60 ± 7.81 vs 26.20 ± 4.72 and 43.60 ± 5.37 vs 20.25 ± 1.45; Figs. 1 and 2). We used the CK7 stain to identify the thickness of the bladder urothelial layer. This revealed the relatively thick and intact urothelium of the control group. Conversely, biopsies from the study group exhibited thinning and denudation of the urothelium (Fig. 3A, 3D). Both immunostainings for HIF-1α showed stronger immunoreactivity in the muscle and urothelial layers of study group than in those of the control group (Fig. 3B, 3E and Fig. 4A, 4D). IHC staining and confocal microscopy revealed a marked overexpression of VEGF in the umbrella (apical) cells of the IC group compared with controls (Fig. 3C, 3F and Fig. 4B, 4E). The significant

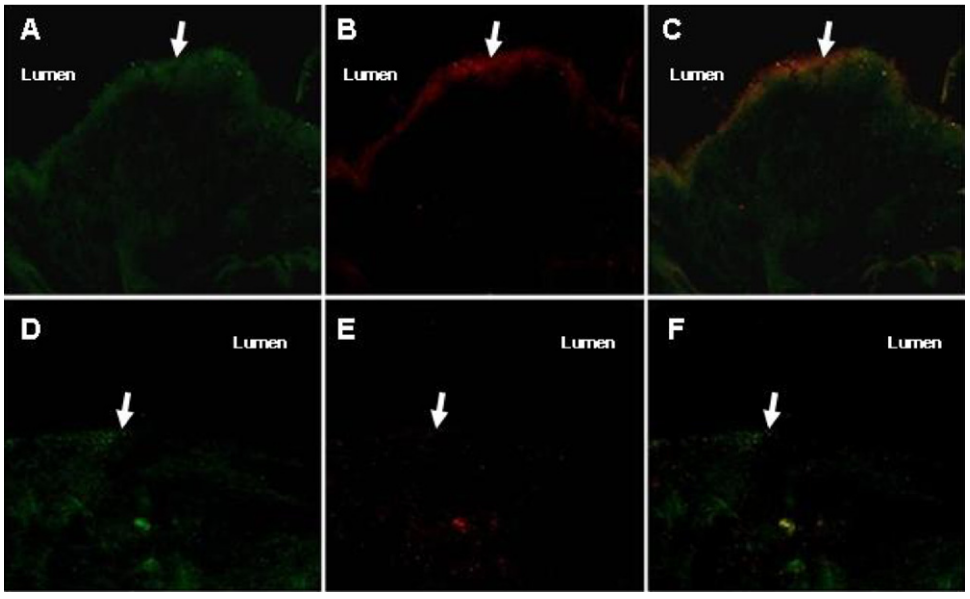

Figure 4. Representative sections of patients with IC **(A-C)** and control **(D-F)** groups after double IF staining and confocal laser scanning microscopy for HIF-1α (green, **A** and **D)**, VEGF (red, **B** and **E)**, and merged image **(C** and **F)**. L = lumen; arrows = endothelium. Original magnification x100.

difference in the distribution and expression of VEGF between the 2 groups is evident in the merged images (Fig. 4C, 4F).

COMMENT

Disruption of the urothelial barrier in cases of IC may initiate a cascade of events in the bladder, leading to clinical symptoms of bladder pain, urgency, frequency, and the formation of glomerulations during hydrodistention.[18,19] Normal bladder impermeability is lost in IC, allowing the migration of urinary solutes. Potassium in particular may cause depolarization of nerve and muscles[1,18,19] and lead to detrusor muscle contractions, resulting in urgency and frequency of urination. Hypoxia would change the expression of tight junction proteins and increase permeability of the bladder urothelium, allowing the passage of small ions across the blood-urine barrier in patients with IC.[20] We reasoned that this mechanism regulates cation balance in cells and keeps the sodium-potassium balance intact, because the urinary levels of these compounds are very different from the serum levels. It has been hypothesized that potassium fulfills this role because its levels in urine are high, ranging from 24-133 mEq/L.[19] Conversely, the potassium concentration in human serum is only 3.5-4.5 mEq/L. Such high levels not only depolarize nerves and muscles,[19] they may also cause the clinical symptoms of urgency, frequency, and bladder pain when potassium leakage occurs in IC patients.

Irwin et al reported that bladder perfusion is lower in IC bladders, especially during the filling phase.[21] This decrease in the perfusion induces hypoxia or ischemia in bladder tissue, which might lead to the upregulation of VEGF expression in urothelium, and this may be associated with glomerulation formation during hydrodistention in patients with IC.[12] In addition, it has been documented that increased VEGF expression promotes neovascularization in umbrella cells, although these newly formed vessels consist of relatively immature and fragile capillaries.[4] Therefore, it seems reasonable to assume that glomerulations result from petechial bleeding caused by bladder hydrodistention, higher pressures, and ischemia.[4]

Our study shows a higher expression of HIF-1α over muscle and urothelial layers of bladder in patients with IC compared with normal controls. This suggests that bladder tissue hypoxia induces a significant overexpression of VEGF in umbrella (apical) cells, which may be associated with glomerulations and petechial hemorrhage during hydrodistention in IC bladders. These molecular findings could offer the therapeutic mechanism for hyperbaric oxygenation application to patients with IC.[22-24]

CONCLUSIONS

Our results showed increased expression of HIF-1α in bladder tissue and overexpression of VEGF in umbrella cells causing immature vascularization, which may be associated with the formation of glomerulations during hydrodistention in IC bladders. Thus, these molecular findings could offer the therapeutic mechanism for hyperbaric oxygenation application to patients with IC.

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