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Original Article: Laboratory Investigation

Metallothionein overexpression of bladder biopsies associated with tissue hypoxia in patients with interstitial cystitis/painful bladder syndrome

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Abbreviations & Acronyms
HIF = hypoxia-inducible factor
IC/PBS = interstitial cystitis/painful bladder syndrome
IF = immunofluorescent
IHC staining = immunohistochemical staining
MT = metallothionein
PUF = pain and urgency/frequency
ROS = reactive oxygen species
SU = suburothelium

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Objective: To study the relationship between hypoxia and metallothionein expression in bladder biopsies of interstitial cystitis/painful bladder syndrome patients.
Methods: The study group consisted of 41 patients with interstitial cystitis/painful bladder syndrome, and the control group consisted of 12 volunteers without any interstitial cystitis/painful bladder syndrome symptoms. All biopsy specimens were analyzed for both proteins of hypoxia-inducible factor-1α and metallothionein expression by immunoblotting, immunostaining and confocal laser scanning microscopy. Data were analyzed using the Mann–Whitney *U*-test.
Results: An increased expression of hypoxia-inducible factor-1α and metallothionein was noted in the study group compared with the control group ($P < 0.05$). Both proteins of hypoxia-inducible factor-1α and metallothionein mainly distributed over bladder urothelium by immunohistochemical staining, and showed co-localization under confocal microscopy.
Conclusions: High expression and co-localization of metallothionein and hypoxia-inducible factor-1 alpha in the bladder mucosa of patients with interstitial cystitis suggest that overexpression of metallothionein is associated with the bladder hypoxia related to this disease.

Key words: hypoxia, interstitial cystitis, metallothionein, painful bladder syndrome.

Introduction

IC/PBS 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] Previous studies have reported that there was decreased blood perfusion and ischemia in bladder tissues of patients with IC/PBS.[4–6]

Hypoxia is partially regulated by transcription factors of the HIF family. HIF-1 is considered the master regulator of the response to hypoxia. Under hypoxia, HIF-1 binds to hypoxia response genes to restore oxygen homeostasis by activating hypoxia-sensitive genes involved in vasodilatation, angiogenesis, erythropoiesis and glycolysis.[7–9] The activity of HIF-1 is mainly determined by HIF-1α.[9–11]

MT is a heavy metal-binding protein with a low molecular weight. It has physiological functions, including the abilities to detoxify metals, to combat oxidative stress, to regulate essential biometals such as zinc and copper,[12,13] and to protect cells against apoptosis under oxidative stress or hypoxic conditions.[14,15]

However, there is no report of MT expression in bladder tissues of IC/PBS patients. To our knowledge, this is the first report to study the relationship between hypoxia and MT in bladder biopsies of humans. These findings might provide us with more understanding of the protective response of MT under tissue hypoxia of this bladder disease.

Methods

Patient and tissue samples

Bladder specimens were obtained from 41 patients (36 females and 5 males) with IC/PBS who were undergoing cystoscopy under anesthesia for diagnosis or therapeutic bladder distention. Patients met the basic and cystoscopic criteria established by the National Institute of Diabetes, Digestive and Kidney Diseases,[11] including moderate to severe disease symptoms of greater than 6 months' duration, and an average age of 39.8 years (range 20–60 years). The demographics of

Table 1 IC/PBS patient demographics

	Value
Age (years)	39.8 ± 13.1
No. 24-h frequency	13.73 ± 6.23
No. nocturnal frequency	3.52 ± 1.78
Mean symptomatic duration	8.47 ± 7.20 years
Maximum cystometric bladder capacity	237.07 ± 116.96 mL
Glomerulation	Mild: 1, Moderate: 3, Severe: 37†
Comorbidity	Negative
Urine cytology	Negative
O'Leary–Sant symptom index	≥11
O'Leary–Sant problem index	≥9
PUF score	≥15

†Definition of severity of glomerulation: Mild, smaller than 2 quadrants localized glomerulation; Moderate, diffuse than 2 quadrants localized glomerulation; Severe, splotch and waterfall bleeding.

enrolled patients are shown in Table 1. All patients had received history taking, physical examination, urinalysis and culture for exclusion screening, then they stopped their intravesical instillation drugs at least 1 month before biopsy. The IC/PBS symptoms were evaluated by questionnaires of O'Leary–Sant symptoms and problem index, and the pelvic PUF.[16] In the study group, a cold-cup biopsy was taken at the site showing glomerulations after bladder hydrodistention. Control specimens were obtained by bladder biopsy from 12 patients (median age 48.7 years, range 42–58 years; 10 females and 2 males whom had received transurethral resection of prostate more than 1 year) undergoing a bladder neck suspension procedure for stress urinary incontinence. There was no evidence of IC/PBS or bladder mucosal disease in all control patients. All tissues were stored at −80°C for immunoblotting or fixed in 10% formalin for immunostaining. All specimens were removed only after obtaining informed written consent from the patients. This prospective study was approved by the institutional review board of Tri-Service General Hospital.

Antibodies

Five types of primary antibody were used in the present study: (1,2) MT: two mouse monoclonal antibodies for immunoblotting (M3009-10; United States Biological, Swampscott, MA, USA; dilution 1:4000) and immunostaining (MS-1175; Thermo Fisher Scientific, Fremont, CA, USA; dilution 1:100); (3,4) HIF-1α: two rabbit polyclonal antibodies for immunoblotting (#3716, Cell Signaling, Beverly, MA, USA; dilution 1:500) and immunostaining (sc-10790, Santa Cruz, Santa Cruz, CA, USA; dilution 1:50); and (5) actin: a rabbit polyclonal antibody (sc-1616-R; Santa Cruz) that served as a loading control for immunoblotting (dilution 1:5000).

The secondary antibodies for immunoblotting were horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G or goat anti-rabbit immunoglobulin G (#0031430 or #0031460, respectively; Pierce, Hercules, CA, USA). The secondary antibodies for immunostaining were provided in the commercial kit (PicTure; Zymed, South San Francisco, CA, USA) used for IHC staining and the Alexa-Fluor 546 conjugated goat anti-rabbit (Molecular Probes, Eugene, OR, USA; dilution 1:200) or the Alexa-Fluor 488 conjugated goat anti-mouse antibodies (Molecular Probes; dilution 1:50) for IF staining. Preliminary experiments of negative controls (sections stained with only primary or secondary antibodies) showed that non-specific staining and overstaining of the background were not found (data not shown) in IF staining.

Immunoblotting

The method used was modified from that of our previous studies.[17,18] Each sample stored at −80°C was homogenized on ice and then centrifuged at 13 000 *g* at 4°C for 20 min. The supernatants were used for determination of protein concentrations and for immunoblotting. Protein concentrations were measured using reagents from the BCA Protein Assay Kit (#23 225; Pierce), using bovine serum albumin as the standard (#23 209; Pierce).

For immunoblotting, the antibodies for HIF-1α, MT and actin detected bands with molecular weights of approximately 110, 54 and 42 kDa, respectively. The blots were cut into upper and lower portions at feasible sites for incubation, incubated at 4°C overnight with the diluted primary antibodies, and then incubated with diluted secondary antibody for 1 h. Finally, protein bands were detected using the SuperSignal West Pico Detection Kit (#34 082; Pierce) and imaged using a cooling-CCD image sensor (ChemiDoc XRS+; Bio-Rad) with software (Quantity One version 4.6.8; Bio-Rad). Immunoreactive bands were analyzed using Image Lab software version 3.0 (Bio-Rad). The results were converted to numerical values in order to compare the relative protein abundance of the immunoreactive bands.

Immunohistochemical staining

This method was modified from a protocol used in our previous studies.[18,19] The deparaffinized bladder tissues sections (4 μm) were rinsed with phosphate-buffered saline. Endogenous peroxidase was inactivated by incubating the sections with 3% hydrogen peroxide. The sections were stained with primary antibody before being analyzed using a commercial kit. Finally, the sections were counterstained with hematoxylin (Catalog no. 1.05175.0500; Merck, Darmstadt, Germany) and rinsed with tap water. The immunostained sections were observed using a fluorescent microscope (BX50; Olympus, Tokyo, Japan) with a cooling-CCD (DP72; Olympus) using software (CellSens standard version 1.4; Olympus).

Double immunofluorescent staining and confocal laser scanning microscopy

The method used for IF staining and microscopy was a modified version of that used in our previous studies.[5,19] In brief, the paraffin sections were incubated at 4°C overnight with the diluted primary antibodies (HIF-1α or MT), and then exposed to the respective secondary antibodies for 1 h. Finally, the sections were covered by a slip with mounting solution (#0100-20; SouthernBiotech, Birmingham, AL, USA). To identify and compare the localization of specific proteins, IF-stained samples were observed with a confocal laser scanning microscope (Leica Lasertechnik, Heidelberg, Germany). The micrographs taken from each photomultiplier were subsequently

Table 2 Expression of HIF-1α and MT in both groups

Groups	*n*	HIF-1α	MT
Study	41	0.57 ± 0.05*	0.52 ± 0.04*
Control	12	0.24 ± 0.03	0.22 ± 0.03

*Significant difference ($P < 0.05$).

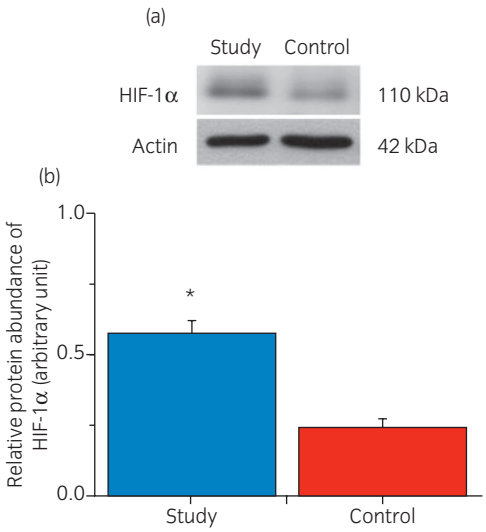

Fig. 1 The (a) representative immunoblot and (b) relative intensity of HIF-1α protein in the study (IC/PBS) and control groups. Actin was used as loading control. The asterisk indicates a significant difference ($P < 0.05$).

merged so that the different-colored labels could be visualized simultaneously.

Statistical analysis

The differences between the groups were analyzed using the Mann–Whitney *U*-test, and all values are expressed as the mean ± SE. A significant difference was established when $P < 0.05$. The analyses were carried out with the SPSS 12.0 software (SPSS, Chicago, IL, USA).

Results

The immunoblots of HIF-1α and MT were higher in the study group than in the control group (0.57 ± 0.05, 0.52 ± 0.04 and 0.24 ± 0.03, 0.22 ± 0.03, respectively, $P < 0.05$; Table 2 and Figs 1,2). Under microscopy, the denudation and thinner bladder urothelium of IC/PBS patients than in the control group were found. Conversely, the stronger immunostaining of HIF-1α and MT in the study group than in the control group were shown by IHC staining (Fig. 3). Confocal laser scanning microscopy showed co-localization of HIF-1α (red color) and MT (green color), which mainly distributed in the bladder urothelium of IC/PBS patients (Fig. 4a–c), and this was not found in the control group (Fig. 4d–f).

Discussion

The most common findings of bladder in patients with IC/PBS are denudation or thinning urothelium and a loss of its normal impermeability to urinary solutes, resulting in bladder pain,

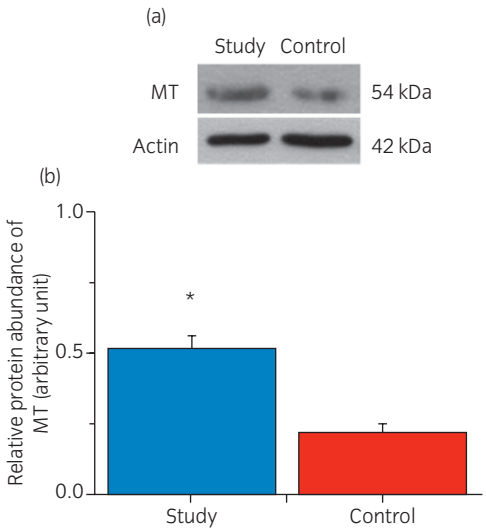

Fig. 2 The (a) representative immunoblot and (b) relative intensity of MT protein in the study (IC/PBS) and control groups. Actin was used as loading control. The asterisk indicates a significant difference ($P < 0.05$).

urgency, and urinary frequency.[1–3] Previous studies have reported decreased blood perfusion and hypoxic changes in bladder tissues of patients with IC/PBS.[4–6] The present study showed higher expression of HIF-1α in the bladder biopsy of the study group, especially in the urothelial layer, which means hypoxia occurrence in the bladder mucosa, and it might cause an increase of permeability then lose its barrier function resulting in a leaky urothelium of this diseased bladder. Higher MT and HIF-1α expression mainly colocalized in urothelium, suggesting MT overexpression is associated with bladder hypoxia in IC/PBS patients. Thus, these molecular findings could provide the treatment mechanism for hyperbaric oxygen therapy of IC/PBS disease.[20,21]

Low oxygen concentration (tissue ischemia) would induce ROS in mitochondria, and the release of cytochrome c and Apaf-1 mediated caspase-9 activation, then leading to cell apoptosis.[22,23] Increased cell apoptosis of bladder urothelium in IC/PBS patients has been reported.[24,25] HIF-1 is considered the master regulator of the response to hypoxia.[7–9] The activity of HIF-1 is mainly determined by HIF-1α, which can aid cells in adapting to hypoxia by enhancing cell survival and proliferation.[9–11] Additionally, the hypoxic factor is a co-activator of MT-I gene expression to reduce the production of hypoxia-induced ROS, which plays a role in cell damage and apoptosis.[6,26–28]

MT have been identified to be responsive to mitochondrial dysfunction. Their various functions and protective roles include: (i) regulating the activity of transcription factors, involved in cell proliferation, differentiation and apoptosis; (ii) controlling the redox status by reducing oxyradicals and nitrogenoxides; and (iii) maintaining metal homeostasis, heavy metal detoxification and free radical scavenging to protect cells from ROS damage and apoptosis.[15,29]

Furthermore, many studies have recently shown that MT is upregulated under hypoxic conditions to protect cells from apoptosis.[26,27,30] Kang *et al*. have reported the anti-apoptosis effect of MT through the inhibition of cytochrome c release and

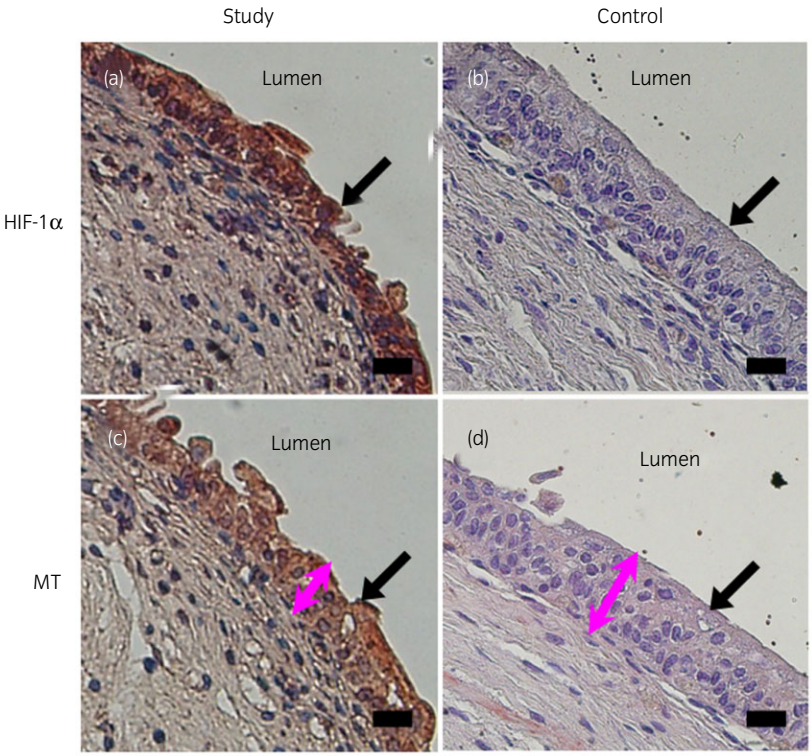

Fig. 3 Micrographs of (a,c) representative sections of study and (b,d) control groups after (a,b) HIF-1α or (c,d) MT immunostaining. Black arrows, urothelium; pink arrows, bladder urothelial thickness. Bar, 20 μm.

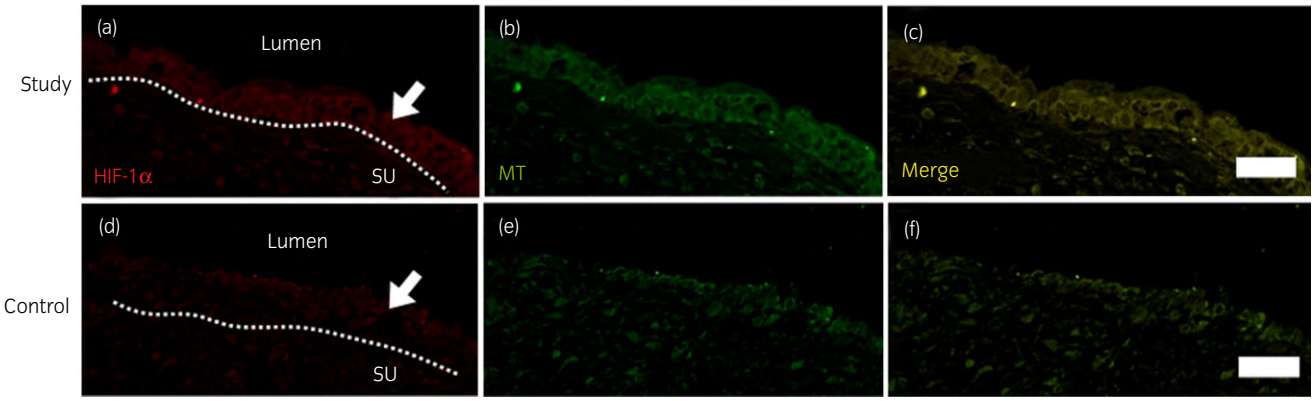

Fig. 4 Representative sections of (a–c) study and (d–f) control groups after double IF staining and confocal laser scanning microscopy for (a,d), HIF-1α (red), (b,e) MT (green) and (c,f) merged image. White arrows, urothelium; SU, dashed lines, the boundary between urothelium and SU. Bar, 20 μm.

caspase-3 activation.[27] Caspase-3 is considered the most crucial executioner protease, as it is essential for apoptotic death in mammalian cells.[23] Zinc has the function to activate the p53, to prevent DNA fragmentation and inhibit many proteins connected to apoptosis. MT also plays an important role in the regulation of apoptosis by distributing the intracellular zinc concentration.[15]

The present study shows higher expression of HIF-1α and MT (more than twofold) in the bladder tissues of IC/PBS patients compared with the control group, and mainly co-localized in the urothelium. It would be of great interest to investigate the change of MT expression as a therapeutic marker for IC/PBS patients in the future.

Significantly higher expression and co-localization of MT and HIF-1α in the bladder mucosa of IC/PBS patients, suggesting MT overexpression, is associated with bladder hypoxia of this disease.

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Conflict of interest

None declared.

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