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Full Length Research Paper

Aberrant expression of monocyte chemoattractant protein-1 (mcp-1) in interstitial cystitis patients

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Our present study investigated monocyte chemoattractant protein-1 (MCP-1) expression in bladder tissue and urine from interstitial cystitis (IC) patients. Totally 35 patients with IC, 20 patients with urinary tract infection (UTI) and 25 healthy subjects were recruited into this present study. IC was diagnosed according to the NIDDK IC Diagnosis Standard. All IC patients received 24 h voiding diaries, O'Leary-Sant IC questionnaires, potassium sensitivity test (PST) and cystoscopy. RT-PCR and ELISA were used to determine the levels of MCP-1 in bladder tissues and urine specimens of IC patients, UTI patients and healthy controls. Immunohistochemistry staining was used to determine the MCP-1 distribution in bladder tissue. ELISA results showed that urinary MCP-1 was 74.1 ± 36.9 pg/ml and 280.6 ± 68.9 pg/ml in IC and UTI patientsand10.8 ± 6.9 pg/ml in healthy controls ($p < 0.01$), respectively. RT-PCR analysis also revealed a moderate expression of MCP-1 mRNA in bladder tissue of IC patients. The severity of IC clinical symptoms was correlated with the levels of MCP-1. IC patients exhibited increased levels of MCP-1 in urine and bladder tissue. Increased MCP-1 may suggest IC after exclusion of UTI.

Key words: Interstitial cystitis, monocyte chemoattractant protein-1, urinary infection.

INTRODUCTION

Interstitial cystitis is a chronic inflammatory disease of the bladder characterized by increased urinary frequency/ urgency and pelvic pain (Peters, 2000; De Juana and Everett, 1977). IC has been described as an obscure chronic clinical syndrome and consequently, specific or effective treatment is lacking (Ajibona and Kehinde, 2003; Messing and Stamey, 1978). The etiology of interstitial cystitis remains still uncertain and may involve multiple co-existing and reinforcing mechanisms. Prevalent theories include possible infectious origin, bladder permeability defects, local neurogenic and histamine-induced inflammation, as well as a more generalized vulnerability to visceral hypersensitivity due to genetic or acquired abnormalities of the immune or neuroendocrine system (Holm-Bentzen, 1989; Gamper et al., 2009). However, regardless of the origin, the majority of IC patients appear to have damaged urothelium or bladder lining. Though IC diagnosis has been improved recently due to emergence of Pelvic Pain Urgency/Frequency (PUF) Patient Survey and Potassium Sensitivity Test (PST) (Parsons et al., 2002). However, both PUF and the PST are reported to have shortcomings in regards to sensitivity and specificity. The IC diagnosis based on such method-logies is often confused with other similar diseases. The spectrum of severity of symptoms and the difficulties encountered with PUF and PST are such that biomarkers have been evaluated as an aid to diagnosis. Antiproliferative factor (APF) appeared to be the most specific for interstitial cystitis, suggesting its potential use in diagnostic testing (Erickson et al., 2002). Unfortunately, the development of a reliable commercial assay for APF has not yet to be developed and the control of its production and its role in actually causing symptoms remain uncertain. Additionally, many potential biomarkers for IC diagnosis were found, including interleukin-1b, glycoprotein-51,

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Abbreviation: IC, Interstitial cystitis; **UTI,** urinary tract infection **PST,** potassium sensitivity test; **PUF,** pelvic pain urgency/ frequency; **APF,** antiproliferative factor; **PCR,** polymerase chain reaction; EGF, epidermal growth factor.

Tamm-Horsfall protein, leukocyte chemotactic factors, etc (Stone et al., 1992; Walzak et al.,1991; Smaldone et al.,2009; Hurst et al.,1993).

In this study, during the period of February 2006 to November 2007, 35 female IC patients in Renji Hospital were recruited into this study. We evaluated cytokines and chemokines expression in the urine of these patients and found that monocyte chemoattractant protein-1 (MCP-1) was the most predominant factor among the cytokines and chemokines tested. We further confirmed the aberrant expression of MCP-1 mRNA in the bladder tissues of IC patients. In addition, we also located MCP-1 expression in the bladder tissue. Together, our results suggested that MCP-1 may serve as a potential biomarker for IC diagnosis.

MATERIALS AND METHODS

Patients

According to the NIDDK IC diagnosis criteria, we recruited 35 female IC patients aged 31 - 65 years (mean 47 ± 16.5 years). All these patients complained of pain and distention on lower abdomen, nocturia and/or accompanied by urinary urgency and frequency. The disease course of these patients ranged from 10 to 52 months with a mean length of 19.7 ± 12.8 months. Among these patients, 30 were parous. All cases had no pelvic cavity and gynecologic operations. Patients were recorded for 24 h urination and symptoms according to the O'Leary-Sant IC questionnaire. Urine routine, B-ultrasound, potassium sensitivity testandcystoscopy after water expansion were also performed on these patients. All cases were excluded from urinary tract infection (UTI), calculi, bladder neoplasms (including carcinoma *in situ*), chemical cystitis, glandular cystitis, tuberculous cystitis and digestive or gynecologic diseases. Oral administration of anticholinergic medicines showed no effects on these patients. For comparison, we also recruited 20 female urinary tract infection (UTI) patients aged 35 - 61 years (mean 44.7 ± 12.9 years). These patients showed > 10 white blood cells per high power field in urine and positive bacterial culture of mid stream urine. In addition, we also recruited 20 female healthy donors aged 29 - 55 years (mean 43.3 ± 13.1 years) as asymptomatic controls. Written informed consent was obtained from each subject and the study was approved by ethical committee of our hospital.

Sample collection

Cystoscopic samples were randomly obtained from the bladder of IC, UTI patients and healthy donors and stored in the RNA-Later solution (Ambion) before RNA extraction. All above procedures were performed after permission from all patients and donors. The urine samples of IC, UTI patients and healthy donators were collected from the mid emiction after the perineum and urethra were sanitized. All above samples were frozen in liquid nitrogen until use.

Immunohistochemistry

The bladder tissues were collected and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (pH 7.4) at 4℃ for 4 h, embedded in paraffin wax (Sigma), sectioned (10 μm sections) and mounted on slides. The sections were dewaxed in xylene (2 × 10 min) and rehydrated through ethanol. ABC kit (Sino-American Biotechnology Company) was used to detect the stained signals according to the manufacturer's instruction.

RNA extraction and quantitative RT-PCR

Total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA integrity was evaluated by denaturing agarose gel electrophoresis. The purity of the RNA was determined by the ratio of absorbance at 260 to 280 nm. The ratio was between 1.9 and 2.0. cDNA was synthesized from the total RNA samples using oligo dT20 and ReverTra Ace First Strand cDNA Synthesis Kit (TOYOBO) according to the manufacturer's instructions. All primers for the genes of interest were designed using Premier 5.0 (PRIMIER Biosoft International) software and synthesized at Sangon Company Ltd (Shanghai). The primer sequences for MCP-1 were:

5'- AACACTCACTCCACAACCCAAG -3'
and
5'- TGTGGTTCAAGAGGAAAAGCAAT -3'

and the product length is 230 base pairs. GAPDH was used as normalizers. Its primers were:

5'- CCAGCAAGAGCACAAGAGGAA -3'
and
5'- ATGGTACATGACAAGGTGCGG-3'

and the product length was 157 bp. Quantitative real-time PCR was conducted using QuantiTect SYBR Green PCR reagent (Qiagen). PCR reactions were performed on DNA Engine Opitcon 2.0 (Bio-rad) in a 96-well format. The PCR program was as follows: incubation at 95℃ for 15 min, followed by 40 cycles of denaturalization at 94℃ for 15 s, reannealing at 60℃ for 20 s and extension at 72℃ for 20 s. Melting curve analysis was conducted from 72 to 95℃. The specificity of proliferation was checked every 0.5℃. Each sample in triplicates was evaluated by three independent assays. Relative quantitation analysis was performed based on the Standard Curve Method.

ELISA assay

MCP-1 ELISA kit (R and D Systems) was used to determine the level of MCP-1 in the urine according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using Sigmaplot 9.0 by t-test. A value of $p<0.01$ was considered statistically significant. Correlation analysis was performed with One-Sample Kolmogorov-Smirnov Test.

RESULTS

As shown in Figure 1, the expression of MCP-1 in the bladder was significantly higher in IC group than that in the normal group ($p < 0.01$) and the highest amount of MCP-1 was detected in the urinary tract infection samples via quantitative RT-PCR. Moreover, the amount of *MCP-1* in the urines of the three groups was assayed by using ELISA. As indicated in Figure 2A, MCP-1 amount

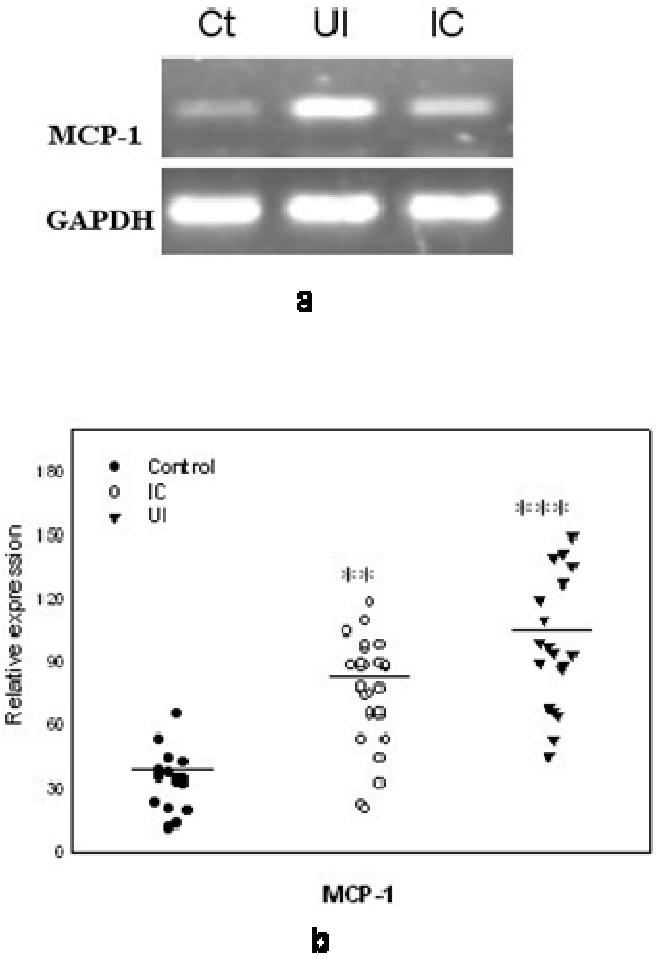

Figure 1. MCP-1 mRNA expression in three groups via RT-PCR. (A) Quantitative RT-PCR. (B) Normal, asymptomatic group, UTI, urinary tract infection, IC, interstitial cystitis. The MCP-1 expression was normalized to GAPDH expression.

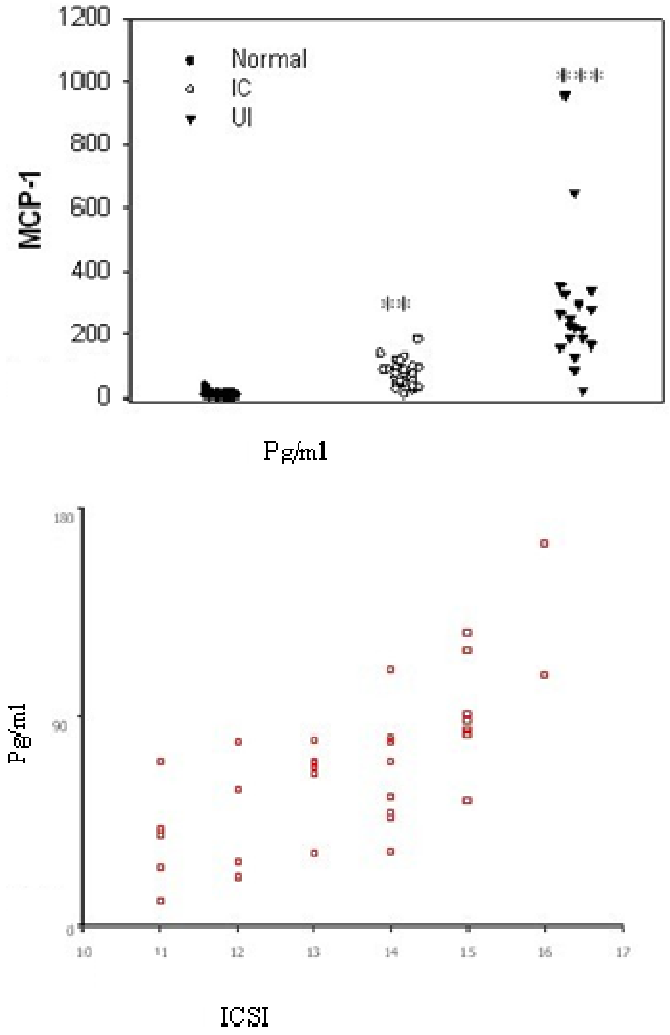

Figure 2. A: ELISA assay for MCP-1 in urines from three groups. Normal: healthy group. UTI: urinary tract infection patients. IC: interstitial cystitis patients. The MCP-1 concentrations in the urine of normal, IC and UTI group were 10.8±6.9, 74.1±36.9 and 280.6±68.9 pg/ml, respectively. B: The correlation analysis for MCP-1 expression with IC symptom evaluation score (r=0.686, p=0.0018). The symptom score were calculated according to O'Leary-Sant (OLS) IC questionnaire of 35 IC pat.

in the urine of normal, IC and UTI group were 10.8 ± 6.9, 74.1 ± 36.9 and 280.6 ± 68.9 pg/ml, respectively. The highest expression of MCP-1 was detected in UTI group and IC patients showed significantly greater expression of MCP-1 than asymptomatic controls.

Moreover, the severity of IC clinical symptoms was correlated with the levels of urine MCP-1 amount ($r = 0.686$, p value < 0.001) according to one-sample Kolmogorov-Smirnov Test (Figure 2B). MCP-1 of IC patients' urine was lower than that of UTI patients, but higher than that of healthy group, which was consistent well with mRNA expression results detected by quantitative RT-PCR. To further locate the expression of MCP-1 at cellular level, immunohistochemisty staining was performed. As shown in Figure 3, MCP-1 expression in IC patients was detected in the interstitial tissue of bladder (such as vascular endothelial cells, fibroblast cells), mucosa cells and smooth muscle cells.

DISCUSSION

Because of complicated causes and unclear patho-genesis and being lack of unique clinical symptom and effective therapies, IC becomes an intractable disease for urologists (Chakravarti et al., 2004). According to a series of diagnostic criteria established by NIDDK, sufferers can be easily diagnosed as IC. However, these identified cases are usually at the advanced stage because these diagnostic criteria are over strict. More and more evidence revealed that a large number of patients (expectedly 60%) at early-middle stages were omitted during diagnosis based on NIDDK criteria (Erickson et al., 2005). To that point, an effective diagnose biomarker like

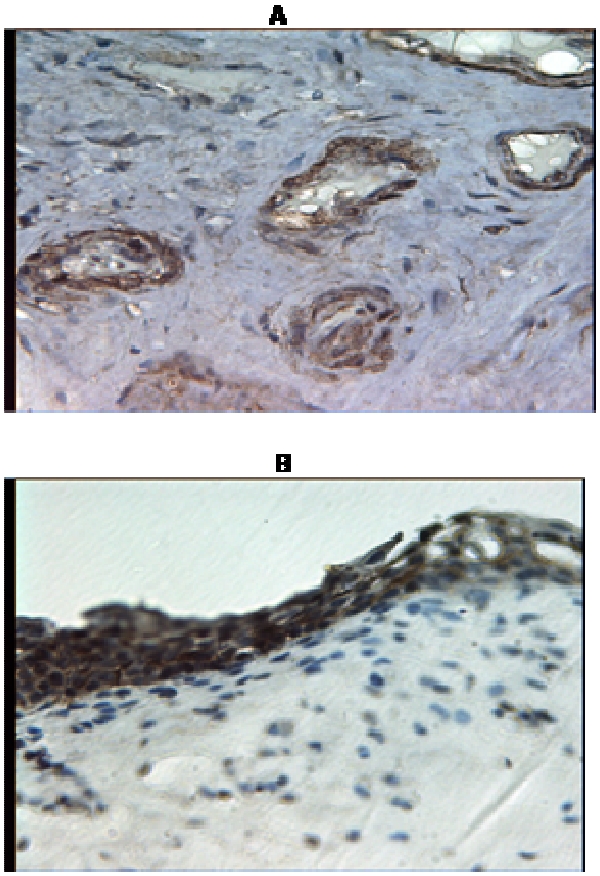

Figure 3. MCP-1 expression in the bladder confirmed by IHC. The arrow indicates its expression in bladder mucosa cells (A) and vascular endothelial cell of bladder interstitial tissue (B).

a specific molecule is particularly needed. Up till now, it is still reported that many genes showing aberrant expression in the urine, serum and bladder of IC patient. *APF*, heparin-binding epidermal growth factor-like growth factor (*HB-EGF*), epidermal growth factor (EGF), insulin-like growth factor 1 and insulin-like growth factor binding protein 3 have been shown to be correlated to PBS/IC. APF, HB-EGF and EGF seem to be the best in discriminating between patients with PBS/IC and asymptomatic controls (Erickson et al., 2002). MCP-1, a member of chemotactic factors, is a 76-amino-acid basic protein, synthesized and secreted by many types of cells including monocytes, macrophages, epithelia cells and smooth muscle cells, etc (Suzuki et al., 2002; Bouchelouche et al., 2006). MCP-1 promotes monocyte chemotaxis and activation and plays roles in host defense, inflammation and antitumor activities. During the acute inflammation period of cystitis, chemoattractant mononuclear macrophage induced by MCP-1 move to inflaming part and release lysosomal enzyme, produce inflammatory mediators like oxygen free radical, active nitrogen, protease, cytokine and growth factor and finally contributing to damage to surrounding tissues. At the chronic stage, mononuclear macrophages secret cytokines like TGF, leading to the interstitial fibrosis and clinic symptoms of pain and decrease of bladder volume (Bouchelouche et al., 2004; Luo, 2005; Malik et al., 2002). Previous research on mouse early-stage IC model in Bladder and Mucosa Diseases Center in University of Iowa revealed that in the urine of IC mice, the MCP-1 level is obviously increased compared with other immune factors (MCP-1: 52, IL-6: 20, chemokine ligand-10: 24, granulocyte-macrophage colony-stimulating factor (GMCSF): 13, interleukin-8: 6, interferon: 4, IL-2: 3, *IL-10*: 0%) (Luo, 2005). Based on the mouse model research, in this study, we reported that MCP-1 level in urine of IC, UTI and normal groups were (74.1 ± 36.9) pg/ml, (280.6 ± 68.9) pg/ml and (10.8 ± 6.9) pg/ml, respectively. On the mRNA level, the quantitative RT-PCR results were well consistent with the urine assay. Statistical analysis showed the difference among three groups was significant ($p < 0.01$). The increased MCP-1 expression in IC patients suggested that MCP-1 might participate in the pathophysiological process of IC. According to the result of immunohistochemistry, bladder stromal cells, mucosa epithelial cells and smooth muscle cells secret MCP-1, leading to the chemotaxis of mononuclear macrophage and consequently resulting in pain for patients. At the same time, the secretion of cytokines like TGF may contribute to fibrosis of bladder interstitial and finally decrease the volume of bladder, consequently contributing to micturition frequency, urgency in urination and increased nocturia.

The MCP-1 level in bladder and urine of patients of bacteria infectious cystitis was increased in contrast to asymptomatic controls, suggesting that MCP-1 expression is somehow related to severity of cystitis. Stimulation of bacteria and inflammation factors arouse bladder MCP-1 expression increase, which leads to chemotaxis and activation of mononuclear phagocyte system, as a result, the ability of host defense against outside invasion is improved (Malik et al., 2002). Concentration of MCP-1 in IC group and UTI group urine and bladder are both higher than that in control group, which means that bladder MCP-1 expression is increased during cystitis whether the inflammation is caused by bacteria infection or not. Besides, MCP-1 level in the cystitis patients accompanied with bacteria infection is much higher. Based on these observations, MCP-1 is a non-specific biomaker of IC. After the exclusion of urinary tract infection by inspection of urine routine, urine micro-organism culture (bacteria, mycoplasma, tracomatis and mycobacterium tuberculosis), increased MCP-1 is a helpful potential early diagnosis marker for non-infectious IC patients. Besides, relationship between degree of IC clinic symptoms and immune makers attracts much attention, because immune marker can serve as an important judging index of therapy evaluation and prognosis judgment for IC patients. Recently, it is reported that inflammatory factors like *IL-6* and *IL-8* might

be correlated to IC clinic symptoms (Lamale et al., 2006). In this study, symptom evaluation scores of O'Leary-San (t OLS) IC questionnaire of 35 IC patients was 14.9 ± 1.8. We compared MCP-1 and severity of IC patients and found they were obviously correlated ($r = 0.686$, $p = 0.0018$), suggesting MCP-1 is one of potential immune indexes for evaluating severity and treatment prognosis for IC patients.

The MCP-1 expression of bladder and urine of IC patient lies between those of UTI and control group, which suggests that this inflammatory factor may serve as one of non specific immune makers for diagnosis of IC at early stage after exclusion of urine infection. Bladder inflamemation and non infectious inflammation are regulated by many regulatory factors, thus discovering and understanding the changes of such factors may give clues for further studies on IC etiology.

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