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Original article

S-allyl cysteine ameliorates cyclophosphamide-induced downregulation of urothelial uroplakin IIIa with a concomitant effect on expression and release of CCL11and TNF-α in mice

Sayed Aliul Hasan Abdi[a,b], Mohd Amir Afjal[a], Abul Kalam Najmi[b], Sheikh Raisuddin[a,*]

[a] Department of Medical Elementology and Toxicology, Jamia Hamdard (Hamdard University), New Delhi, India
[b] Department of Pharmacology, Jamia Hamdard (Hamdard University), New Delhi, India

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ABSTRACT

Background: The aim of this study was to evaluate the modulatory effect of *S*-allyl cysteine against cyclophosphamide-induced changes in uroplakin IIIa, CCL11 and TNF-*α*.
Methods: Mice were treated with cyclophosphamide (200 mg/kg × 7 d, *ip*). *S*-allyl cysteine (150 mg/kg × 7d, *ip*), and comparator compound mesna (40 mg/kg × 7d, *ip*) were administered 1 h before and 4 h after each cyclophosphamide dose. The urinary bladder was analysed for mRNA and protein changes in uroplakin IIIa, CCL11 and TNF-α and histopathological findings.
Results: Cyclophosphamide caused hemorrhagic cystitis formation and downregulation of UPIIIa. These changes were accompanied by upregulation of CCL11 and TNF-α. *S*-allyl cysteine attenuated these changes including protection at histological level. Mesna which was used as a comparator drug also showed protection. However, relatively *S*-allyl cysteine showed a stronger protective effect than mesna.
Conclusion: These findings highlight a correlation between downregulaion of UPIIIa and enhanced production of inflammatory biomarkers and protective effects of *S*-allyl cysteine which has been reported to be a potent uroprotective agent. The present study strengthens its role which could be clinically exploited in chemotherapy regimen.

Introduction

Hemorrhagic cystitis (HC) is a very common pathological response associated with cyclophosphamide (CP) chemotherapy [1,2]. Role of chemokines in physiopathology of HC is unraveling and chemokines such as CX3CL1 (fractalkine), CXCL12 and CXCL10 have been reported to be upregulated in CP-induced HC [1–3]. The upregulation may come through functional variation in the bladder and intense inflammatory changes [1,2]. It is reported that tissue damage caused by CP leads to the release of TNF-α, a key mediator of inflammatory responses, and several other inflammatory and hyperalgesic mediators including chemokines [1–3].

CCL11 is an inflammatory chemokine contributing to patho-physiological development in diverse tissues [4]. CP-induced HC also shows damage to the urothelium and transmembrane proteins, uroplakins (UPs) [5]. UPs have important role in the permeability of the mammalian urinary bladder. Various isoforms of UPs collectively form uroplaques in the bladder urothelium acting as a biological membrane barrier to solutes in the urine and also regulate bladder function [5]. Transient reduction in the expression of UP has been reported in pathogenic changes of urinary bladder including urothelial damage and HC [5,6]. Specificity of UPs to their expression for pathogenic changes in urinary bladder and their utility as markers of bladder cancer have been recently investigated [6]. Among UP family UPIIIa has the largest mass in terms of molecular weight [7]. However, there is no information on expression profile of UPIIIa under CP treatment, its correlation with HC induction and its exudation in serum under CP treatment. There is also no data showing a relationship between UPs expression, CCL11and TNF-α.

Mesna (mercaptoethane sulphonic acid) is approved by the US-Food and Drug Administration for HC treatment [8]. It has been observed that in spite of positive clinical effect of mesna recurrence of HC has been reported [9,10]. Other observable adverse drug reactions such as chest pain, and facial edema have also been reported [8]. *S*-allyl cysteine (SAC), a constituent of aged garlic extract is known for its antioxidant activities which affords protection against CP toxicity in mice [5,11].

* Corresponding author.
E-mail address: sraisuddin@jamiahamdard.ac.in (S. Raisuddin).

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In the present study we evaluated effect of SAC against CP-induced changes in expression profile of UPIIIa in mice and also studied its effect on CCL11 and TNF-α in urinary bladder and serum to decipher mechanism of action and implication of inflammatory cascade in HC. Additionally, efficacy of SAC was compared with that of mesna.

Materials and methods

Chemicals

CP, SAC and mesna were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). UPIIIa monoclonal antibody, β-actin and horseradish peroxidase-conjugated secondary antibody were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). RNA isolation kit, cDNA synthesis kit, Sybr® green and protease inhibitor were purchased from Roche (Basel, Switzerland). UPIIIa ELISA kit and TNF-α and CCL11 ELISA kit were from MyBioSource, Inc. (San Diego, CA, USA), and Life Span Bioscience Inc. (Seattle, WA, USA), respectively. TNF-α cytometric Bead Array (CBA) Flex Set C8 was from BD Biosciences (Franklin Lakes, NJ, USA).

Animals

Adult male Swiss albino mice (25 ± 3 gm) were used in the study. The study protocol was approved by the Institutional Animal Ethics Committee of the University (Project # 1029). The animals were maintained in controlled temperature (22 ± 2 °C) and humidity (60–70%) under a 12-h light–dark cycle with food and water *ad libitum*.

Experimental protocol

Animals were randomly divided into six groups ($n = 6$). Control animals (Group I) were administered normal saline and group II with CP (200 mg/kg × 7d, *ip*). Animals of group III and IV were treated with CP along with SAC (150 mg/kg × 7d, *ip*) or mesna (40 mg/kg × 7d, *ip*) 1 h before and 4 h after each CP dose, respectively. Animals of group V and VI were administered with SAC and mesna, respectively. Normal saline was used to suspend CP, SAC and mesna. Doses and treatment schedule were based on previous studies [5,11].

Histological analysis

Slides were prepared from urinary bladder tissues, stained with haematoxylin-eosin (HE) and observed under light microscope. Hisptopathological changes were graded using criteria of Gray et al. [12].

Real time quantitative PCR for mRNA analysis of UP IIIa, CCL11 and TNF-α

For determination of real time PCR efficiency cDNA templates were serially diluted to generate a standard curve by absolute quantification method. The PCR efficiency was calculated with Roche LightCycler® 480 [13]. This efficiency was used to calculate the crossing point (Cp) for relative quantification of mRNA.

Bladder tissue RNA was extracted by using high-pure RNA tissue Roche® kit as per manufacturers' protocol including a digestion step with DNase I to prevent subsequent amplification of genomic DNA during PCR run. RNA purity and concentration were verified by determining the A_{260nm}/A_{280nm} ratio using Nanodrop Spectrophotometer (Thermo Scientific, Waltham, MA). The 260/280 nm ratio was ~2.0 in all cases. cDNA was synthesized using Roche Transcriptor First Strand cDNA Synthesis Kit. Primers were designed using Primer Quest Tool of Integrated DNA Technology for UPIIIa, CCL11, TNF-α and β-actin (Table 1). The thermocycler parameters were as follows: 95 °C for 5 min, 50 cycle of 95 °C for 10 s, 60 °C for 10 s 72 °C for 20 s, one cycle of melting curve 95 °C for 10 s 60 °C for 10 s, 72 °C for 20 s and cooling one cycle 49 °C for 30 s.

Table 1
Detail of primers used in the expression study.

Gene	Sequence (5′ → 3′)	Reference
Uroplakin IIIa	F- ACATGGGCAGTTCTGATGG R- CGGTTCACAGATGAGTAGGAAG	NM_023478.2
TNF-α	F- CTACCTTGTTGCCTCCTCTTT R- GAGCAGAGGTTCAGTGATGTAG	NM_001278601.1
CCL11	F- TGTAGCTCTTCAGTAGTGTGTTG R- CTTCTATTCCTGCTGCTCACG	NM_011330.3
β-actin	F- ATGCCGGAGCCGTTGTC R- GCGAGCACAGCTTCTTTG	NM_007393

LightCycler® 480 Multi well plate-96 was run on Roche LightCycler® 480 with three independent real-time PCR reactions of three independent samples in triplicate. Quantification was done by comparative Cp method (calculated as $2^{-\Delta\Delta Ct}$ fold change ± SD) [14]. Melting curve analysis was done to reveal primer dimer formation and as per the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines. Non-template control and non-reverse transcriptase controls were also included during real – time PCR run [13].

Western blot analysis of UPIIIa

Radioimmunoprecipitation buffer was used to prepare bladder tissue lysate. Urinary bladder tissue was homogenized by sonication in lysis buffer and centrifuged at 10,000×*g* for 30 min at 4 °C and supernatant was recovered. Protein concentration was measured by the Bradford method. Normalized protein was separated and transferred onto 0.45 mm nitrocellulose membrane, blocked with 3% bovine serum albumin in Tris-buffered saline and Tween-20 (TBST) for 1 h. Membranes were incubated with monoclonal primary antibody at 4 °C overnight after washing in TBST it was incubated with the secondary IgG HRP-antibody for 1 h at room temperature. Colorimetric HRP system using 3,3′ diaminobenzidine was used for detection of protein expression. The densitometry of protein bands (UPIIIa/β-actin) was also conducted.

Enzyme-linked immunosorbent assay

UPIIIa and CCL11 in serum and CCL11 and TNF-α in mouse urinary bladder tissue homogenate were measured by ELISA as per manufacturers' assay procedure.

Estimation of TNF-α in serum by flowcytometry

TNF-α in serum was measured by BD TNF-α cytometric Bead Array (CBA) Flex Set C8 as per manufacturers' protocol. All the flow cytometry data were acquired on BD LSR II flow cytometer and analysis was carried out using BD FCAP ArrayTM Software. The FACS analysis was performed at Jamia Hamdard - BD Biosciences FACS Academy.

Statistical analysis

Data enumerated as means + SEM were analysed by using Student's *t*-test and $p < 0.05$ were considered as significant.