Published in final edited form as:

Urology. 2009 November ; 74(5): 1163–1168. doi:10.1016/j.urology.2009.02.066.

Changes in ATP Stimulated ATP Release Suggests an Association between Cytokine and Purinergic Signaling in Bladder Urothelial Cells

Yan Sun^{a,*}, Susan Keay^{b,c}, Todd J. Lehrfeld^a, and Toby C. Chai^a

^a Department of Surgery, Division of Urology, University of Maryland School of Medicine, Baltimore, MD 21201

^b VA Maryland Healthcare System, University of Maryland School of Medicine, Baltimore, MD 21201

^c Department of Medicine, Division of Infectious Diseases, University of Maryland School of Medicine, Baltimore, MD 21201

Abstract

Objective—To determine whether antiproliferative factor (APF) or epidermal growth factor (EGF) can induce changes in purinergic signaling in normal bladder urothelial cells (BUC) and/or whether antagonizing EGF activity or blocking ATP-purinergic receptors can induce changes in purinergic signaling in interstitial cystitis (IC) cells.

Methods—IC and normal BUC were obtained from patients' bladder biopsies. IC BUC were treated with genistein, which antagonizes EGF's activity, while normal BUC were treated with EGF, mock APF, or APF. Suramin, which antagonizes ATP activity, was used to treat APF-treated normal BUC. ATP release was determined by stimulating BUC with 30 μ M ATP and then collecting supernatant over a 3-hour period. ATP quantification was measured by luciferin-luciferase assay. P2X₃ expression on BUC was determined by fluorescence activated cell sorting (FACS).

Results—Genistein treatment of IC BUC resulted in significantly decreased ATP release, thus reverting IC cells to a normal purinergic signaling phenotype. Conversely, normal BUC treated with EGF or APF resulted in significantly increased ATP release and P2X₃ expression, converting normal BUC to an IC phenotype. Suramin treatment of APF-treated normal BUC significantly reduced ATP release.

Conclusions—Genistein and suramin reversed the augmented ATP release in IC BUC and APF-treated normal BUC respectively, suggesting the possibility of intravesical use of these agents in IC treatment. EGF and APF induced augmented purinergic signaling in normal BUC as determined by increased ATP release and increased P2X₃ expression. These data suggest an association between cytokines and purinergic signaling in human BUC that should be explored further.

*Correspondence: Yan Sun, Ph.D., 10 S. Pine Street, MSTF 4–11, University of Maryland School of Medicine, Baltimore, Maryland 21201, (telephone: 410-706-7318; FAX: 410-706-0311; e-mail: ysun@smail.umaryland.edu).

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Keywords

anti-proliferative factor (APF); adenosine triphosphate (ATP); Bladder urothelial cells (BUC); epidermal growth factor (EGF); heparin-binding epidermal growth factor-like growth factor (HB-EGF); interstitial cystitis (IC)

Introduction

Symptoms of interstitial cystitis (IC) are characterized by urinary urgency, increased frequency, and bladder pain. Because the etiology is still unknown, treatment is empiric. Recent studies have indicated that explanted bladder urothelial cells (BUC) from IC patients have several stably heritable abnormalities compared to normal controls. These include unique expression of a small sialoglycopeptide, antiproliferative factor (APF) ¹; altered expression of cytokines such as increased epidermal growth factor (EGF) and decreased heparin-binding epidermal growth factor-like growth factor (HB-EGF) ^{2,3}; augmented ATP release in response to stretch and exogenous ATP stimulus ^{4,5}; and increased expression of P2X₃ and P2X₂ purinergic receptors ^{6,7}.

A new paradigm is that the bladder urothelium plays an active role in bladder sensory function and is not merely a barrier compartment. We previously demonstrated that exogenous ATP stimulation of IC BUC significantly increased ATP release by these cells *in vitro* ⁵. For the sake of brevity, this phenomenon will be referred to as ATP stimulated ATP release or ASAR. ASAR reflects the purinergic signaling pathway in BUC which is important for determining the apical urothelial cell surface area ⁸. We previously showed that treatment of IC BUC with HB-EGF decreased ASAR ⁵. We hypothesized that altering the cytokine milieu could affect ASAR in a parallel fashion. We therefore asked the questions: 1) could treatment of normal BUC with APF increase ASAR, thus converting normal BUC to IC phenotype? and 2) could treatment of normal BUC with EGF increase ASAR? Because the main downstream effect of EGF is protein phosphorylation, we also asked could genistein, a non-specific tyrosine kinase inhibitor, reduce ASAR in IC BUC? Also, could suramin, a non-specific purinergic P2X receptor antagonist, decrease the ASAR in APF-treated normal BUC (as it was previously shown to do in IC BUC) ⁵? Finally, could the expression of P2X₃ in normal BUC be altered by treatment with APF or EGF? The measurement of the reciprocal relationship between cytokines and purinergic signaling will not only shed light into the pathophysiology of IC, but also establish a cell model by which potential novel agents for IC can be tested.

Materials and Methods

Materials

All chemicals were purchased from Sigma (Sigma, St. Louis, MO). P2X₃ antibody was purchased from Neuromics (Neuromics, Edina, MN). Media were purchased from Invitrogen (Invitrogen, Carlsbad, CA).

Methods

1. Definition of IC and normal subjects—IC patients met National Institute of Diabetes and Digestive and Kidney Diseases criteria⁹. Control patients had neither voiding nor pelvic pain symptoms. The ethics and study protocols were approved by the University of Maryland Baltimore Institutional Review Board.

2. Bladder Biopsies and BUC Culture—Bladder biopsies were obtained from IC and normal subjects using the cold cup biopsy technique. The techniques of BUC culture from

cystoscopic biopsies have been described in detail previously^{4, 11}. After outgrowth became established, BUC were counted using a hemocytometer and evenly distributed onto 6-well cell culture plates at a density of 1×10^5 cells per well for each ASAR experiment.

3. ATP-Stimulated ATP Release (ASAR)—BUC were plated as described above. ATP (30 μ M) was added to cell culture media⁵. Cell supernatants were collected periodically over a three hour period (0, 1, 2 and 3 hrs after addition of ATP with time 0 representing sample collected immediately after addition of ATP).

4. ATP Assay—ATP assays were performed using a Sigma luciferin-luciferase assay kit as previously prescribed in detail⁴.

5. Preparation APF and Mock APF—APF was harvested and purified from the supernatant of cultured IC BUC using molecular weight fractionation, ion exchange chromatography, hydrophobic interaction chromatography, and reversed-phase high-performance liquid chromatography (HPLC), as previously described¹. Mock APF was prepared by using the supernatant of normal BUC and the same purification procedure.

6. Treatment of BUC by Genistein, EGF, AG1478, APF, and Suramin

Genistein: IC BUC were pretreated with 100 μ M genistein for 24 hours prior to ASAR measurement. Control experiments were normal BUC and IC BUC or vehicle underwent ASAR.

EGF: Normal BUC were pretreated with 10ng/ml or 20ng/ml EGF for 10 minutes prior to ASAR measurement. Control was normal BUC treated with medium alone.

AG1478: Normal BUC were pretreated with 1 μ M of AG1478, a selective tyrosine kinase inhibitor, for 2 hours before adding 20ng/ml EGF for 10 minutes prior to ASAR measurement. Controls were BUC underwent ASAR or treated with 20 ng/ml EGF for 10 minutes.

APF: Normal BUC were plated as described above. The next day, cells were serum-starved and pretreated with 30 μ l/cc media of HPLC-purified APF for 48 hours prior to ASAR measurement. As controls, normal BUC either underwent ASAR or treated with 30 μ l/cc media of HPLC purified mock-APF for 48 hours prior to ASAR measurement.

Suramin: Normal BUC were plated as described above, serum-starved, and treated with APF for 48 hours. After the first 24 hours' of APF incubation, suramin (40 μ M) was added to treat the cells for an additional 24 hours in the presence of APF prior to ASAR measurement.

Genistein, AG1478, and suramin concentrations were selected based on previous publications 11–13.

7. Fluorescence Activated Cell Sorting (FACS) Analyses—FACS analysis was performed using 3-color FACScan™ Flow Cytometer (BD Bioscience, NJ) to determine expression of P2X₃ receptor. The detailed methodology of P2X₃ fluorescence labeling and data acquisition has been described previously⁶.

8. Statistical Analysis—For each patient-derived cell line, the experiment was performed in triplicate. Results were expressed as mean values plus or minus standard error of the mean (mean $\pm$ SEM). Analysis of variance (ANOVA) was used to compare the means and p-values were considered statistically significant if $p < 0.05$.

Results

1. Effect of Genistein Treatment on ASAR in IC BUC

The IC BUC derived from six IC patients were treated with genistein for 24 hours and then underwent ASAR measurements. With exogenous ATP stimulation, IC BUC showed significantly augmented release of ATP compared to normal BUC (Fig. 1, line with open diamonds vs. line with open squares). Genistein treatment of IC BUC significantly decreased ASAR at 0 and 1 hour after addition of ATP (Fig. 1, line with closed squares).

2. Effect of EGF and AG1478 Treatment on ASAR in Normal BUC

Treatment of normal BUC from three control subjects with EGF at 10 ng/ml and 20ng/ml for 10 minutes, resulted in significantly increased ASAR (Fig. 2A, 0 hr time point), similar to the ASAR seen in IC cells. Pretreatment of normal cells with the selective tyrosine kinase EGFR inhibitor AG1478 significantly decreased ASAR in the presence of EGF (Fig. 2B, 0hr and 1hr time points, line with closed diamonds), suggesting that EGF stimulation of ASAR in normal BUC occurs through EGF/EGFR signaling.

3. Effect of APF Treatment on ASAR in Normal BUC

Normal BUC from five control subjects were then treated by APF and normal BUC from three control subjects were treated with mock APF. As shown in Figure 2C, APF treatment of normal BUC resulted in an increased ASAR pattern similar to IC BUC. In comparison, mock APF had no effect on ASAR (Fig. 2C, line with closed squares).

4. Effect of Suramin on ASAR in APF-treated Normal BUC

We had previously published that suramin reduced the augmented ASAR detected in IC BUC⁵. In this study, normal BUC were first treated by APF, followed by suramin, and then followed by ASAR. Suramin treatment also significantly decreased APF-stimulated ASAR in normal BUC (Fig. 2D, closed squares). In fact, suramin treatment reduced ASAR to a level even below that measured in untreated normal cells. (Fig. 2D, open diamonds).

5. Effects of EGF or APF Treatment on P2X₃ Receptor Expression in Normal BUC

Studies have demonstrated that ATP can stimulate the purinergic P2X₃ signaling pathway in bladder urothelium and BUC^{6,14}. To determine whether the purinergic receptor P2X₃ might be augmented as a result of increased ASAR, FACS analysis was performed on EGF-treated, APF-treated and mock APF-treated normal BUC 3 hours following treatment with ATP. Representative FACS histograms were shown in Figure 3. Regions of higher fluorescence (region M1) were seen after normal BUC were treated with EGF or APF (Fig. 3C, 3D), suggesting cytokine-stimulated ASAR can lead to higher purinergic receptor expression in BUC.

Table 1 shows the mean percentages of positive P2X₃ receptor fluorescence density per 2,500 cells for each group. EGF and APF treatment both resulted in significantly increased expression of P2X₃, (Table 1 rows C and D) whereas mock APF had no effect (Table 1 row E).

Discussion

It was previously reported that BUC grown *in vitro* from IC patient bladder biopsies have abnormalities including altered cytokine production and augmented purinergic signaling¹⁻⁷. Consistent with the *in vitro* cell culture phenotype, IC urine specimens also have increased EGF, decreased HB-EGF, increased APF activity¹⁻³ and increased ATP levels⁴. Since changes in phenotypic expression persist in cultured IC BUC, it is possible to investigate the link

between cytokine expression and purinergic signaling in IC BUC. In this report, we demonstrate that EGF treatment of normal BUC results in a significant increase in ASAR, while AG1478 pretreatment of normal BUC in response to EGF (Fig. 2B) and genistein treatment of IC BUC (Fig. 1) resulted in a significant decrease in ASAR, suggesting that EGF and mechanisms related to EGF-receptor activation/tyrosine phosphorylation may be able to modulate purinergic signaling. EGF was previously shown to change BUC behavior in several ways. It acutely suppressed the inward potassium current presumptively mediated by the Kir2.1 channel¹³, an effect that might result from increased tyrosine phosphorylation of Kir2.1 which closes the channel¹¹

We showed previously that exposure to suramin changed the ASAR phenotype of IC BUC to normal⁵. Suramin has multiple mechanisms of action. It is a non-specific P2X and P2Y receptor blocker^{15, 16}; thus having an effect on purinergic signaling. Suramin has also been shown to inhibit the growth of urothelial carcinoma cells *in vitro* through inhibition of EGF binding¹⁷ suggesting an effect on cytokine signaling. Although mechanisms underlying genistein and suramin treatment are different and are not clearly known, their effects on ASAR are similar, suggesting that intravesical genistein or suramin could be a potential therapeutic agent for IC.

The IC BUC phenotype includes decreased *in vitro* expression of HB-EGF, and possible decreased *in vivo* expression as manifested by decreased urine HB-EGF levels. Treatment of IC BUC with 20 ng/ml HB-EGF, which is the upper limit of normal concentration found in urine from asymptomatic normal subjects², was previously shown to lower the augmented ASAR *in vitro*⁵. Our current data show that APF increases the ASAR in normal BUC suggesting a link between APF and purinergic signaling also.

In summary, this is the first report describing a link between the cytokines EGF and APF, and purinergic signaling, in bladder urothelial cells. To date, our studies have demonstrated that EGF, APF and HB-EGF alter the purinergic signaling phenotype of bladder urothelial cells in response to an ATP stimulus. Indeed, the ASAR characteristic of normal BUC can be converted to that of IC BUC after treatment of normal BUC with EGF or APF. Conversely, the ASAR characteristics of IC BUC can be converted to that of normal BUC by treating IC BUC with HB-EGF or genistein. Additional study should be focused on EGF- or HB-EGF-targeted pathways by using widely-available and well-studied specific inhibitors for these pathways (such as those that inhibit EGFR phosphorylation). Future treatments for IC could target urothelial abnormalities in the cytokine and/or purinergic pathways.

Conclusion

ASAR is augmented in IC BUC when compared to normal BUC. ASAR can be decreased in IC BUC by treating IC BUC with HB-EGF or genistein. Conversely, increased ASAR can be induced in normal BUC by treatment of normal BUC with EGF or APF. The expression of the purinergic receptor, P2X₃, on normal BUC can be significantly increased by treating normal BUC with EGF or APF. These findings suggest a novel link between cytokine (EGF, HB-EGF, APF) and purinergic (ATP, P2X₃) signaling pathways in human BUC. The ability to induce phenotype switching offers insight into therapeutic testing models for potential future treatments for IC.

Acknowledgments

Funding Sources: NIH R01-DK059441, R01 DK075728 and R01-DK52596.

References

1. Keay SK, Szekely Z, Conrads TP, et al. An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proc Natl Acad Sci USA* 2004;101:11803–08. [PubMed: 15282374]
2. Keay S, Kleinberg M, Zhang CO, et al. Bladder epithelial cells from patients with interstitial cystitis produce an inhibitor of heparin-binding epidermal growth factor-like growth factor production. *J Urol* 2000;164:2112–18. [PubMed: 11061938]
3. Keay S, Zhang CO, Kagen DI, et al. Concentrations of specific epithelial growth factors in the urine of interstitial cystitis patients and controls. *J Urol* 1997;158:1983–8. [PubMed: 9334654]
4. Sun Y, Keay S, DeDeyne P, et al. Augmented stretch activated adenosine triphosphate release from bladder urothelial cells in patients with interstitial cystitis. *J Urol* 2001;166:1951–56. [PubMed: 11586266]
5. Sun Y, Chai TC. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. *Am J Physiol Cell Physiol* 2006;290:C27–C34. [PubMed: 16107499]
6. Sun Y, Chai TC. Up-regulation of P2X3 receptor during stretch of bladder urothelial cells from patients with interstitial cystitis. *J Urol* 2004;171:448–52. [PubMed: 14665953]
7. Tempest HV, Dixon AK, Turner WH, Elneil S, Sellers LA, Ferguson DR P2X2 and P2X3 receptor expression in human bladder urothelium and changes in interstitial cystitis. *BJU Int* 2004;93:1344–48. [PubMed: 15180635]
8. Wang EC, Lee JM, Ruiz WG, et al. ATP and purinergic receptor-dependent membrane traffic in bladder umbrella cells. *J Clin Invest* 2005;115:2412–22. [PubMed: 16110327]
9. Gillenwater JY, Wein AJ. Summary of the National Institute of Arthritis, Diabetes, Digestive and Kidney Diseases Workshop on Interstitial Cystitis, National Institutes of Health, Bethesda, Maryland, August 28–29, 1987. *J Urol* 1987;140:203–06. [PubMed: 3379688]
10. Trifillis AL, Cui X, Jacobs S, et al. Culture of bladder epithelium from cystoscopic biopsies of patients with interstitial cystitis. *J Urol* 1995;153:243–48. [PubMed: 7966781]
11. Wischmeyer E, Doring F, Karschin A. Acute suppression of inwardly rectifying Kir2.1 channels by direct tyrosine kinase phosphorylation. *J Biol Chem* 1998;273:34063–68. [PubMed: 9852063]
12. Martínez-García E, Irigoyen M, Ansó E, et al. Recurrent exposure to nicotine differentiates human bronchial epithelial cells via epidermal growth factor receptor activation. *Toxicol Appl Pharmacol* 2008;228(3):334–42. [PubMed: 18262213]
13. Sun Y, Chen M, Lowentritt BH, et al. EGF and HB-EGF modulate inward potassium current in human bladder urothelial cells from normal and interstitial cystitis patients. *Am J Physiol Cell Physiol* 2007;292:C106–14. [PubMed: 16837648]
14. Cockayne DA, Hamilton SG, Zhu QM, et al. Urinary bladder hyporeflexia and reduced pain-related behaviour in P2X3-deficient mice. *Nature* 2000;407(6807):1011–5. [PubMed: 11069181]
15. Hourani SM, Hall DA, CJ. Effects of the P2-purinoreceptor antagonist, suramin, on human platelet aggregation induced by adenosine 5'-diphosphate. *Br J Pharmacol* 1992;105:453–57. [PubMed: 1559134]
16. Mallard N, Marshall R, Sithers A, et al. Suramin: a selective inhibitor of purinergic neurotransmission in the rat isolated vas deferens. *Eur J Pharmacol* 1992;220:1–10. [PubMed: 1330614]
17. Gansler T, Vaghmar N, Olson JJ, et al. Suramin inhibits growth factor binding and proliferation by urothelial carcinoma cell cultures. *J Urol* 1992;148:910–14. [PubMed: 1512858]

Figure 1. Genistein Decreases ASAR in IC BUC

Genistein treated IC BUC (squares) resulted in decreased ASAR compared to untreated IC BUC (diamonds). In this and all subsequent figures, dotted line represents concentration of exogenously added ATP (30 μ M). * = $p < 0.05$ comparing genistein treated vs. untreated IC BUC at 0 and 1 hour time points. BUC from 6 IC subjects and BUC from 6 normal subjects were used in the experiment.

A

B

C

D

Figure 2.

A. EGF Increases ASAR in Normal BUC – EGF treatment, at both 10 ng/ml (squares) and 20 ng/ml (triangles) of normal BUC increased ASAR compared to untreated normal BUC (diamonds). * = $p < 0.05$ comparing EGF (20 ng/ml) treatment to untreated normal BUC at time 0. \$ = $p < 0.05$ comparing EGF (10 ng/ml) treatment to untreated normal BUC at time 0. BUC from 3 normal subjects were used in this experiment.

B. AG1478 Decreases ASAR in EGF-treated Normal BUC – AG1478 treatment of normal BUC (line with closed diamonds) decreased ASAR compared to APF pretreated normal BUC (line with triangles). * = $p < 0.05$ comparing EGF (20 ng/ml) treatment to untreated normal BUC

at time 0 hr and 1 hr. \$ = $p < 0.05$ comparing AG1478 treatment to EGF (20 ng/ml) treatment to normal BUC at time 0 hr and 1 hr. BUC from 3 normal subjects were used in this experiment.

C. APF Increases ASAR in Normal BUC – APF treated normal BUC (triangles) increased ASAR compared to untreated normal BUC (diamonds). Mock APF treated normal BUC (squares) had similar ASAR compared to untreated normal BUC. * = $p < 0.05$ comparing APF treated vs. untreated normal BUC at time 0. \$ = $p < 0.05$ comparing APF treated vs. mock-APF treated BUC at time 0. BUC from 5 normal subjects were used in APF treatment experiment and BUC from 3 normal subjects were used in mock APF treatment experiment.

D. Suramin Decreases ASAR in APF-treated Normal BUC – Similar to previous experiment, APF treatment of normal BUC (triangles) resulted in augmented ASAR compared to untreated normal BUC (diamonds). However, suramin treatment of APF-treated normal BUC (squares) decreased ASAR to that below untreated normal BUC. * $p < 0.05$ comparing suramin-, APF-treated normal BUC vs. APF treated normal BUC at 0 and 1 hour time points. \$: $p < 0.05$ comparing suramin-, APF-treated normal BUC vs. untreated normal BUC at 0 hour and 2 hour time points. BUC from 3 normal subjects were used in this experiment.

Figure 3. FACS of P2X₃ Expression in Normal BUC after EGF and APF treatment

Histograms of fluorescence intensity were plotted against cell numbers. Representative FACS histograms of normal BUC treated with EGF (20ng/ml), APF, or mock APF. A: Untreated normal BUC. B: Normal BUC 3-hours after ASAR. C: EGF treated normal BUC 3-hours after ASAR, D: APF treated normal BUC 3-hours after ASAR, E: Mock-APF treated normal BUC 3-hours after ASAR. For each run, 2,500 cells were collected. Histograms of fluorescence intensity were plotted against cell numbers (events). The percentages of distribution of high fluorescence intensity field over total field (i.e. M1 region over total field in Figure 3) were compared across all experiment conditions BUC from 3 normal subjects were used in this experiment.

Table 1
Flow Cytometry Analysis of P2X₃ Expression in Normal BUC after EGF and APF treatment

Summary of mean percentages of distribution of P2X₃ receptor in high fluorescence density region (i.e. M1 region over total field in Figure 3) in normal BUC (n=3 subjects) after different treatment conditions.

Cell Condition	P2X ₃ Receptor Expression (%positive) (mean $\pm$ SEM)	p-value (compared to row B)
A. Normal BUC prior ASAR	3.5 $\pm$ 1.2	0.01
B. Normal BUC after 3-h of ASAR	9.9 $\pm$ 0.9	-
C. EGF treated normal BUC after 3-h of ASAR	15.7 $\pm$ 0.3	0.009
D. APF treated normal BUC after 3-h of ASAR	21.4 $\pm$ 1.0	0.001
E. Mock-APF treated normal BUC after 3-h of ASAR	9.0 $\pm$ 0.7	0.39