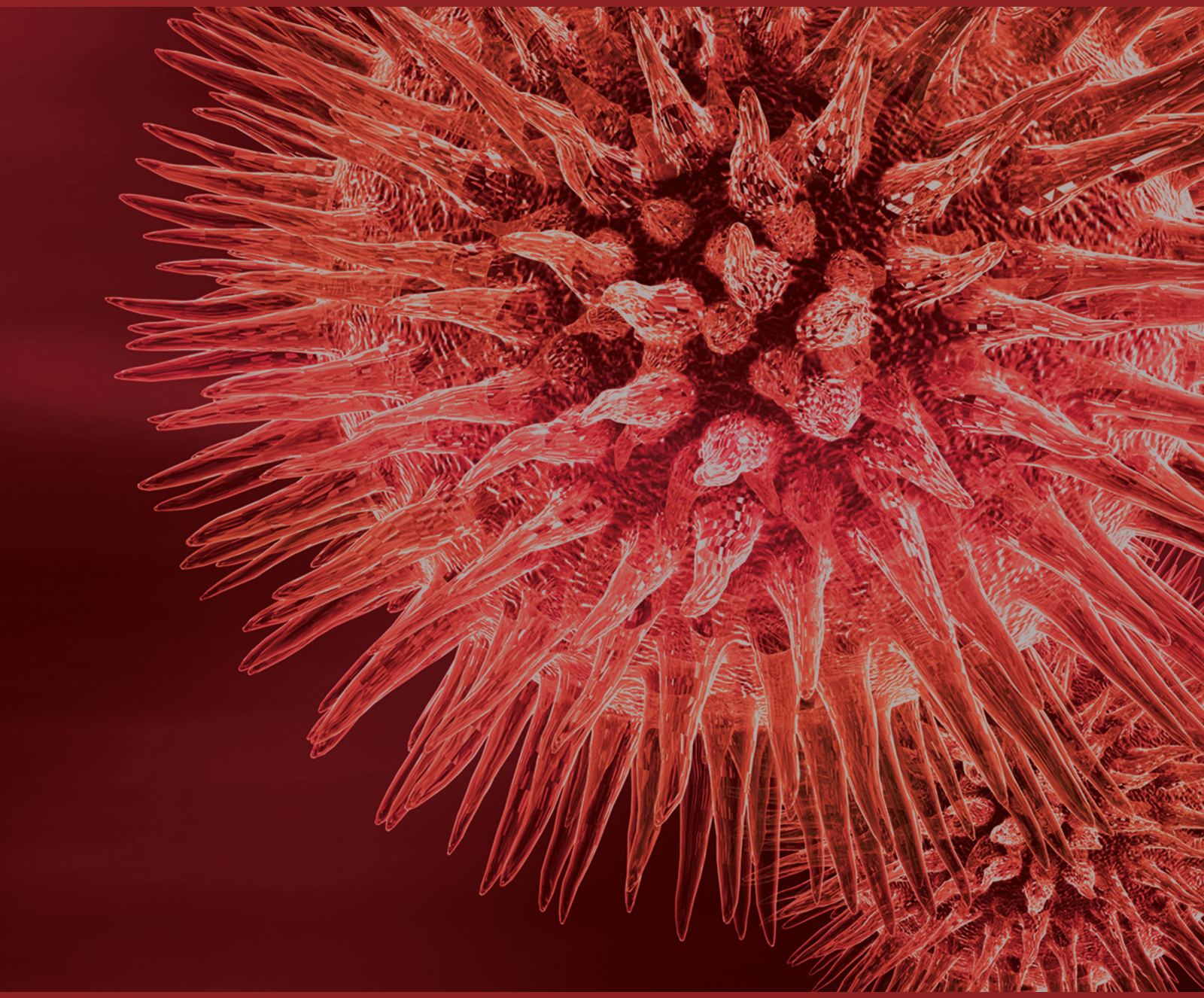
BioMed Research International

Recent Advances in the Biology of the Urothelium and Applications for Urinary Bladder Dysfunction

Guest Editors: Rok Romih, Michael Winder, and Gilho Lee



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Editorial

Recent Advances in the Biology of the Urothelium and Applications for Urinary Bladder Dysfunction

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The mammalian urinary bladder is a hollow organ adapted to short-term storage of urine, which contains waste products of metabolism, some of them being potentially toxic. During the micturition cycle, the urinary bladder is gradually distended by a flow of urine from ureters and then at a certain degree of fullness it is relatively rapidly contracted by voiding. One of the tasks of the bladder is to prevent the exchange of waste products and water between urine and tissue fluids/blood. The other task of the bladder is a precise regulation of the micturition cycle. Both tasks greatly depend on the structure of the urinary bladder wall compartments, epithelium (urothelium), nerve tissue (afferents and efferents), cells in the connective tissue (myofibroblasts, interstitial cells, and others), and muscles (detrusor), and the communication between them.

The first task, the so-called blood-urine permeability barrier, is achieved mainly by the unique specialization of the apical plasma membrane of superficial urothelial cells. This plasma membrane is composed of so-called urothelial plaques. Urothelial plaques are 2D crystals of hexagonally packed 16 nm particles consisting of integral membrane proteins, that is, uroplakins. Uroplakins are directly responsible for formation and maintenance of the functional permeability barrier. Moreover, they are involved in urinary tract infections since one of them, uroplakin Ia, serves as a receptor for uropathogenic *E. coli*. The specialized apical plasma membrane develops gradually during differentiation of urothelial cells from basal, intermediate to superficial layer and by maturation processes within membrane compartments of superficial cells.

Over the last two decades it has become clear that urothelium may also sense changes in the extracellular environment and transduce signals to nerves and muscles in the bladder wall, therefore contributing to the precise regulation of the micturition cycle.

Original research and review articles published in this special issue bring new knowledge and discussions on the complexity of urinary bladder wall structure/function and communication between different types of cells/tissues of the bladder wall. The articles reveal the importance of research for understanding many urinary bladder-related diseases and symptoms (cystitis, bladder pain syndrome, overactive bladder, inflammation, and neoplastic growth) and also suggest possible treatments for these conditions.

S. K. Keay and coworkers review urothelial cell abnormalities, including structural changes, altered gene and protein expression, functional abnormalities, abnormal cell proliferation, and others, in different lower urinary tract symptoms. They discuss a challenge to target the most relevant pathophysiology to treat these symptoms.

In the review by G. Lee et al., basic urothelial cell biology is discussed in the view of its role in cystitis. Different forms of cystitis and current knowledge on their treatments are discussed. Animal model of cyclophosphamide as a tool for induced cystitis studies is also presented.

P. Aronsson and colleagues study whether blockade of muscarinic receptors or nitric oxide synthase inhibition affects the induction of cyclophosphamide-induced cystitis. They suggest that such treatments may have a therapeutic potential.

K. J. Smith and coworkers describe effects of dimethyl sulfoxide on urothelium and consequences for interstitial cystitis/bladder pain syndrome.

In the review article, E. J. Gonzalez et al. discuss the potential of two chemokine/receptor pairs and the cytokine/receptor pair as targets for pharmacologic therapy for treatment of bladder dysfunction and for reduction of somatic sensitivity associated with urinary bladder inflammation.

Two research articles by K. A. J. Kuijpers and coauthors investigate interstitial cells found in the lamina propria and detrusor layer of human bladder wall. They describe discriminatory markers for interstitial cells and their role in detrusor overactivity.

Detrusor overactivity was studied also by Y. Cheng et al. They show that bladder stretch-induced ATP release may play an important role in early sensation of urgency in female patients with detrusor overactivity.

Stretch-induced ATP release by inflammatory mediators (bradykinin, histamine, and serotonin) is examined by K. J. Mansfield and J. R. Hughes. Their study challenges the hypothesis that there is a direct interaction between the release of inflammatory mediators and increased ATP release. In another article, the same authors demonstrate the importance of the purinergic system, particularly P2Y receptor activation for ATP release in urothelium.

F. Mistretta and coworkers discuss the possibility of using curcumin, which might work as TRPV1 agonist, for the management of bladder inflammation and neoplastic cell growth.

I. Sterle and coauthors investigate possible correlation between urothelial cell differentiation and urothelial sensory properties. They compare apical plasma membrane structure with uroplakin, purinergic receptors (P2X3, P2X5), and transient receptor potential vanilloid channels (TRPV1, TRPV4) expression in normal human urothelium and in papillary carcinomas.

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Research Article

Inhibition of Nitric Oxide Synthase Prevents Muscarinic and Purinergic Functional Changes and Development of Cyclophosphamide-Induced Cystitis in the Rat

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Nitric oxide (NO) has pivotal roles in cyclophosphamide- (CYP-) induced cystitis during which mucosal nitric oxide synthase (NOS) and muscarinic M5 receptor expressions are upregulated. In cystitis, urothelial muscarinic NO-linked effects hamper contractility. Therefore we wondered if a blockade of this axis also affects the induction of cystitis in the rat. Rats were pretreated with saline, the muscarinic receptor antagonist 4-DAMP (1 mg/kg ip), or the NOS inhibitor L-NAME (30 mg/kg ip) for five days. 60 h before the experiments the rats were treated with saline or CYP. Methacholine-, ATP-, and adenosine-evoked responses were smaller in preparations from CYP-treated rats than from saline-treated ones. Pretreatment with 4-DAMP did not change this relation, while pretreatment with L-NAME normalized the responses in the CYP-treated animals. The functional results were strengthened by the morphological observations; 4-DAMP pretreatment did not affect the parameters studied, namely, expression of muscarinic M5 receptors, P1A1 purinoceptors, mast cell distribution, or bladder wall enlargement. However, pretreatment with L-NAME attenuated the differences. Thus, the current study provides new insights into the complex mechanisms behind CYP-induced cystitis. The NO effects coupled to urothelial muscarinic receptors have a minor role in the development of cystitis. Inhibition of NOS may prevent the progression of cystitis.

1. Introduction

In rodents, as well as in humans, cyclophosphamide (CYP) treatment induces cystitis, which includes alterations both at functional and histological levels [1, 2]. Specifically, the urothelium/mucosa is affected, both regarding morphology and expression of receptors and signaling molecules. Functional changes occur via hampered efferent and afferent effects [3–6]. In conscious rats, this results in frequent micturitions of smaller volumes [7–9]. In the rat urinary bladder both acetylcholine and adenosine-5′-triphosphate (ATP) mediate the parasympathetic contractile response [10, 11]. In addition, the ATP metabolite adenosine evokes relaxations [12, 13]. The reduction of the parasympathetic contractile response in CYP-treated rats depends partly on an increased production of nitric oxide (NO) due to sensitization of urothelial muscarinic receptor stimulated NO effects [6, 14, 15]. The expression of nitric oxide synthase (NOS) in the mucosa has been reported to increase after CYP treatment [6, 16, 17]. CYP-induced morphological changes include bladder wall thickening [18], mast cell appearance in the smooth muscle [19], and upregulation of the expression of urothelial muscarinic M5 receptors [6, 20].

The CYP-induced cystitis in the rat is a commonly used disease model since it shares many features with the cystitis

occurring in patients treated with CYP, but also with bladder pain syndrome/interstitial cystitis (BPS/IC) [21, 22]. BPS/IC is a noninfectious inflammatory condition with unclear etiology [23], including pelvic pain and voiding disturbances such as urinary urge and frequency. Although the pathophysiology of BPS/IC is largely unknown, it has been observed that the release of ATP and NO are associated with the condition [24, 25]. NO, in particular, is considered to have a pivotal role in this disease and mucosal NOS is upregulated in patients with BPS/IC corresponding to the findings in CYP-induced cystitis in the rat [6, 20]. Also, a pathognomonic mast cell infiltration into the detrusor muscle occurs [26–29]. Another factor suggested to be correlated to the degree of the disease is macrophage migration inhibitory factor (MIF) [30]. In CYP-induced cystitis in the rat increased levels of MIF occur in the urine and MIF can also be detected in all areas of the urothelium, not only in the basal part as is the case in healthy bladders [16].

We have recently demonstrated that pretreatment with the P1A1 antagonist DPCPX alleviate the symptoms to CYP-induced cystitis [31], and the aims of the current study were thus to examine if muscarinic receptors and NO are also involved in the inflammation in experimental CYP-induced cystitis, as well as to investigate if there exists a link between the two. Therefore, rats were pretreated with a NOS inhibitor or a muscarinic receptor antagonist before the induction of inflammation. Functional cholinergic and purinergic responses, tissue changes and the degree of mast cell infiltration, muscarinic M5 receptor, P1A1 purinoceptor, and MIF expressions were evaluated in saline and CYP-treated rats with particular care taken to the effects in the mucosa.

2. Materials and Methods

The ethics committee at the University of Gothenburg approved the study design, in which 38 male rats (300–400 g) of the Sprague-Dawley strain were used. For five days before sacrifice (starting at −120 h relative to sacrificing), the rats received daily intraperitoneal administrations of either saline, L-NAME (30 mg/kg), or 4-DAMP (1 mg/kg; Figure 1 shows a schematic layout of the study design). Cystitis was chemically induced (at −60 h relative to sacrificing) by a single intraperitoneal injection of CYP (100 mg/kg), while controls received the same volume of saline (9 mg/mL ip; 1 mL/kg). Both injections were conducted in the presence of the analgesic buprenorphine (10 μg/kg ip). Sixty hours subsequent to the saline/CYP treatment (at the peak of inflammation [6]), the rats were anaesthetized with medetomidine (Domitor 0.3 mg/kg ip) then gassed and killed with an overdose of carbon dioxide. From the sacrificed rats, the urinary bladder was excised and thereafter divided into two identical parts; one half was used for functional examinations, while the other half was used for morphological and biochemical examinations.

2.1. In Vitro Functional Examinations. From the part of the bladder designated for functional examination, full thickness transverse strips were prepared (6×2 mm). The weights of the strips were 9.7 ± 0.3 (saline-saline; $n = 10$), 13.7 ± 0.5 (saline-CYP; $n = 12$), 14.0 ± 1.3 (4-DAMP-saline; $n = 8$), 19.0 ± 1.5 (4-DAMP-CYP; $P < 0.001$ versus saline-saline; $n = 10$), 12.5 ± 2.9 (L-NAME-saline; $n = 8$), and 16.2 ± 2.1 (L-NAME-CYP; $n = 8$) mg. The strips were excised from the detrusor proximal to the orifices of the two ureters in accordance to a standard procedure. Two strip preparations were taken from each bladder. The preparatory methods were generally as described previously for full thickness urinary bladder strip preparations with intact urothelium [12]. The detrusor strip was mounted between two steel rods of which one was fixed and the other adjustable and connected to an isometric force transducer (Linton). The strips were immersed in 25 mL organ baths containing Krebs solution of the following composition (mM): NaCl 118, KCl 4.6, $CaCl_2$ 1.25, KH_2PO_4 1.15, $MgSO_4$ 1.15, $NaHCO_3$ 25, and glucose 5.5, and which was gassed with 5% CO_2 in O_2 at 37°C. The preparations were repeatedly stretched in order to obtain a stable tension of about 5 mN after 45–60 min. The agonists utilized in the current studies, namely, methacholine (nonselective muscarinic agonist), ATP (nonselective P2 purinoceptor agonist), and adenosine (nonselective P1 purinoceptor agonist), were dissolved in distilled water and added to the organ baths at a volume of 125 μL. The total amount of agonist did not exceed 3% of the organ bath volume. Data were recorded using a MP100WSW data acquisition system and Acquire software (Biopac). A high K^+ solution (124 mM K^+ obtained by exchanging Na^+ for equimolar amounts of K^+) was administered in the beginning of each experiment in order to assess the viability of each strip preparation. Strip preparations that did not respond to potassium were omitted (<15 mN). Before continuing with the experiment the organ baths were washed three times with normal Krebs solution and equilibrated for 10 min. Relaxations to adenosine were studied on urinary bladder strip preparations precontracted with a medium K^+ solution (50 mM K^+ obtained by exchanging Na^+ for equimolar amounts of K^+). The precontractile tensions were 18 ± 1 (saline-saline; $n = 10$), 16 ± 1 (saline-CYP; $n = 12$), 15 ± 1 (4-DAMP-saline; $n = 8$), 13 ± 1 (4-DAMP-CYP; $n = 10$; $P < 0.05$ to saline-saline and L-NAME-CYP), 16 ± 1 (L-NAME-saline; $n = 8$), and 18 ± 1 (L-NAME-CYP; $n = 8$) mN. The differences in precontractile values were not statistically significant within the groups analyzed in the Results section. All drug concentrations presented in the Results section are based on pilot experiments or on previous studies [32, 33].

2.2. Morphological Examinations. The bladder tissues not used in the functional examination were fixed in phosphate buffered paraformaldehyde (4%; pH 7.0) and then embedded in paraffin for further investigation by immunohistochemistry or staining with toluidine blue used as a mast cell marker.

2.3. Immunohistochemistry. Sections of the tissues were investigated by immunohistochemistry using subtype specific muscarinic antibodies for the M5 receptor, the P1A1 purinoceptor, and MIF. Four μm sections were deparaffinized in xylene and rehydrated in ethanol. Any protein cross-linking that may have been induced by formalin was broken by incubation in citrate buffer (10 mM; pH 6.0), in a steamer

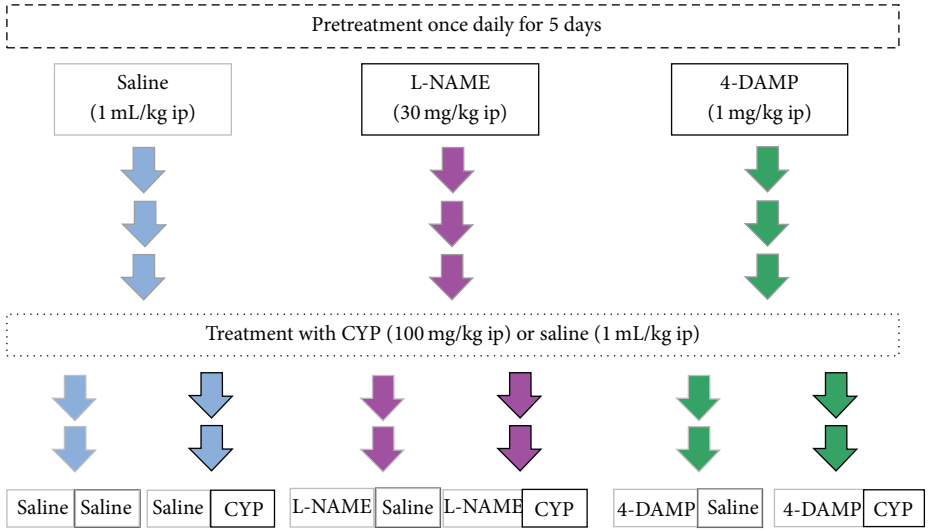

Figure 1: Study design. The rats were pretreated every morning for five days, starting at 120 hours before sacrifice. Sixty hours before the experiment, the rats received saline or CYP treatment and the rats were grouped into matched groups (saline-saline and saline-CYP, 4-DAMP-saline and 4-DAMP-CYP, L-NAME-saline and L-NAME-CYP; each group included 5-6 rats). Arrows indicate the five pretreatment events for each animal.

at 95–100°C for 30 min. Further incubation in goat serum in PBS (5%, 1 h) was performed to block nonspecific background staining. Sections were subjected to primary polyclonal rabbit antibodies, diluted 1 : 100 in PBS containing 1% goat serum at 4°C overnight. The following day the samples were incubated with secondary antibody Alexa Fluor 488 goat anti-rabbit IgG (1 : 250 in PBS containing 1% goat serum) and rhodamine conjugated phalloidin (15 μL/mL; dissolved in methanol 1 mg/1.5 mL). Finally, dehydration by ethanol was performed and the slices were mounted with Prolong gold antifade reagent with DAPI and were then viewed by microscopy (Eclipse 90i, Nikon, Tokyo, Japan). Photos were taken using camera DS-Fil (Nikon, Tokyo, Japan) and software NIS-Elements D 3.10 (Nikon, Tokyo, Japan). Negative controls were handled at the same occasion in the same fashion, but without addition of the primary antibody.

Although the antibodies are regarded by their manufacturers to be specific, cross-reactions with other peptides can never be ruled out and the resulting staining will hence be referred to as "P1A1-like," "M5-like," and "MIF-like."

2.4. Toluidine Blue Staining. Sections of the paraffin embedded bladders (4 μm) were deparaffinized by three 10 min intervals in 100% xylene and then rehydrated by serial incubations in 99.5% (2 × 5 min), 95% (5 min), 70% (5 min), and 50% (5 min) ethanol in water, followed by deionized water (10 min). Subsequently, they were stained in toluidine blue working solution (consisting of 20 mL of 1 g toluidine blue + 100 mL 70% ethanol; 180 mL of 1% sodium chloride in deionized water, pH = 2.3) for 2.5 min. Thereafter they were washed in deionized water for 4 × 30 sec and dehydrated through 10 dips in 95% ethanol, 10 dips in 99.5% ethanol, and 2 × 3 min in xylene.

2.5. Materials. The following substances were employed in the pretreatment of the rats and in the contraction experiments: acetyl-β-methylcholine chloride (methacholine), adenosine, adenosine-5′-triphosphate (ATP), 4-diphenylacetoxy-N-methylpiperidine methobromide (4-DAMP), N_ω-nitro-L-arginine methyl ester HCl (L-NAME), and cyclophosphamide monohydrate (CYP). All these substances were purchased from Sigma-Aldrich, St Louis, MO, USA. Buprenorphine (Temgesic, Schering-Plough, Brussels, Belgium) and medetomidine (Domitor Vet., Orion Pharma, Espoo, Finland) were purchased from Apoteket Farmaci, Apoteket AB, Stockholm, Sweden.

The following substances were used for the immunohistochemistry: Alexa Fluor 488 goat anti-rabbit IgG (purchased from Molecular Probes, Eugene, Oregon, US), rabbit polyclonal muscarinic M5 receptor antibody (Research and Diagnostic Antibodies, Berkley, US), ABC Staining system sc-2018 for use with rabbit primary antibody (Santa Cruz Biotechnology, USA), prolong gold antifade reagent with DAPI and PBS tablets (Invitrogen, Burlington, Canada), xylene (Histolab, Gothenburg, Sweden), and ethanol (Kemetyl, Stockholm, Sweden). Rabbit polyclonal P1A1 purinoceptor antibody, goat serum, rhodamine conjugated phalloidin, rabbit MIF antibody (Sigma-Aldrich, St Louis, USA), citrate buffer (citric acid and sodium citrate tribasic dehydrate), and methanol were all purchased from Sigma-Aldrich, St. Louis, MO, USA.

The following substances were used in the mast cell staining: hydrochloric acid, sodium chloride and toluidine blue O (Sigma-Aldrich, St Louis, USA), ethanol 99.5% and 95% (Kemetyl, Haninge, Sweden), pertex and xylene (Histolab Products AB, Gothenburg, Sweden), and phosphate buffered paraformaldehyde 4% (Apoteket Produktion & Laboratorier AB, Gothenburg, Sweden).

2.6. Calculations and Statistics. Statistical significance was determined by Student's t-test for unpaired data. When multiple comparisons with the same variable were made, statistical significance was determined by two-way analysis of variance or one-way analysis of variance (ANOVA) followed by the Bonferroni correction. Pharmacodynamic modeling was performed by applying built-in models (log (agonist) versus response (variable slope), biphasic or bell-shaped equations) for nonlinear and linear regression (GraphPad Software Inc., San Diego, USA). In the model fitting procedure, comparisons were based on visual inspection of observed versus predicted plots, value of the objective function, assessment of parameter correlation precision, and the Akaike information criterion. The measurements of bladder wall thicknesses were performed at four randomly chosen places at each specimen, and the average value was employed in the analyses. In the assessment of mast cell occurrence, all mast cells occurring in the whole half bladder segment (the half separated for morphological examination) were counted except for those in the serosa. P values of 0.05 or less were regarded as statistically significant. Data are presented as mean ± SEM. Graphs were generated and parameters computed using the GraphPad Prism program (GraphPad Software Inc., San Diego, USA).

3. Results

3.1. In Vitro Contractile Responses. Methacholine (5×10^{-8} – 5×10^{-3} M) evoked concentration-dependent contractions in saline- (normal and controls) and CYP-treated rats (Figure 2(a)). In the rats, which had only received saline as pretreatment (in contrast to L-NAME or 4-DAMP pretreatments), CYP-treatment (60 h before experiment) reduced the contractions. In saline-treated rats, the maximum methacholine response was 43±8 mN (4.2±0.4 mN/mg strip weight; 5×10^{-4} M; $n = 10$), whereas the maximum response was 26 ± 3 mN in CYP-treated rats (1.8 ± 0.2 mN/mg strip weight; 5×10^{-5} M; $P < 0.001$; $n = 12$; Figure 2(d)). The pEC50 values were 4.80 and 5.43 in saline pretreated and saline-treated rats and saline pretreated and CYP-treated rats, respectively (Table 1). The corresponding responses to methacholine at 5×10^{-6} M (close to EC_{50}) were 15±2 and 18±2 mN, in saline and CYP-treated animals, respectively. Administration of Krebs solution with a high concentration of potassium (124 mM) tended to evoke larger contractions in saline-treated (36 ± 5 mN; $n = 10$) than in CYP-treated rats (30 ± 4 mN; n.s.; both pretreated with saline; $n = 12$). Likewise, the ATP-evoked (5×10^{-6}–5×10^{-4} M) contractions seemed to be larger in the saline-treated bladders than in the CYP-treated (6±2 ($n = 10$) versus 4 ± 1 mN ($n = 12$); Figure 3(a)), but no statistical significance was attained. When expressed as contraction per mg of tissue, the response was significantly larger in the saline-treated than in the CYP-treated group at 5×10^{-4} M ATP (0.75 ± 0.21 and 0.22 ± 0.04 nN/mg, respectively; $P < 0.01$, Figure 3(d)). The adenosine-evoked (5×10^{-5} M) relaxations to precontracted (K^+; 50 mM) strip preparations were significantly larger in the saline-treated bladders than in the CYP-treated ones (−2.4 ± 0.1 ($n = 10$) versus −0.7 ± 0.1 ($n = 12$) mN; −0.26 ± 0.04 versus −0.04 ± 0.01 mN/mg; $P < 0.001$; Figure 4).

Pretreatment with the muscarinic receptor antagonist 4-DAMP (1 mg/kg ip) for five days (saline/CYP 60 h before experiment) did not change the relation between the maximum responses in the two groups of rats (maximum response in saline- and CYP-treated: 32 ± 2 ($n = 8$) and 24 ± 3 ($n = 10$) mN, respectively; $P < 0.05$; Figure 2(b)). At methacholine concentrations less than 10^{-4} M, the CYP-treated preparations seemed to be somewhat more sensitive than the saline-treated. However, no statistical significance was attained. The responses to 5×10^{-6} M of methacholine were 4 ± 2 and 12 ± 1 mN, respectively. Also the contractile responses to ATP showed great resemblance after the 4-DAMP pretreatment (2.8 ± 0.6 ($n = 8$) versus 2.4 ± 0.4 ($n = 10$) mN; n.s.; Figure 3(b)), while the adenosine relaxations were still different (−1.3 ± 0.1 ($n = 8$) versus −0.5 ± 0.1 ($n = 10$) mN; $P < 0.01$; Figure 4). However, pretreatment with the NOS inhibitor L-NAME (30 mg/kg ip) normalized the cholinergic contractile response in the CYP-treated animals (Figure 2(c)). Almost identical contractile responses to methacholine appeared in the two groups (maximum response in saline- and CYP-treated: 31 ± 4 and 34 ± 2 mN, respectively; n.s.; $n = 8$ in each group). At 5×10^{-6} M, the response to methacholine was even significantly larger in the CYP-treated rats than in control (20 ± 2 and 11 ± 1 mN, respectively; $P < 0.05$). Also, the Hill coefficients were close to 1 in both groups (Table 1). No significant differences occurred regarding the ATP contractions after the L-NAME pretreatments (2.5±0.4 versus 3.8±0.6 mN; n.s.; $n = 8$ in each group; Figure 3(c)). The adenosine-evoked relaxations were almost identical in the saline- and CYP-treated bladders after L-NAME pretreatment (−1.3 ± 0.3 versus −1.2 ± 0.2 mN; n.s.; $n = 8$; Figure 4).

The responses to potassium (124 mM) were still smaller, if anything, in the 4-DAMP pretreated CYP-treated rats than in the saline-treated (29 ± 2 ($n = 8$) versus 23 ± 4 ($n = 10$) mN; n.s.). In the L-NAME pretreated rats, the response in the saline-treated group was 25 ± 3 mN and in the CYP-treated group 26 ± 2 mN ($n = 8$ in each group; n.s.). The comparison of the overall maximum contractile responses to methacholine showed no significant differences within neither the saline-treated groups (43 ± 8 ($n = 10$), 32 ± 2 ($n = 8$) and 31 ± 4 ($n = 8$) mN; saline, 4-DAMP and L-NAME pretreatment, resp.) nor the CYP-treated groups (26 ± 3 ($n = 12$), 24 ± 3 ($n = 10$) and 34 ± 2 ($n = 8$) mN, saline, 4-DAMP and L-NAME pretreatment, resp.), although the methacholine responses tended to be smaller after 4-DAMP and L-NAME pretreatments in saline-treated rats, and to be larger in the L-NAME pretreated group in CYP-treated rats. Neither did the overall maximum contractile responses to ATP differ significantly in the saline-treated group (5.5 ± 1.8 ($n = 10$), 2.5±0.6 ($n = 8$) and 2.2±0.5 ($n = 8$) mN; saline, 4-DAMP and L-NAME pre-treatment, resp.) nor did they differ in the CYP-treated (4.0 ± 1.0 ($n = 12$), 2.4 ± 0.6 ($n = 10$) and 3.8 ± 0.6 ($n = 8$) mN; saline, 4-DAMP and L-NAME pre-treatment, resp.) groups. However, the adenosine relaxations were significantly larger in the saline-treated and saline pretreated group than

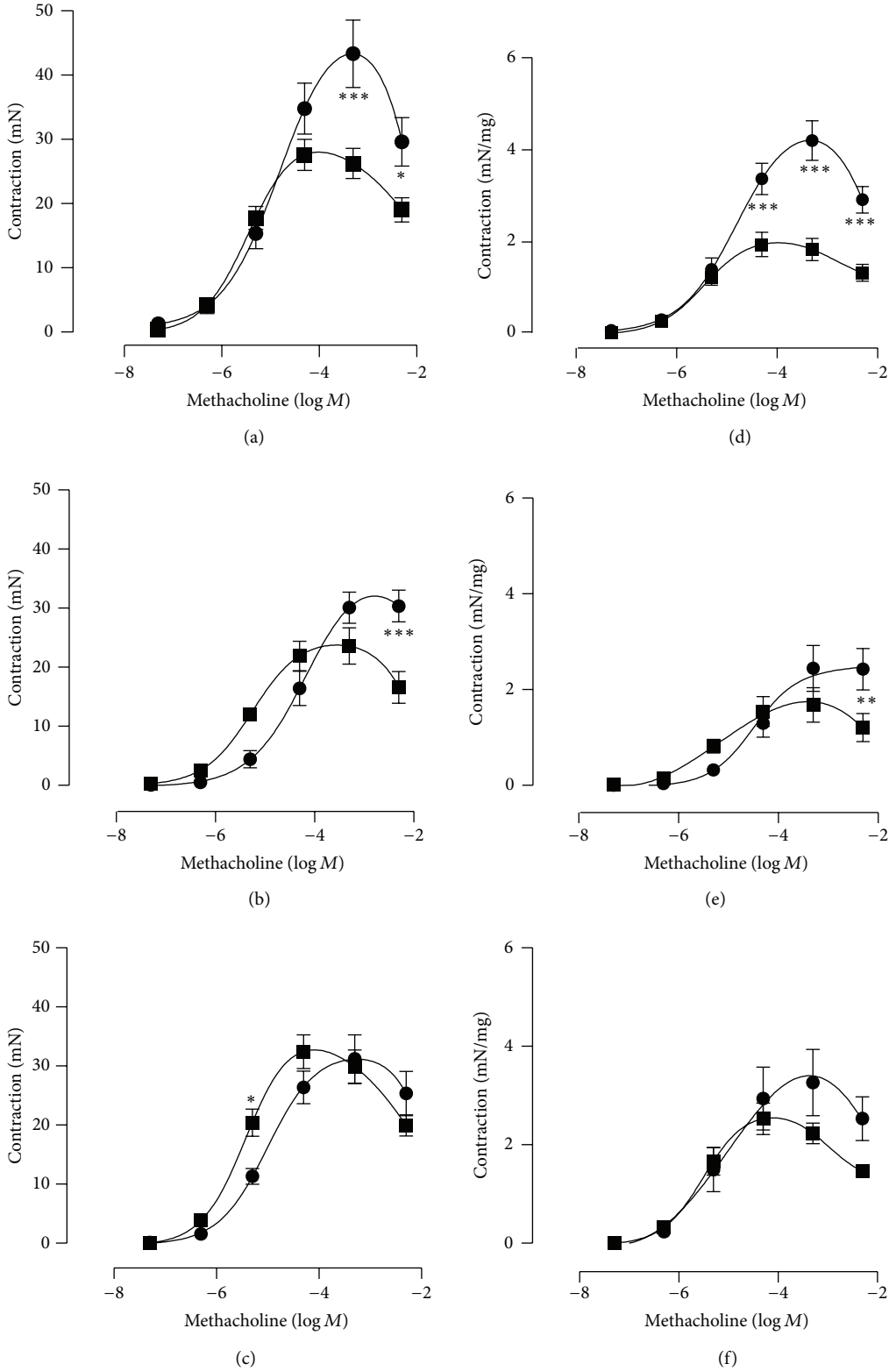

Figure 2: Mean contractions to methacholine of isolated urinary bladder strip preparations from control (saline-treated; ●) rats and rats with CYP-induced cystitis (■), pretreated with saline ((a), (d); $n = 10$ and 12), 4-DAMP ((b), (e); $n = 8$ and 10) or L-NAME ((c), (f); $n = 8$ in each group). The left column ((a)–(c)) expresses data as absolute values (mN) and the right column ((d)–(f)) as mN/mg. The vertical bars represent the SEM. * denotes $P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

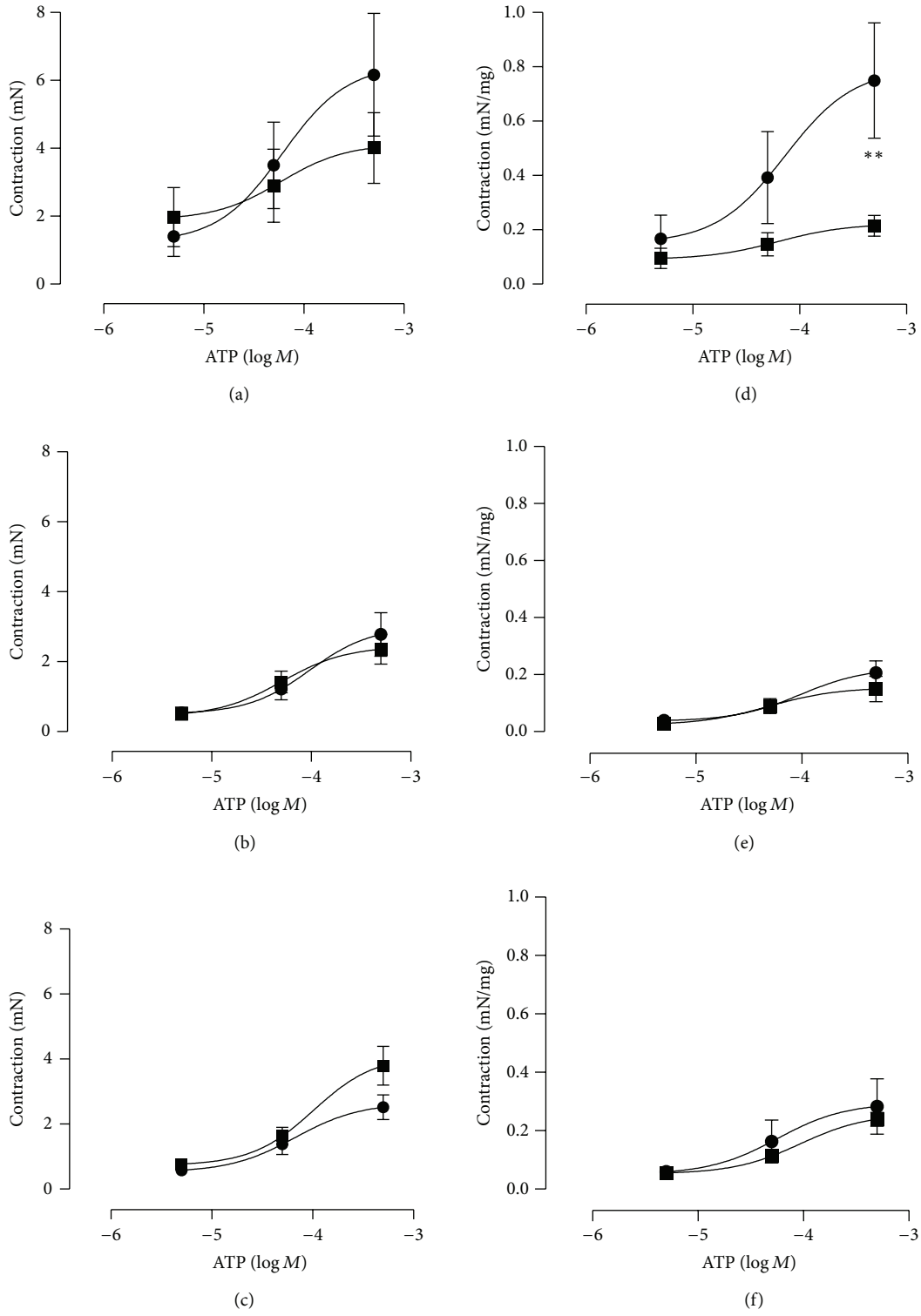

Figure 3: Mean contractions to ATP of isolated urinary bladder strip preparations from control (saline-treated; ●) rats and rats with CYP-induced cystitis (■), pretreated with saline ((a), (d); $n = 10$ and 12), 4-DAMP ((b), (e); $n = 8$ and 10) or L-NAME ((c), (f); $n = 8$ in each group). The left column ((a)–(c)) expresses data as absolute values (mN) and the right column ((d)–(f)) as mN/mg. The vertical bars represent the SEM. ** denotes $P < 0.01$.

Table 1: Methacholine concentration-response curve characteristics. Nonlinear analyses according to bell-shaped response-curves.

Treatment	n_H (first part of biphasic curve)	log EC_{50}	R^2	Curve differences (F, P)
Saline-saline	0.74	−4.80	0.71	4.38, 0.001
Saline-CYP	0.89	−5.43	0.75	
4-DAMP-saline	0.75	−4.13	0.85	7.26, <0.0001
4-DAMP-CYP	0.89	−5.25	0.64	
L-NAME-saline	0.91	−5.00	0.79	2.55, 0.035
L-NAME-CYP	0.97	−5.41	0.83	

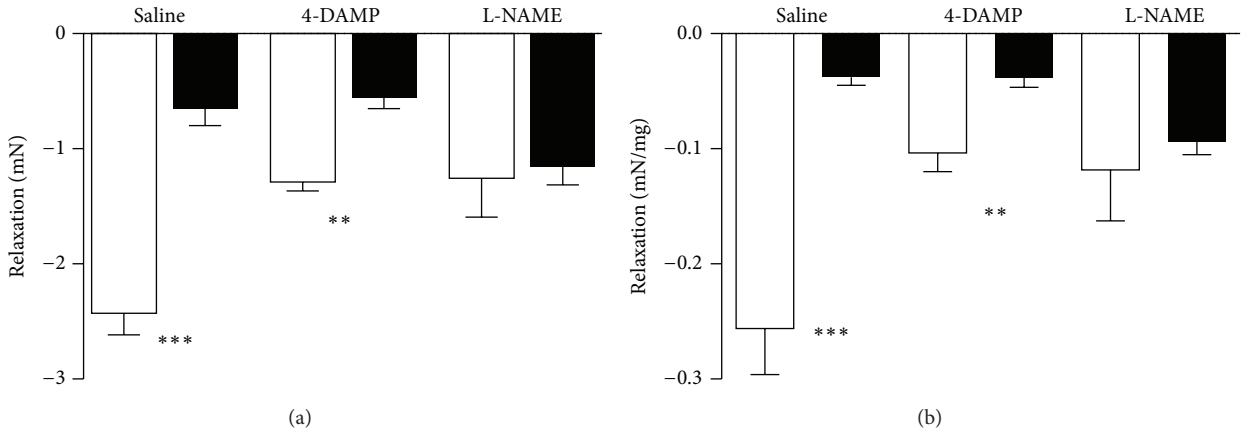

Figure 4: Mean relaxations to adenosine (5×10^{-5} M) of precontracted (K^+; 50 mM) isolated urinary bladder strip preparations from control (saline-treated; white) rats and rats with CYP-induced cystitis (black), pretreated with saline (n = 10 and 12), 4-DAMP (n = 8 and 10), or L-NAME (n = 8 in each group). (a) expresses data as absolute values (mN) and (b) as mN/mg. The vertical bars represent the SEM. ** denotes $P < 0.01$ and *** $P < 0.001$.

in the 4-DAMP and L-NAME pretreated groups ($P < 0.01$ (4-DAMP) and $P < 0.001$ (L-NAME)). The corresponding responses in the CYP-pretreated groups were not significantly different.

3.2. Morphological Examinations. The inflamed bladder specimens showed a dramatic thickening of the bladder wall in comparison to the normal, saline-treated specimens. In urinary bladders from saline pretreated and saline-treated rats, the total wall thickness was 1.2 ± 0.3 mm, while in the corresponding CYP-treated group the wall thickness was 3.3 ± 0.5 mm ($P < 0.05$; $n = 5$; Figure 5(a)). Particularly, the urothelium/mucosa was enlarged, which is reflected by the mucosa versus total wall thickness ratio demonstrated in Figure 5(b) (20.4 ± 2.0 versus 42.6 ± 2.2% in saline-treated and CYP-treated groups, respectively; $P < 0.001$; $n = 5$). Also, the urothelium was less folded in the bladders from CYP-treated rats (Figure 6). In the muscularis layer, more stromal structures appeared and the muscle seemed less well organized. Both 4-DAMP and L-NAME possibly induced a slight bladder thickening in the saline-treated animals, however, statistical significance was not attained. No effects by the 4-DAMP and L-NAME pretreatments were observed on the total bladder wall thickness in the CYP-treated animals. However, the L-NAME pretreatment caused the CYP-treatment to induce less thickening of the mucosa (42.6 ± 2.2 versus 26.8 ± 4.1%; $P < 0.01$; $n = 5$; Figure 5(b)). Also the 4-DAMP pretreatment may possibly had prevented some of the urothelial/mucosal enlargement (34.1±3.3%; n.s.; $n = 5$).

3.3. Mast Cell Infiltration. No differences in the total number of mast cells in the half bladder specimen (the assessment included the mucosa and the muscularis layers and not the serosa/adventitia) could be detected between the groups, but the variations between the individual rats were large (17–71 mast cells). However, the mast cells were differently distributed in the bladders of saline- and CYP-treated rats. In the saline-treated rats, only few mast cells occurred in the muscularis layer, which was reflected by the ratio of the number of mast cells in the muscularis expressed as percentage of the total number of mast cells in the bladder half. In the CYP-treated rats, there was a 4.5-fold increase of the ratio in comparison to saline-treated rats (32±5 versus 7± 3%, $P < 0.01$; Table 2, Figure 5(c)). In the 4-DAMP pretreated animals, the ratio was even higher in the CYP-treated rats (6.5-fold; $P < 0.001$). However, the L-NAME pretreatment eliminated the difference in mast cell occurrence between saline- and CYP-treated rats.

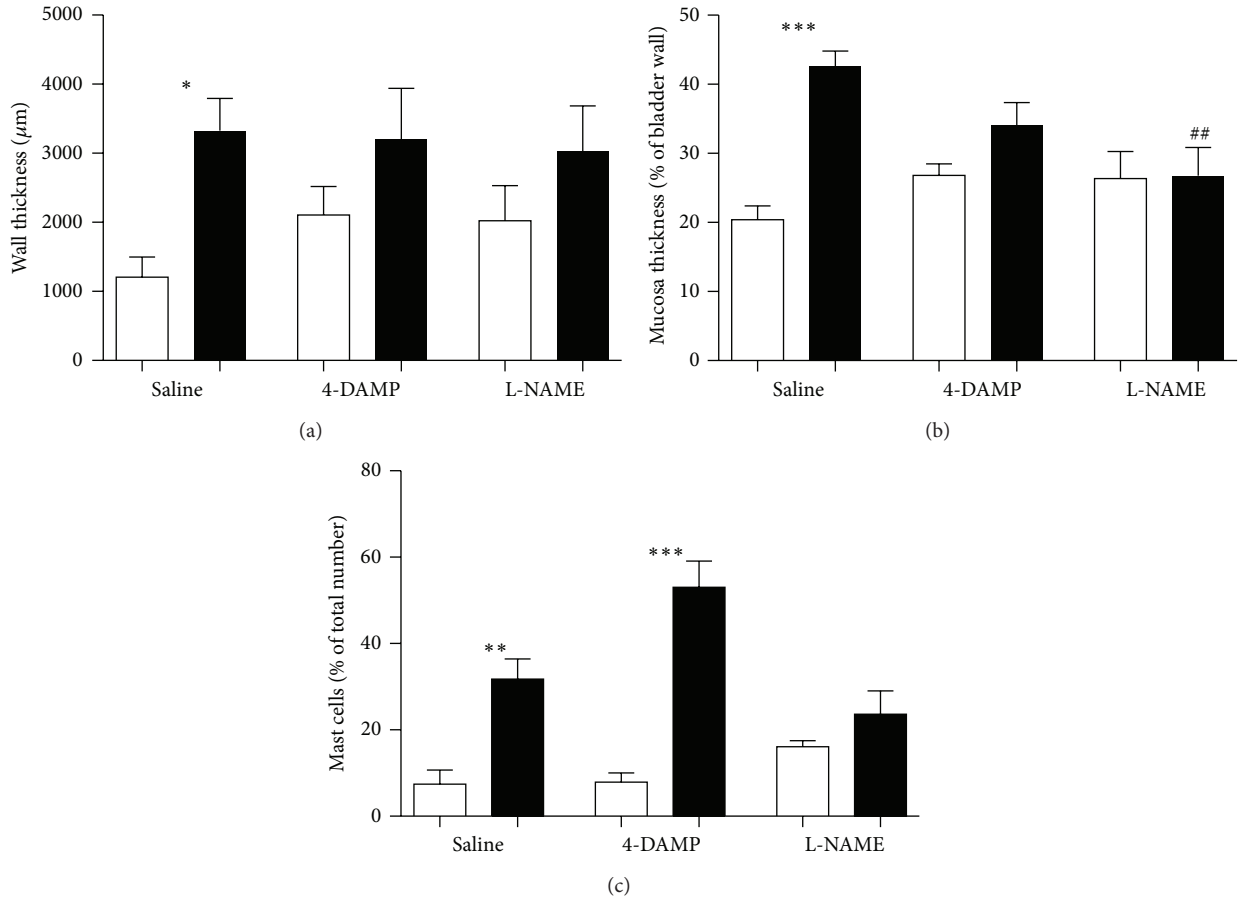

Figure 5: Bladder wall thickness (a), mucosa thickness (b), and ratio of mast cell occurrence in the detrusor (c), expressed as % of total number of mast cells ($n = 3$ in all groups) in saline-treated (white) and CYP-treated (black) rats. The vertical bars represent the SEM. * denotes $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ (comparisons between saline- and CYP-treated groups). ## denotes $P < 0.01$ (comparison with saline pretreated and CYP-treated group).

Table 2: Receptor expression and mast cell occurrence as judged by muscarinic M5 receptor-like, P1A1 purinoceptor-like and MIF-like staining in the detrusor muscle in saline and CYP-treated rats that had received saline, 4-DAMP, or L-NAME pretreatment ($n = 5$ in each group). 0, (+), +, ++ refer to none (or almost none), possibly occurring, occurring, and obvious, respectively.

	Muscarinic M5 receptor-like staining	P1A1 purinoceptor-like staining	MIF-like staining
Saline-saline	+	+	(+)
Saline-CYP	++	(+)	++
4-DAMP-saline	+	+	(+)
4-DAMP-CYP	++	(+)	++
L-NAME-saline	+	+	(+)
L-NAME-CYP	(+)	+/(+)	+

3.4. Immunohistochemistry. In rats that had received saline pretreatment, the mucosal muscarinic M5 receptor-like staining was obvious in CYP-treated rats (Table 2, Figure 6). The specimens of saline pretreated and saline-treated rats showed little staining for muscarinic M5 receptor-like reactivity. Pretreatment with 4-DAMP did not affect the expression, neither in the normal nor in the cystitis group. However, pretreatment with L-NAME eliminated the difference in mucosal muscarinic M5 receptor-like expression between the saline- and CYP-treated groups. P1A1 purinoceptor-like expression showed little variance between saline-treated and CYP-treated bladders but tended to be less in the CYP-treated if anything. While 4-DAMP pretreatment had no effect on the expression, the L-NAME pretreatment may possibly have prevented some part of the CYP-induced P1A1 purinoceptor-like expression decrease. MIF-like staining occurred in both CYP-treated and saline-treated rats, which also showed a substantial variation within each groups. The staining seemed

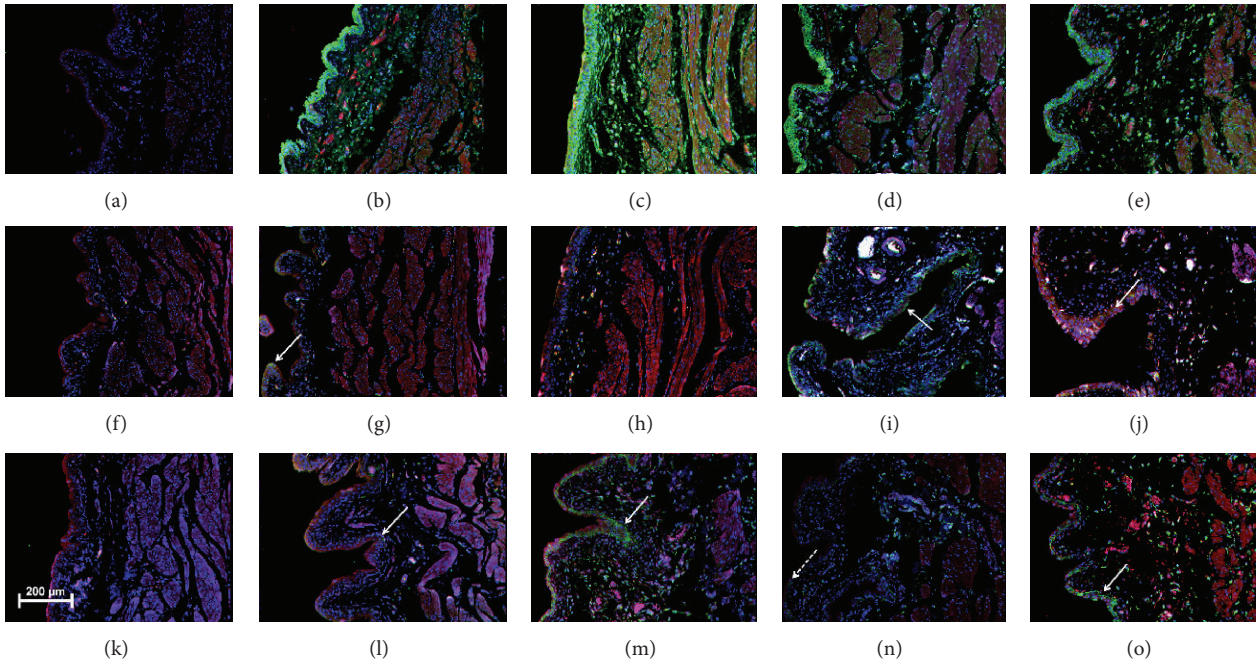

Figure 6: Representative images of immunohistochemical muscarinic M5 receptor-like (upper row; (a), (b), (c), (d), and (e)), P1A1 purinoceptor-like (middle row; (f), (g), (h), (i), and (j); solid arrows indicate staining) and MIF-like (lower row; (k), (l), (m), (n), and (o); solid arrows indicate staining, dashed arrow indicates possibly occurring staining) staining. Left columns show saline pretreated and saline treated (control), negative ((a), (f), (k)) and positive staining ((b), (g), and (l)). The middle column ((c), (h), and (m)) shows saline pretreated and CYP treated and the second column from the right ((d), (i), and (n)) shows L-NAME pretreated and saline treated bladders. The right column ((e), (j), and (o)) shows staining from L-NAME pretreated and CYP treated bladders. The horizontal bar represents 200 μm for all images. All sections are counterstained with DAPI (blue) and phalloidin (red). Green color represents positive staining.

to occur more superficially in the urothelium in the tissue from CYP-treated rats. Neither 4-DAMP nor L-NAME pretreatment seemed to affect the MIF-like expression.

4. Discussion

Noxious stimuli to a tissue induce a battery of effects in which mediation of a number of different signaling molecules participates. In CYP-induced cystitis, urothelial NO and ATP play pivotal roles [24, 34, 35]. While the focus for the former has been in the aspect of inflammation progression, the latter has been discussed in relation to sensory mechanism. Consequently, it may be of clinical relevance to determine if inhibition of NOS generally impedes the induction of cystitis or just affects some parts of the inflammation. Effects of similar experimental approaches as being employed currently, that is, NOS inhibition in parallel with CYP treatment, have been reported previously in the literature [36, 37]. These studies, in which no functional parameter has been assessed, show that pretreatment with a NOS inhibitor prevents increased CYP-induced extravasation, bladder weight, and plasma nitrite-nitrate levels. In the mentioned studies, the NOS inhibition was administered only a few hours before the CYP treatment. In our pilot experiments, trying to determine suitable doses, the pretreatment was administered close to the CYP injection. However, no effect could be then seen on the CYP-induced functional alterations. Consequently, a longer pretreatment dosage regime was employed. Anyhow, the differences in the results of the pretreatment indicate that inflammatory changes do not all have the same sensitivity towards pharmacological modulation of the NO system.

The results of the current study are in accordance with what is mentioned in the introduction. Namely that, in the rat, concomitantly to a mucosal NOS upregulation, muscarinic M5 receptors are upregulated 60 hours after the CYP treatment [6], which has been suggested to be involved in the urothelial/mucosal production of NO. Noteworthy, even though this is not the only source for NO production [38], it seems to dominate the muscarinic coupled release [14]. Furthermore, P1A1 purinoceptors, suggested to exert proinflammatory effects, show pronounced plasticity and, based on experiments on urothelial cell lines, seem to be downregulated during the course of inflammation [39]. This phenomenon is supported by observations made in the rat, in which P1A1 purinoceptors in the urinary bladder are downregulated 60 h after CYP treatment [32]. As also mentioned above, muscarinic receptor-induced contractions to methacholine are reduced after CYP treatment, and, further, the contractile impairment is greater than what could be explained by a muscular insufficiency only [40]. However, the major part of the contractile decrement depends on a general alteration of the tissue responsiveness.

Before the effectuation of the current study, the observations mentioned above led us to a tentative standpoint

for how the bladder inflammatory key molecules interact. Namely, an increased formation and release of purines and of adenosine in particular enhance an inflammatory defense including stimulation of the release of acetylcholine of nonneuronal origin [15]. The acetylcholine induces an upregulation of NOS and the release of NO that aggravates the condition. During the course of inflammation, the P1A1 purinoceptors are downregulated, which may seem logical in view of protecting the tissue from negative effects of an exaggerated inflammatory response. The inflammatory alterations cause functional disturbances, which may be counteracted by muscarinic antagonists or NOS inhibitors [6], as well as by P1A1 purinoceptor antagonist [31]. But is this the only effect of the substances? Can they even prevent the development of the condition into an aggravated state?

In the current study, the results of the pretreatment with the NOS inhibitor were clear-cut regarding functional responses, showing that pretreatment with L-NAME tended to normalize the responses in the CYP-treated animals. This may be particularly obvious when considering the responses close to the EC_{50}. In the case of 4-DAMP, a dose of 1 mg/kg was used, which possibly may have been too low since the blockade of muscarinic receptors had minor or no direct effect on the inflammatory process. However, in similar experiments doses of other antagonists within this range have proven to be effective [31]. Also, the general reduction of methacholine-evoked responses may be an effect of a remaining 4-DAMP blockade, suggesting a sufficient dose. Both pretreatments (4-DAMP and L-NAME) seemed to reduce the contractility in the saline-treated animals, which was particularly obvious when analyzing the ATP contractile responses. However, ATP responses seem to be less robust than methacholine-evoked responses. One reason to this is of course the magnitude of the ATP contractile responses, which is substantially smaller than the methacholine-evoked contractions. Nevertheless, in spite of the general pretreatment decrementing effect, the contractions in the L-NAME pretreatment group that had been CYP-treated seemed to respond better than any other CYP-treated group did. However, comparisons between the pretreatment groups should be made with caution. This is because saline and CYP treatments, rather than antagonist pretreatments, were performed in parallel; thus, comparisons between saline and CYP treated animals are more relevant. Generally, the ATP-evoked contractions tended to be reduced in the saline-treated rats, but not in the CYP-treated ones (particularly not in the L-NAME pretreated). Nevertheless, the reduction in the saline-treated bladders thwarts the interpretation of the data. Also, the adenosine relaxations were smaller in the 4-DAMP and L-NAME pretreated preparations in the saline-treated group. However, the L-NAME pretreatment revealed the same overall pattern for adenosine-evoked relaxations as for methacholine-evoked contractions.

The assumption of the upstream urothelial muscarinic M5 receptor regulation of NO inflammatory effects now has to be rephrased. The reported effect on NO mechanisms by muscarinic receptor blockade, probably occur in the acute micturition situation. In the course of inflammation, the muscarinic receptor mechanism seems to be of less importance. Part of the stimulus for NO formation has to be searched for elsewhere. Importantly, recent studies confirm that increased levels of NOS are of great importance in the development of cystitis, regardless of experimental procedure, making this enzyme a fundamentally important pharmacological target [41]. It has also previously been demonstrated that interferon gamma, among other factors, can increase the expression of inducible NOS in the rat, which may constitute a nonmuscarinic NOS stimulation [42]. The morphological signs in the current study of the cystitis included an upregulation of muscarinic M5 receptors and possibly a downregulation of P1A1 purinoceptors, which confirm previous findings [6, 32, 40]. In agreement with the effects of the pretreatment on the functional responses, 4-DAMP pretreatment had no effect on receptor expression, while L-NAME pretreatment prevented the upregulation of muscarinic M5 receptors and possibly the downregulation of the P1A1 purinoceptors in cystitis.

The morphological correlations to the functional changes were also sought for in bladder wall changes, since one reason for the general alteration of the bladder tissue responsiveness in the CYP-treated rats may be found in the disorganization of the smooth muscle layer. Concerning the total thickness, a small increase might have occurred by the 4-DAMP and L-NAME pretreatments in the saline-treated animals, while no effect of the pretreatments could be observed in the CYP-treated animals. However, the examination of mucosal thickness revealed that L-NAME pretreatment prevented CYP-induced changes. In this context it is interesting to consider the small, but not significant, reduction that may have occurred in the CYP-treated animals that were pretreated with 4-DAMP. Namely, as mentioned above, it has previously been demonstrated that there exists a link between muscarinic receptor stimulation and NO effects in the CYP-treated rat [6], which may play some minor role even for structural inflammatory effects. The present results thus support an upstream muscarinic receptor regulation of NO production to be important for some inflammatory changes in the bladder in the rat.

In order to evaluate how severe the CYP-induced cystitis may be, the occurrence of mast cells in the detrusor muscle was counted. The number of mast cells was fairly the same outside the detrusor smooth muscle layer in the different pretreatment groups. In the healthy rat bladder almost no mast cells occurred in the muscle layer, whereas in the CYP-treated rats, a small, but definite number appeared in the muscle layer. This corresponds to what has been reported in BPS/IC in humans [43]. The effects of the pretreatment with 4-DAMP and L-NAME showed the same pattern on the mast cell distribution as it did on functional responses and on receptor expression. That is, while 4-DAMP had no effect, L-NAME eliminated the mast cell appearance in the detrusor muscle in cystitis.

In contrast to the L-NAME effect on function, muscarinic M5 receptor expression, P1A1 purinoceptor expression, mast cell detrusor occurrence, and mucosal enlargement, L-NAME did not affect the MIF expression. It showed an identical occurrence after 4-DAMP and L-NAME pretreatments as after saline pre-treatment both in the saline and in

the CYP groups. This underlines that NO cannot be the only mediator of CYP-induced changes. Other key molecules in the pathogenesis are purines, in particular adenosine [31]. Obviously and rather expectedly the pathogenesis is orchestrated by a number of participating molecules of which NO is only one.

5. Conclusions

The current study shows that pretreatment with a NOS inhibitor reduces many of the changes that are induced by CYP-treatment. A muscarinic receptor antagonist, on the other hand, seems to have little effect on the development of the CYP-induced cystitis in the rat. While NO is involved both in the cystitis development and in the direct function impairment, the urothelial muscarinic receptors that via NO regulate part of the hampered bladder contractility during CYP-induced cystitis seem to be less important in the long-term (indirect) regulation of function and morphology during cystitis. Thus, while there exists an urothelial muscarinic receptor-NO coupling in the control of function in cystitis [40], this coupling seems to be of minor importance in the regulation of inflammation development in the rat urinary bladder. In general, the data indicate that urothelial factors, such as NO, affect not only the sensory signaling, but also mucosal morphology as well as detrusor contractility.

Key of Definitions for Abbreviations

4-DAMP:	4-diphenylacetoxy-N-methylpiperidine methiodide
ATP:	Adenosine-5′-triphosphate
BPS:	Bladder pain syndrome
CYP:	Cyclophosphamide
IC:	Interstitial cystitis
L-NAME:	N_ω-nitro-L-arginine methyl ester
NO:	Nitric oxide
NOS:	Nitric oxide synthase.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

ATP during Early Bladder Stretch Is Important for Urgency in Detrusor Overactivity Patients

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ATP is an important mediator of urgency in women with detrusor overactivity (DO). In order to understand how different degrees of bladder stretch elicited ATP release in DO patients compared with controls, sequential aliquots were collected during cystometry and ATP release was measured at each degree of bladder filling, in female patients with DO and controls. In both DO and control groups, ATP release was induced during bladder filling, suggesting that stretch stimulated further ATP release. However, the luminal ATP concentrations were already high at early filling stage (200 mL), which was even greater than those at the later filling stages (400 mL and maximum cystometric capacity, MCC), indicating that a substantial ATP release has been induced during early filling (200 mL) in both DO and controls. In DO, ATP release at 200 mL was significantly higher in those with low first desire to void (FDV) (≤200 mL) than in those with higher FDV (>200 mL); this may suggest that ATP release at early stretch may play an important role in urgency (early sensation) in DO. ATP concentrations remained unchanged after voiding, suggesting that voiding did not further induce ATP release into intraluminal fluid.

1. Introduction

The urothelium, which was once regarded as an inert, protective barrier, plays a key role in bladder sensory and motor functions [1]. The hypothesis that the purine nucleotide ATP is a neurotransmitter responsible for nonadrenergic, noncholinergic neurotransmission was proposed in 1972 and termed purinergic signalling [2]. More recently, both animal and human studies show that nonneuronal ATP is released from the bladder urothelium in response to stretch [3–6]. Besides urothelial cells, cultured pig myofibroblasts were also reported to release ATP in response to stretch [3]. In rat bladder, intravesical application of ATP has been shown to induce detrusor overactivity [7].

Urothelial derived mediators, such as acetylcholine and ATP, signal the sensation of bladder fullness to the central nervous system, thus triggering the micturition reflex [8]. Such mediators act upon specific receptors such as P2X receptors located on suburothelial afferent nerves [9], with possible involvement of myofibroblasts [10]. In addition to stretch, ATP is also released from animal bladder urothelial cells and tissue strips in response to capsaicin and acid [3, 5]. In human subjects, OAB was shown to be associated with increased ATP release [11, 12].

Previous studies have explored the hypothesis that ATP in bladder luminal fluid could be a marker for the sensation of "urgency" as experienced by patients with overactive bladder symptoms [13, 14]. An inverse correlation between the intravesical concentration of ATP and bladder volume at FDV has previously been reported in two groups: patients with urodynamically proven DO [13] and patients with clinical OAB and low FDV [14]. However, the mechanism responsible for this was not elucidated.

Stretch is considered to be a major stimulus for ATP release in the bladder and it was hypothesized therefore that more ATP should be released as the bladder fills, with the

highest ATP levels occurring at the end of the filling stage in accordance with murine studies which showed increased ATP release with increased intravesical pressure [15]. However, to our knowledge there are no reports indicating exactly when ATP is released during human bladder filling or how ATP release is influenced by the degree of bladder stretch. Therefore, we performed cystometry in women, collecting consecutive samples of bladder fluid at 200 mL and 400 mL and MCC for sequential ATP measurement. We recruited control patients and those with DO in order to characterise and compare the pattern of sequential ATP release in these two groups.

2. Materials and Methods

2.1. Cystometry. Each female patient was catheterised to empty the bladder completely for routine clinical cystometry [16], with the exception that dual lumen catheters were employed. A CSU was sent for culture and patients with proven bacterial cystitis ("UTI" 10^8 cfu/L with pyuria >10/HPF) were excluded from subsequent analysis. Female patients gave informed consent in accordance with approval from the South Eastern Sydney human research ethics committee (SESIAHS HREC 06/11).

Saline at room temperature (approximately 25°C) was infused into the bladder at a filling rate of 75 mL/min (medium-fill) [16] as described previously [13]. As per routine, volumes were noted when the patient felt FDV and reached maximum cystometric capacity (MCC), (the volume at which the patient feels she can no longer delay micturition [16]), when filling was stopped. Tap water stimuli and erect provocation were routinely performed. At the end of cystometry, the patient voided the filling solution into a clean uroflow chamber, in private. The presence of any detrusor contractions during filling, or provocation such as the sound of running tap water or changing supine to erect position, was noted.

Patients were characterised as (a) idiopathic DO, with involuntary detrusor contractions during the filling phase which may be spontaneous or provoked, or (b) control, that is, pure urodynamic stress incontinence, involuntary leakage of urine during increased abdominal pressure in the absence of detrusor contractions, and no symptoms of urgency. Patients with the following were excluded: (a) bladder pain syndrome (FDV < 200 mL with bladder pain, and MCC < 400 mL), (b) voiding dysfunction (detrusor pressure (Pdet) at maximal flow greater than 60 cm H_2O), (c) incomplete voiding (postvoid residual volume > 100 mL), or (d) evidence of spinal cord or central neurological disease.

2.2. Sampling and ATP Measurements. When the filling volume reached 200 mL, 400 mL, and MCC, the infusion was paused for sample collection. These volumes were chosen as fixed values that approximate the commonly observed filling volumes for first desire to void and maximal capacity in patients with DO (see discussion). At each volume, the first 10 mL in the collecting catheter ("deadspace") was aspirated and discarded; then, a further 5 mL of intravesical filling fluid was collected. After voiding, fluid from the uroflow chamber was collected (known as "voided volume," VV). Each woman thus yielded four samples for ATP assay. Immediately after collection, samples were transported on ice from the clinic to the laboratory in the adjacent building and then ATP assayed immediately.

ATP concentration (in nM) was measured in duplicate per sample, using a GloMax 20/20 luminometer and a bioluminescence assay (Sigma). A standard curve was constructed using freshly made standards (10^{-5} to 10^{-10} M and blank) from frozen concentrated ATP stock. The total amount of ATP (in nmoles) contained in each sample was then calculated by multiplying the filling volume (200 mL, 400 mL, MCC, or VV) by its corresponding ATP concentration.

2.3. Statistical Analysis. ATP data were not normally distributed ($P < 0.05$, D'Agostino & Pearson omnibus normality test) and were expressed as median (interquartile range, IQR). Nonparametric statistical analyses were performed using GraphPad Prism 6.0 software (San Diego, USA). Comparison of ATP levels in the different samples was performed by Friedman ANOVA with Dunn's multiple comparisons test (Figures 1 and 4) or using a Mann-Whitney test when two unmatched groups were compared (Figure 2) or a Wilcoxon test when matched groups were compared (Figure 3). Methods, definitions, and units conform to the standards recommended by the International Continence Society except where specifically noted.

3. Results and Discussions

3.1. Stretching Is a Stimulate to Induce Further ATP Release in Both DO and Controls. Although ATP release is induced by urothelial stretch during bladder filling, it is unclear whether stretch-induced ATP is involved in triggering bladder contraction, either at physiological maturation or during pathological conditions such as DO, and the pattern of ATP release in human bladder and its association with bladder sensation have not been clearly understood. Traditional animal models cannot provide this information, because of the small bladder volume in rats and mice and the inability to assess the sensations of bladder filling in the rodent. Results from *in vitro* laboratory studies reporting ATP release from human bladder urothelium/lamina propria strips have shown a very marked increase in stretch-induced ATP release in DO, compared with control [12]. Using urodynamic fluid to investigate ATP release has become a valuable tool to answer these questions under true physiological condition relevant to patients.

To investigate how bladder stretch could induce ATP release, we also chose to collect samples at volumes of 200 mL, which approximates the FDV in most patients (median FDV in DO 167 mL and control 200 mL, Table 1), and a filling volume of 400 mL, which is close to the MCC (median MCC 430 mL) in most DO patients, in addition to sampling at MCC and in voided fluid. We compared ATP release in these consecutive intraluminal fluid samples from each patient. These intraluminal fluid samples have been generated during

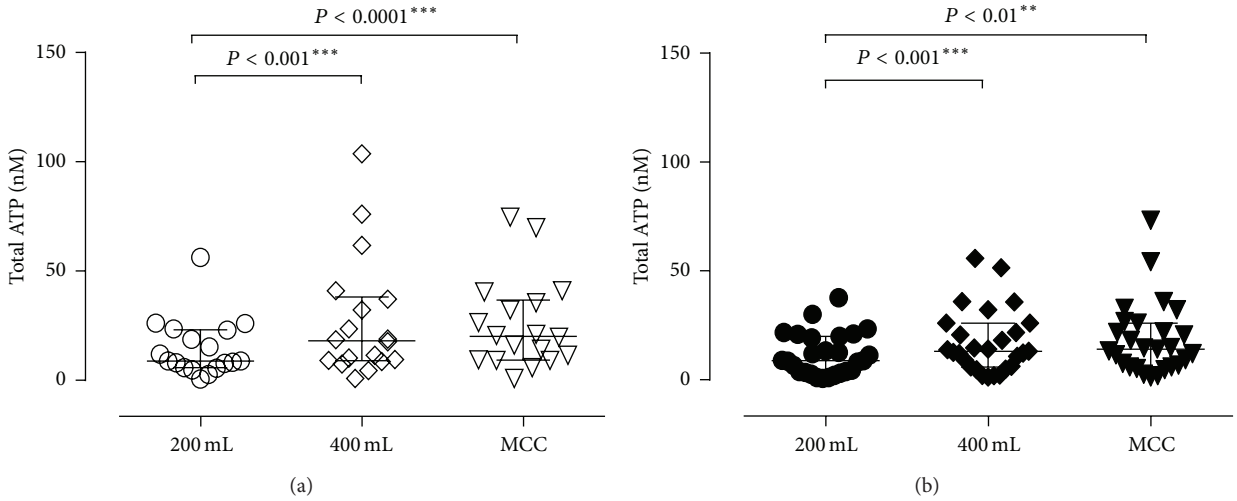

Figure 1: Comparison of total ATP (nmoles) in bladder fluid collected at volumes of 200 mL and 400 mL and at MCC, in control ($n = 18$) (a) and DO ($n = 27$) (b). ATP content significantly increased between filling volumes of 200 mL and 400 mL and between 200 mL and MCC. Data were analyzed by the Friedman test followed by Dunn's multiple comparison test. ($^{**} = P < 0.01$; $^{***} = P < 0.001$).

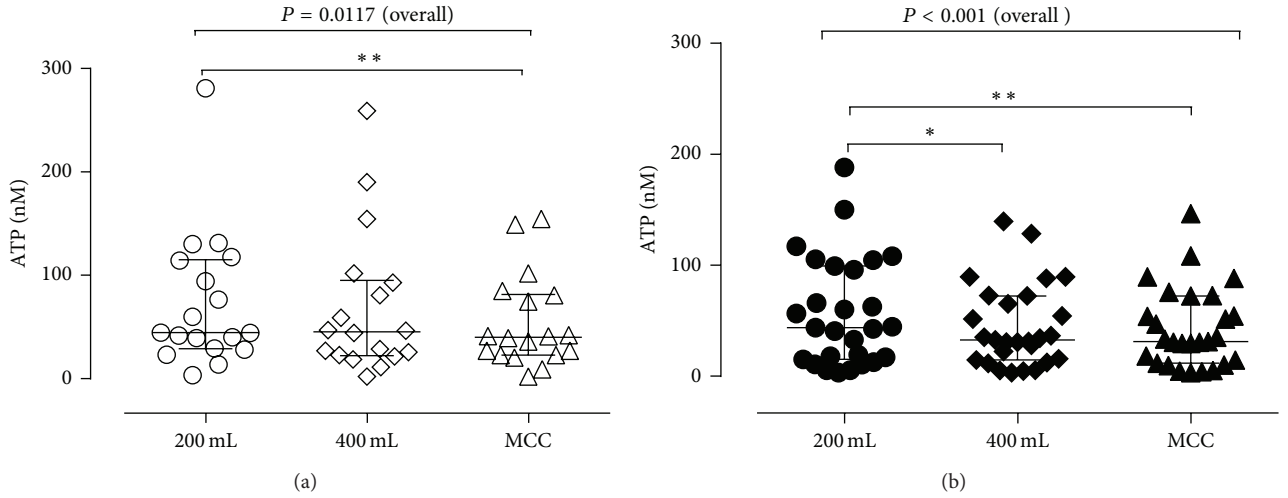

Figure 2: Comparison of ATP concentrations in bladder fluid collected at volumes of 200 mL and 400 mL and at MCC for control ($n = 18$) (a) and DO ($n = 27$) (b) patients. In this and subsequent figures, data points are from individual patients, showing median and interquartile range. Data were analyzed by the Friedman test followed by Dunn's multiple comparison test.

Table 1: Patient urodynamic characteristics.

	Control (median (IQR))	DO (median (IQR))
n	18	27
Age (years)	60.5 (47–70)	59 (54–66)
FDV (mL)	200 (170–265)	167 (100–277)
MCC (mL)	492 (450–500)	430 (400–500)
*Max Pdet during filling (cm H_2O)	6.5 (5–9.25)	33 (17–46)
Detrusor contractions during cystometry	0	27
VV (mL)	485 (415–532.5)	417 (380–500)

*Maximal detrusor pressure during filling or provocation.

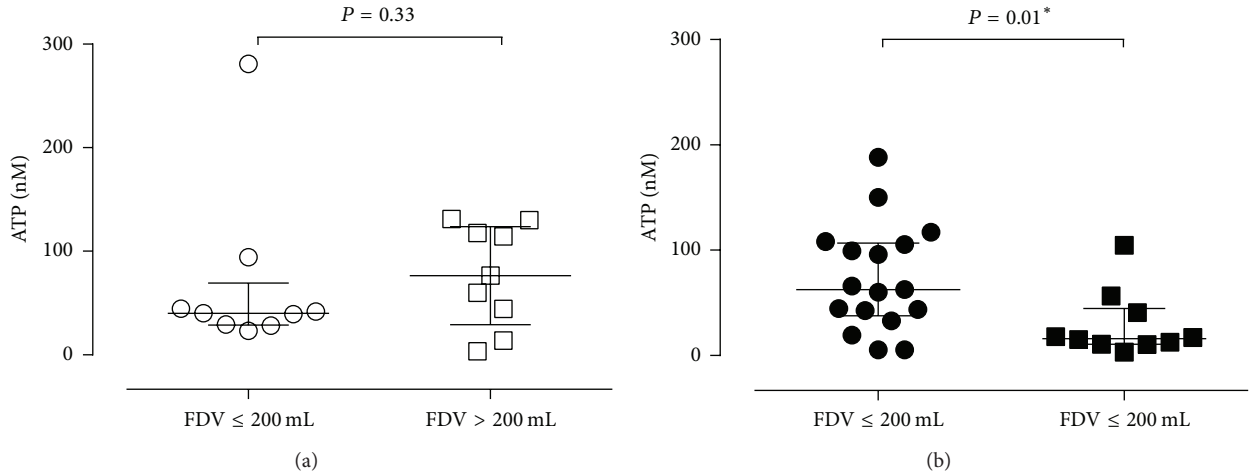

Figure 3: Comparison of ATP release at a bladder volume of 200 mL. For control (a) and DO patients (b), the data have been divided into two groups of low (≤200 mL) and higher FDV (>200 mL). The two control groups of low ($n = 9$) and high ($n = 9$) FDV showed no significant difference ($P = 0.33$), whereas there was a significantly higher concentration of ATP in the DO group with low FDV ($n = 17$), compared to the DO group with higher FDV ($n = 10$) ($P = 0.01$, Mann-Whitney test).

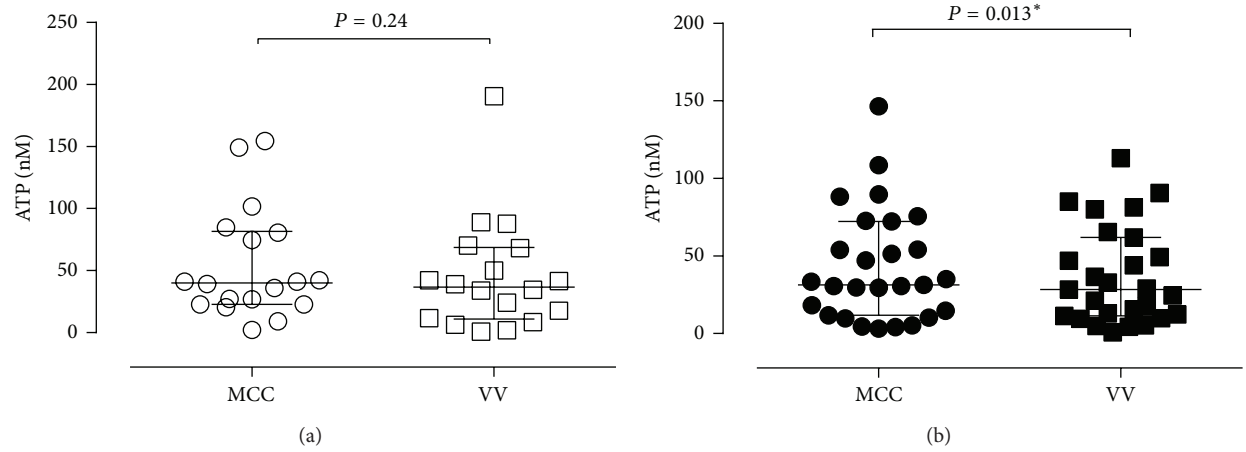

Figure 4: Comparison of ATP concentration before and after voiding in (a) control ($n = 18$) and (b) DO patients ($n = 27$). For control patients, there was no difference in ATP concentration of urodynamic fluid before MCC or after voiding (VV). For DO patients, there was a significant decrease in ATP concentration in VV compared with MCC ($P = 0.01$, Wilcoxon test).

routine urodynamic testing under standard clinical conditions which occurs at a supraphysiological filling rate (75 mL/min) [16].

Cystometry with measurement of ATP at the four filling volumes was performed on 53 women (age 28–87 yrs). Eight subjects with UTI on the test day and 2 subjects with voiding dysfunction were excluded, yielding 27 patients with DO and 18 control patients (Table 1).

The total amount of ATP (in nmoles) at each bladder volume was examined. For both control (Figure 1(a)) and DO (Figure 1(b)), there was a significant increase in the total amount of ATP, in nmoles, between 200 mL and 400 mL, as well as between 200 mL and MCC; the total amount of ATP in the intraluminal fluid (in nmoles) was the lowest at the lowest bladder filling volume (total ATP median values (in nmoles) at 200 mL, 400 mL, and MCC are 8.8, 13.1, and 14, for DO, and 8.9, 18.1, and 20.2, for controls). Note that ATP was released continuously throughout the urodynamic test in both DO and control and that the total ATP content in the intraluminal fluid increased during filling, that is, from 200 mL to 400 mL, which agreed with the initial hypothesis that ATP release was induced by bladder stretch in both DO and controls.

We have also compared the difference in ATP release between DO and control groups at each filling/sampling point, but we did not see any significant difference between the two groups. One of the reasons for this could be the variations in ATP release in individuals, which made it too hard to see the difference between two groups. This is not

a surprise as there was no significant difference in ATP release between DO and control patients with a much larger sample size in a previous study [13].

3.2. Substantial ATP Released at the Early Bladder Filling Stage. We also compared the ATP concentrations according to the filling volumes and determined that in both control (Figure 2(a)) and DO (Figure 2(b)) patients, there was a significant difference in ATP concentration at the three different bladder volumes (Friedman test). Overall, in both control and DO patients the ATP concentration was higher at the early filling stage (200 mL, median ATP 44.5 nM control; 43.8 nM DO) compared with later stages (MCC, median ATP 40.2 control; 31.3.7 nM DO).

This is a surprising and important finding. This indicates that there was substantial ATP release at early filling volumes in both DO and controls, and early ATP release may play an important role in bladder function and dysfunction, that is, early sensation or urgency for DO.

3.3. Early ATP Concentration Is Related to the First Desire Volume (FDV) in DO Patients. In the DO patients, the median FDV was 167 mL, with values ranging between 85 and 400 mL. In order to investigate whether FDV was associated with ATP release, patients were divided into two groups based on low FDV (≤200 mL) and higher FDV (>200 mL). In control patients, no difference in ATP concentration in intravesical fluid at 200 mL was seen between the two groups (FDV less than 200 mL (ATP median = 40.2 nM) and FDV more than 200 mL (ATP median = 76.5 nM)) (Figure 3(a)). However, in DO patients, the concentration of ATP in intravesical fluid at 200 mL was significantly higher in patients with FDV ≤ 200 mL than those with FDV > 200 mL (Figure 3(b)). The same analysis was applied to the data sets from the 400 mL, MCC, and VV samples, but no association was shown between FDV and ATP release at these volumes (data not shown).

The fact that DO patients with a lower FDV (≤200 mL) had higher ATP concentrations (ATP median 62.7 nM) at an earlier bladder volume, compared with DO patients who had a higher FDV (>200 mL) (ATP median 16.1 nM), suggests that the early ATP release may play an important role in DO patients, which may cause urgency. The previous clinical studies have also found that ATP in voided urodynamic fluid was inversely correlated with the FDV in DO [13] and OAB [14] patients, but not controls. This current study supports the supposition that it is the early ATP release which is important to the sensation of urgency in DO patients.

Although ATP can be degraded by membrane-bound ectoATPases, studies have shown that there is little ectoATPase activity associated with the apical surface of the urothelium [17]; thus the intravesical ATP measured may reflect the ATP released from the urothelial cells. Moreover, the source of the ATP is assumed to be from the urothelial cells, but this was not able to be determined from our *in vivo* study. The ATP in bladder intraluminal fluid might be released directly from urothelial cells into the lumen and/or could diffuse into the lumen from various sources within the bladder wall, such as from the suburothelial myofibroblasts. Diffusion into the lumen from suburothelial cells would be limited by the intact bladder urothelium and also by the ectoATPase activity associated with intermediate and basal urothelial cells and also with the lamina propria [17]. However, a reduced ectoATPase activity in laboratory samples of OAB has been reported [18]; thus, more ATP may diffuse into deep sites of bladder wall, enhancing stretch-induced signalling pathways inducing urgency or involuntary contractions. These findings may explain why the early ATP release plays an important role in early sensation (urgency) in DO but not in controls.

3.4. Voiding Did Not Further Induce ATP Release. Previous studies have reported that ATP plays a role in the bladder as a purinergic neurotransmitter in functional motor [6] as well as sensory bladder disorders [12]. Thus ATP might also appear in the bladder as a result of parasympathetic nerve stimulation to the detrusor during voiding [19].

In order to investigate whether a micturition contraction might increase ATP release in the bladder, we compared the ATP concentration before voiding (MCC) and after voiding (VV). The results showed no significant difference in ATP concentration between MCC (ATP median = 40.2 nM) and voided volumes (ATP median = 36.8 nM) in control patients (Figure 4(a)); that is, ATP content remained unchanged after voiding. In DO patients (Figure 4(b)), there was no evidence of any increase in ATP release and in fact the ATP concentration was slightly lower in voided fluid (VV) (ATP median = 28.4 nM) than in bladder intraluminal fluid at MCC (ATP median = 31.3 nM) ($P = 0.01$, Wilcoxon test). This suggests that the voiding contraction does not contribute further to intravesical ATP release in bladder lumen.

This is not surprising as ATP released from motor nerves will be deep in the detrusor muscle layer and unlikely to reach the bladder lumen due to abundant ecto-ATPase in lamina propria and smooth muscle [17]. Instead of increased ATP after voiding, we have noticed a decreased intravesical ATP after voiding in DO patients in our study. It is possible that, during micturition, the intravesical ATP is exposed to ectoATPase located on exfoliated urothelial cells [20] and, therefore, ATP may undergo enhanced degradation during or after voiding.

4. Conclusions

For the first time ATP release during bladder filling has been investigated in sequential intraluminal fluid samples in female patients with or without DO. This study has shown that total amount of ATP in intraluminal fluid (in nmoles) continued to increase from lower bladder volume (200 mL) to higher bladder volume (400 mL and MCC) in both DO and controls. The study demonstrates that stretch (bladder filling) can induce further ATP release in human bladder. Interestingly, we have noticed that there was a substantial ATP release in intraluminal fluid in the early filling stage, that is, bladder filling volume of 200 mL. The results also show that ATP release at 200 mL filling volume is related to FDV only in DO patients but this relationship was not observed in the control group.

These data indicate that ATP release at early bladder filling may play an important role in increased bladder sensations, for example, urgency, in DO patients.

Abbreviations

ATP: Adenosine triphosphate
CSU: Catheter specimen of urine
DO: Detrusor overactivity
FDV: First desire to void
MCC: Maximal cystometric capacity
Pdet: Maximal detrusor pressure
VV: Voided volume.

Conflict of Interests

All authors have no conflict of interests regarding this paper.

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Research Article

Luminal DMSO: Effects on Detrusor and Urothelial/Lamina Propria Function

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DMSO is used as a treatment for interstitial cystitis and this study examined the effects of luminal DMSO treatment on bladder function and histology. Porcine bladder was incubated without (controls) or with DMSO (50%) applied to the luminal surface and the release of ATP, acetylcholine, and LDH assessed during incubation and in tissues strips after DMSO incubation. Luminally applied DMSO caused ATP, Ach, and LDH release from the urothelial surface during treatment, with loss of urothelial layers also evident histologically. In strips of urothelium/lamina propria from DMSO pretreated bladders the release of both ATP and Ach was depressed, while contractile responses to carbachol were enhanced. Detrusor muscle contractile responses to carbachol were not affected by DMSO pretreatment, but neurogenic responses to electrical field stimulation were enhanced. The presence of an intact urothelium/lamina propria inhibited detrusor contraction to carbachol by 53% and this inhibition was significantly reduced in DMSO pretreated tissues. Detection of LDH in the treatment medium suggests that DMSO permeabilised urothelial membranes causing leakage of cytosolic contents including ATP and Ach rather than enhancing release of these mediators. The increase in contractile response and high levels of ATP are consistent with initial flare up in IC/PBS symptoms after DMSO treatment.

1. Introduction

Interstitial cystitis or painful bladder syndrome (IC/PBS) is a chronic inflammatory bladder disease characterised by bladder or pelvic pain and irritative voiding symptoms in the absence of infection or other pathology [1]. However symptoms are variable and often include urinary urgency and frequency and painful urination [2]. A range of oral and intravesical therapies are available for IC/PBS; however, reliable evidence from well-designed clinical studies is generally lacking [3, 4].

Dimethyl sulfoxide (DMSO) has been used since the 1960s to treat the symptoms of IC/PBS [5]. Despite limited clinical trial data, Rimso-50 which is a 50% (v/v) solution of DMSO is used to relieve pain and reduce the inflammation and voiding symptoms observed in patients with this condition [3, 4]. The exact mechanism of action of DMSO is not fully understood; however, it is reported to cause mucosal damage and have analgesic, anti-inflammatory, bacteriostatic, and muscle relaxant properties [3, 6, 7]. Borzelleca et al. reported that 50% DMSO causes desquamation of the urothelium without altering the lamina propria [8]. Recently, studies have identified that DMSO directly affects cellular phospholipid membranes, with DMSO molecules found to occupy positions just below the lipid head groups acting as a spacer increasing average lateral distance favouring the entrance of water into the cell. Higher concentrations of DMSO directly increase lateral expansion of the cellular bilayer and have been known to cause destruction of cellular lipid bilayers [9, 10]. Patients commencing treatment with DMSO often experience an initial flare up of symptoms, which usually subsides after 2 weeks [3].

DMSO is highly permeable and will come into contact with the urothelium but possibly also the underlying lamina propria, detrusor smooth muscle, and the nerves innervating the bladder wall. The urothelium forms a barrier, protecting underlying nerves and muscle from contents of the urine; however, the urothelium and lamina propria also play important roles in bladder sensation, with the urothelium releasing a number of mediators including ATP, acetylcholine (Ach), prostaglandin E2 (PGE2), nitric oxide (NO) [11], and an unidentified diffusible substance known as urothelial-derived

inhibitory factor (UDIF) [12]. These mediators are involved in modulating sensory nerve activity and also detrusor function. DMSO has been reported to depress nitric oxide release from efferent nerves [13].

All 5 muscarinic subtypes are expressed in the human urothelium and stimulation of these receptors in the urothelium releases ATP, NO, and UDIF [14, 15]. Bladder stretch during filling triggers ATP release from the urothelium and also subepithelial myofibroblasts, and this ATP is believed to be the source of primary excitation of the bladder afferents by acting on the P2X receptors [16–18]. The urothelial $P2X_{2/3}$ receptors have been implicated in the sensory role involved with micturition and also nociception in pathological states [19, 20]. Myelinated Aδ afferent fibres are believed to be involved in the nonpainful micturition reflex, whereas high threshold unmyelinated C afferent fibres are activated in painful, pathological conditions.

Intravesical dimethylsulfoxide (DMSO) is used for the treatment of interstitial cystitis/painful bladder syndrome (IC/PBS) although patients initially experience a flare up of symptoms on commencement of treatment. Little is known regarding the effects of treatment on bladder function or the cause of the initial worsening of symptoms. As the urothelium comes into contact with the highest concentrations of DMSO during intravesical administration, changes in urothelial function may be involved in the drugs therapeutic actions but also initial worsening of symptoms. The aim of this study was therefore to investigate possible changes in urothelial/lamina propria and detrusor function using an *in vitro* model to simulate intravesical DMSO treatment.

2. Materials and Methods

2.1. Drugs, Chemicals, and Reagents. Carbachol (carbamylcholine chloride), adenosine triphosphate (ATP), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (Castle Hill, New South Wales, Australia). Solutions were prepared in deionised water and further diluted in Krebs-bicarbonate solution.

2.2. Luminal Treatment of Porcine Bladder with DMSO. Fresh bladders from Large White-Landrace pigs (6 months old, 80 kg) were obtained from a local abattoir and immediately immersed in cold Krebs-bicarbonate solution (composition in mM: NaCl 118, $NaHCO_3$ 24.9, $CaCl_2$ 1.9, $MgSO_4$ 1.15, KCl 4.7, KH_2PO_4 1.15, and D-glucose 11.7). The bladders were opened longitudinally and sheets of full thickness anterior wall from the dome region were set up in a bath where separated gassed (5% CO_2/95% O_2) solutions bathed the luminal and serosal surfaces (see Figure 1), allowing dimethyl sulfoxide (DMSO) to be administered to the luminal surface only, with Krebs-bicarbonate solution bathing the serosal surface. The tissues were incubated at 37°C for 15 min with a therapeutic concentration (50% v/v) of DMSO applied to the luminal surface. Control bladders were incubated for 15 min without the addition of DMSO. Incubation media were collected immediately after the 15-minute incubation to measure release of ATP, Ach, and lactate dehydrogenase (LDH) from the luminal surfaces during the treatment. After the incubation (control or DMSO), tissue strips were then isolated and set up under 1 g resting tension in organ baths containing Krebs-bicarbonate solution at 37°C to allow for the examination of tissue responses. Four sets of tissues were examined:

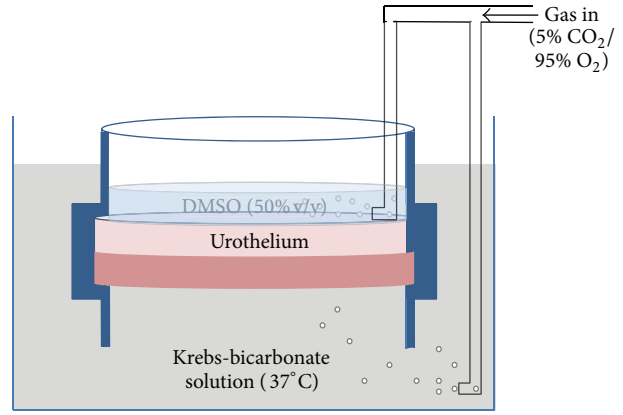

Figure 1: Schematic figure of the incubation chamber. Full thickness sheets of bladder dome were sandwiched between two separated bathing solutions, with each containing gassed (5% CO_2/95% O_2) Krebs-bicarbonate (serosal) or DMSO (urothelial) solution at 37°C. Tissues were incubated with DMSO (50% v/v) applied to the luminal side only for 15 min before isolation of the various tissues for pharmacological analysis.

(i) full thickness bladder wall with an intact urothelium and lamina propria;

(ii) denuded detrusor strips with the urothelium and lamina propria removed;

(iii) strips of urothelium and lamina propria for recording of tissue contraction;

(iv) strips of urothelium and lamina propria for the measurement of stretch-induced ATP and acetylcholine release.

2.3. Functional Organ Bath Studies. To assess the effects of DMSO on tissue responsiveness, contractile response to ATP (1 mM) and cumulative concentration-response curves to the muscarinic receptor agonist carbachol (1 nM–10 μM) were obtained on tissues (i), (ii), and (iii). Isometric contractions of isolated tissue strips were recorded using a Powerlab data acquisition system (ADInstruments).

To investigate the effects of DMSO on nerve mediated responses, detrusor strips denuded of urothelium and lamina propria (tissues (ii)) were set up under 1 g resting tension in organ baths and electrically field stimulated via silver electrodes placed either side of the tissue. Tissues were stimulated at 1, 5, 10, and 20 Hz using 5 s trains of pulses (20 v, 0.5 ms pulse-width) delivered every 100 s. Contractile responses and the release of mediators for tissues from DMSO pretreatment bladders were compared with those of tissues from control incubated bladders.

Tissues (iv) were used to examine the effects of DMSO on basal and stretch-induced release of mediators from the urothelium/lamina propria. These tissues were washed and 2 min later a sample of the bathing medium was collected and frozen for later assay of mediators (basal release). The tissues were then stretched, increasing length by 50%, and the bathing medium again was collected and frozen for assay of mediators (stimulated release).

2.4. Measurement of ATP, Ach, and LDH. ATP was measured using a luciferase-luciferin assay kit (Molecular Probes) according to the manufacturer's instructions. Luminescence was measured using a Modulus microplate reader (Promega). Acetylcholine was measured using a fluorescence-based Amplex Red Acetylcholine Assay kit (Molecular Probes) according to the manufacturer's protocol. Fluorescence was measured on a Modulus Microplate reader (Ex. 540/Em. 590 nm). Leakage of LDH into the incubation media was measured using LDH Cytotoxicity assay (Cayman Chemicals). Absorbance was measured on a Modulus Microplate reader (490 nm).

2.5. Bladder Histology. Sections of control and DMSO pretreated intact bladder dome were fixed (4% neutral buffered formalin), processed, and embedded in paraffin. Tissues were sectioned at 6 μm and placed on uncharged slides. Sections were stained using haematoxylin and eosin to assess urothelial integrity and examined using an Olympus CX31 microscope (Olympus Australia Pty. Ltd.) equipped with an Infinity 2 camera and Infinity Capture software. Image J software was used to measure relative urothelial thickness in control and DMSO pretreated tissues.

2.6. Data Analysis and Statistical Procedures. Mean (±SEM) increases in tension induced by carbachol or electrical field stimulation were calculated. For responses to carbachol, individual −Log EC50 (pEC50) values were determined from the concentration-response curves by the use of GraphPad Prism 5 software (SanDiego, CA) and mean (±SEM) pEC50 values and maximum responses were calculated. Similarly, for the mediator release study, mean (±SEM) concentrations were determined before and after stretch and data for DMSO and control pretreated bladders were compared. Data were analysed using a paired Student's t-test or one-way ANOVA with Dunnett's multiple comparisons test, using Graphpad InStat3 software (SanDiego, CA). Significance levels were defined as $P < 0.05$. n values represent the number of separate pig bladders examined.

3. Results

3.1. Mediator Release during Incubations. At the end of the pretreatment period the incubation medium was collected and assayed for Ach ($n = 5$) and ATP ($n = 4$). Concentrations of Ach were significantly greater than those of ATP during both control ($1.22 \pm 0.05\,\mu$M versus $0.010 \pm 0.003\,\mu$M, $P < 0.001$) and DMSO incubations ($21.3 \pm 2.94\,\mu$M versus $1.12 \pm 0.09\,\mu$M, $P < 0.001$). The presence of DMSO during the incubation produced a significant increase in the levels of both mediators (Figures 2(a) and 2(b)), Ach levels rising 17-fold ($P < 0.001$), and ATP levels rising by >100-fold ($P < 0.001$). LDH (7 mU/mL) was also detected in luminal incubation medium from DMSO treated bladders, but none was detected in matched controls.

3.2. Mediator Release from the Urothelium/Lamina Propria after Incubation. Isolated strips of urothelium/lamina propria prepared from control incubated bladders released both ATP ($n = 4$) and Ach ($n = 8$) under basal and stretch conditions. There was a significant increase in ATP release in response to stretch (Figure 2(c)) in control tissues; however, basal and stretch-induced ATP release from DMSO treated tissues were not detected. Ach was released from control urothelium/lamina propria under basal conditions with no significant increase in response to stretch. Ach release from DMSO treated tissue was significantly reduced (Figure 2(d)).

3.3. Contractile Responses following Incubation with DMSO. Luminal pretreatment of bladders with DMSO (50%) did not significantly affect detrusor, urothelial, or intact tissue response to KCl or ATP ($n = 5$, data not shown). Similarly, DMSO pretreatment did not affect subsequent responses of isolated detrusor smooth muscle strips to the muscarinic receptor agonist carbachol (Figure 3(a), $n = 4$), with both pEC50 values and maximum responses to carbachol being similar in muscle strips from DMSO and control pretreated bladders (Table 1). However responses of urothelium/lamina propria strips and the responses of intact bladder strips (detrusor plus urothelium/lamina propria, $n = 5$) to carbachol were enhanced after pretreatment with DMSO (Figures 3(b) and 3(c), Table 1).

The presence of the urothelium/lamina propria in the intact tissues significantly inhibited contractions of bladder strips (Figure 3, Table 1). This inhibition was significantly ($P < 0.05$) greater in control tissues ($53 \pm 7.8\%$) than in tissues from DMSO pretreated bladders ($33 \pm 4.1\%$).

Detrusor responses to electrical field stimulation (EFS) were frequency dependent and contractions were increased in tissues from DMSO pretreated bladders. Contractions to EFS were greater at all stimulation frequencies examined, with the differences being statistically significant for the responses at 5 Hz, 10 Hz, and 20 Hz (Figure 3(d), $n = 5$). In the presence of atropine (1 μM), detrusor contractions to EFS were depressed by $68 \pm 10\%$ at 20 Hz in control tissues ($P < 0.001$, $n = 5$). The inhibition of responses to EFS by this muscarinic antagonist was similar at all frequencies examined and was not altered significantly by DMSO pretreatment ($75 \pm 6.1\%$ inhibition at 20 Hz, $n = 5$).

3.4. Bladder Histology. Representative H and E stained sections of control and DMSO pretreated bladders (detrusor + urothelium/lamina propria) are shown (Figure 4). Typical histological features were clearly identifiable in sections of control incubated bladder, with the urothelium and lamina propria thrown into folds and overlying a deeper smooth muscle layer. However in DMSO

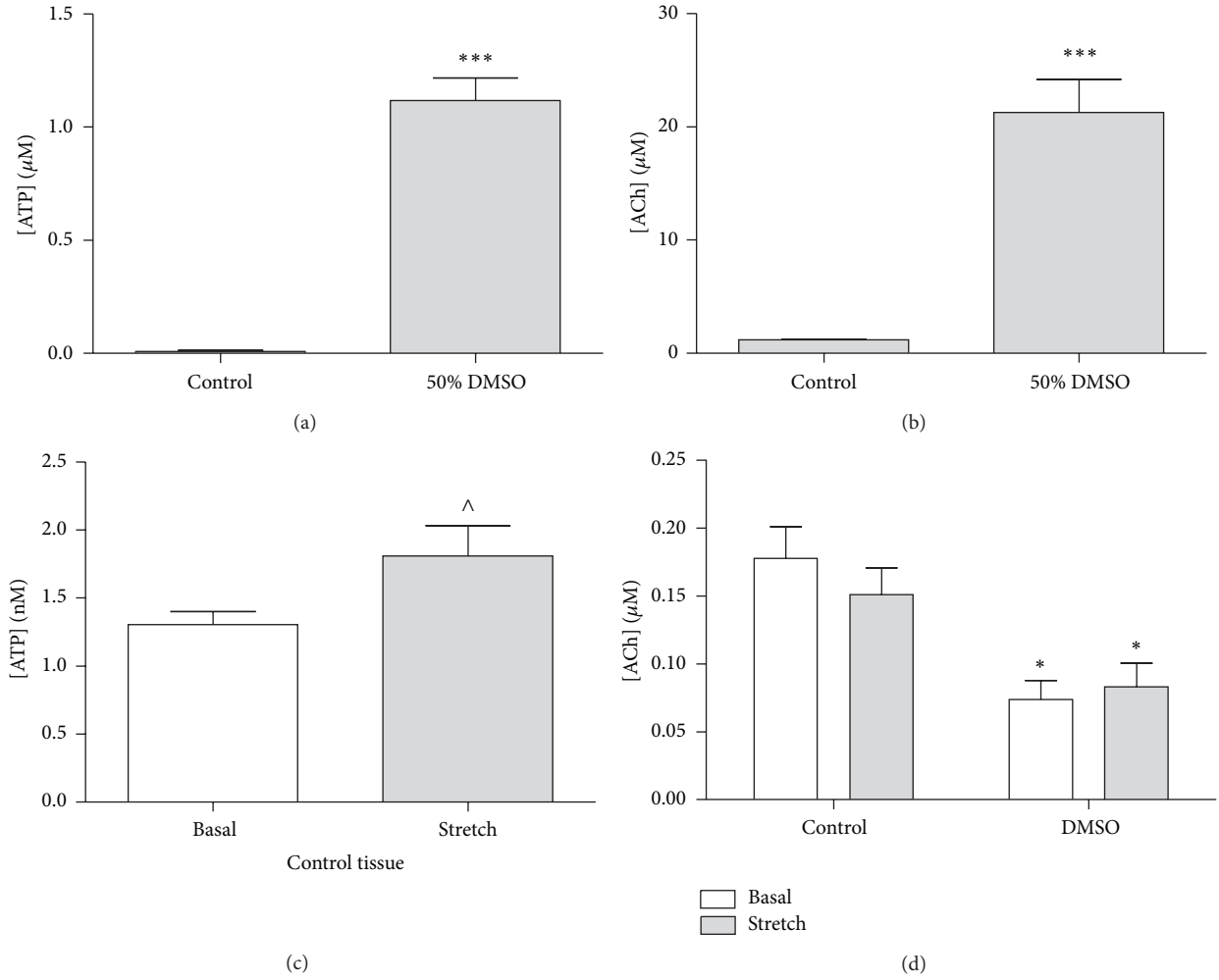

Figure 2: Effect of DMSO (50% v/v) on (a) ATP and (b) Ach release into incubation medium during bladder pretreatment and also on subsequent basal and stretch-induced release of (c) ATP and (d) Ach from strips of urothelium/lamina propria. Data represents mean ± SEM. ($^{***}P < 0.001$, DMSO pretreated versus control incubated bladders. $^{\wedge}P < 0.05$, control basal versus control stretch. $^{*}P < 0.05$, DMSO pretreated versus control incubated tissues).

pretreated tissues, damage to the luminal layers was evident. Urothelial thickness was significantly reduced from $31.1 \pm 1.48\,\mu$m in control tissues to $11.3 \pm 0.45\,\mu$m in DMSO treated tissues ($n = 15$, $P < 0.001$). In addition, the folding of the urothelium/lamina propria observed in control bladders was absent from DMSO pretreated bladders.

4. Discussion

Although the exact mechanism by which DMSO relieves symptoms associated with IC/PBS is unclear, when applied to the human skin, it penetrates rapidly and produces pharmacological effects such as anti-inflammation, analgesia, and bacteriostasis [21]. For IC/PBS, DMSO is administered intravesically and, due to the highly absorptive nature of DMSO, it is likely that not only the urothelium but also the underlying lamina propria, detrusor smooth muscle, and nerves innervating the bladder wall will be affected by DMSO. However, it is the urothelial layer that comes into direct contact with DMSO and is subject to the highest concentrations.

Treatment with DMSO is usually biweekly for 3 months and this has been found to be effective for approximately 16 to 72 months [22]. Previous studies have reported urothelial desquamation, mucosal damage, and interference of cellular phospholipid membranes to be associated with the application of DMSO [3, 6–10]. Chemical injury and subsequent loss of urothelial layers exposed to DMSO over a 3-month period may explain some of its effectiveness in treating IC/PBS as it has been reported that the removal of a diseased urothelium by laser treatment leads to nonrecurrence of pain for 6–12 months [23]. In the present study, loss of urothelial layers and mucosal folding were also detected following DMSO treatment. In addition, the detection of LDH in the luminal treatment effluent suggests that DMSO permeabilised the urothelial membranes causing leakage of cytosolic contents. The high levels of ATP and Ach detected in the treatment effluent may therefore reflect leakage of these mediators

Table 1: Mean (±SEM) maximal responses (g) and pEC50 values for carbachol on tissue strips prepared from DMSO or control pretreated bladders.

Carbachol	Urothelium/lamina propria		Detrusor smooth muscle		Intact bladder strips	
	Control	DMSO	Control	DMSO	Control	DMSO
Maximum response (g)	4.2 ± 0.28	6.6 ± 0.35***	17.8 ± 1.4	17.1 ± 0.7	8.3 ± 0.63	11.5 ± 0.94*
pEC_{50}	4.14 ± 0.09	4.4 ± 0.08	4.37 ± 0.12	4.39 ± 0.07	4.35 ± 0.14	4.45 ± 0.52

* $P < 0.05$ and *** $P < 0.001$ when comparing control versus DMSO (50% v/v).

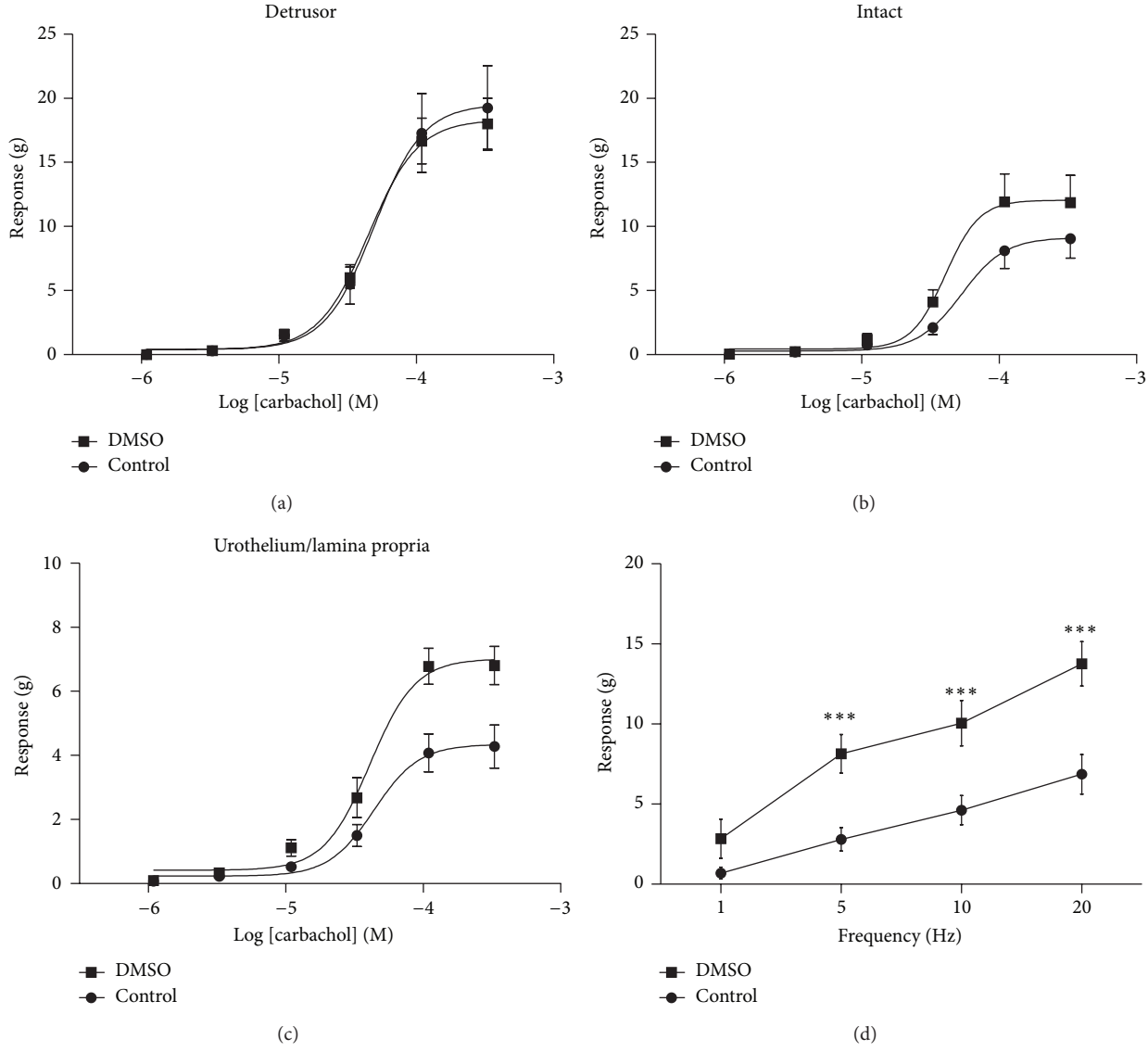

Figure 3: Cumulative carbachol concentration-response curves ($n \geq 4$) for DMSO (50% v/v) preincubated and control preincubated strips of (a) denuded detrusor, (b) intact tissue, and (c) urothelium/lamina propria. Detrusor strip responses to electrical field stimulation (d) are also shown ($n = 5$). Data represents mean ± SEM. *** $P < 0.001$ compared to responses of control incubated tissues.

from the urothelium due to physical damage rather than enhanced levels of release.

High levels of ATP and Ach were observed during luminal treatment with DMSO, and urothelial mediator release after treatment was also investigated. Following exposure to DMSO neither subsequent basal release nor stretch-induced release of ATP could be detected. Ach release was also significantly reduced following DMSO treatment. This is likely due to depletion of ATP and Ach stores during DMSO treatment and loss of urothelial cells from the mucosal surface. The inhibition of ATP release in response to stretch from urothelium treated with DMSO is consistent with

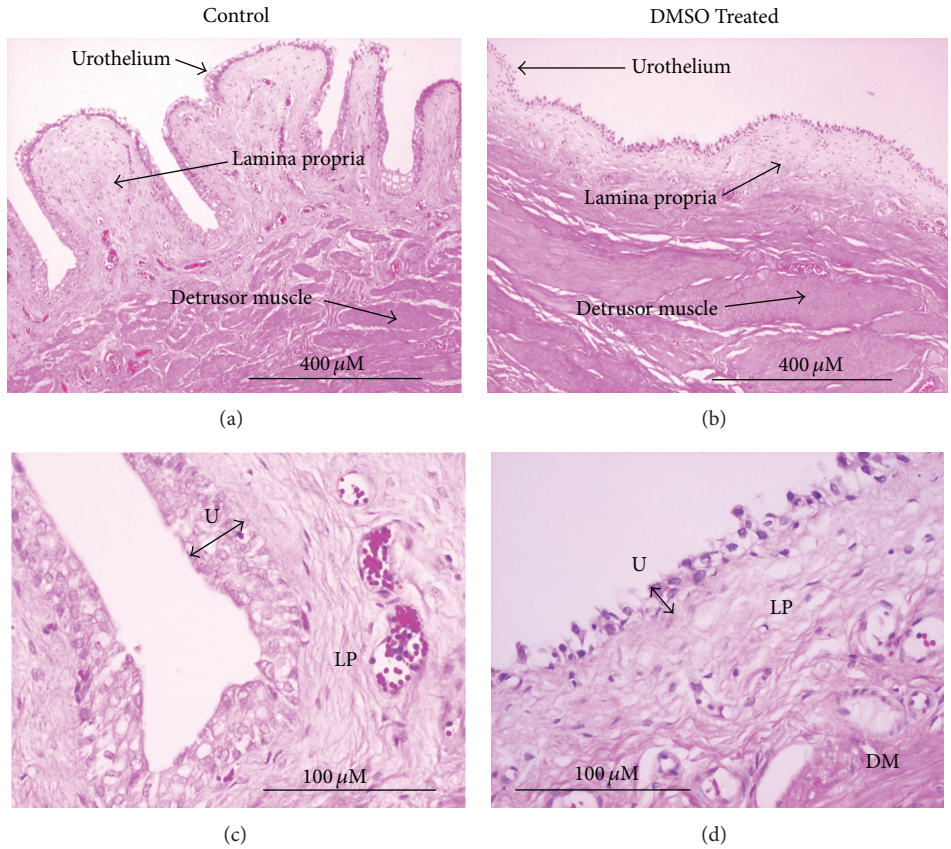

Figure 4: Hematoxylin and eosin (H and E) staining of control (a) and (c) and DMSO pretreated (b) and (d) bladders. H and E staining at (a) and (b) 10x and (c) and (d) 40x.

previous reports which have identified a significant decrease in ATP release from the urothelium treated with DMSO in response to stretch [24].

Augmented release of urothelial ATP and changes to urothelial purinergic P2X and P2Y receptor profiles are common features of patients suffering from IC/PBS [14]. It is well established that ATP, released in response to stretch, acts on afferent nerve $P2X_{2/3}$ receptors, playing a sensory role in the micturition cycle and also nociception in pathological states [20]. Therefore, lower levels of urothelial ATP release after treatment with DMSO may be beneficial in correcting the augmented ATP release and the enhanced afferent nerve activity observed in IC/PBS.

It has been reported that in the porcine and human bladder the urothelium/lamina propria releases a factor that inhibits detrusor contraction [12, 25]. This inhibitory effect of the urothelium was evident in tissues from both control and DMSO pretreated bladders, but the inhibitory effect was significantly reduced following DMSO pretreatment. The consequences of this change are unknown, but a similar reduction in inhibitory mechanisms has been observed in the human neurogenic overactive bladder [26]. Thus the observed reduction in urothelial inhibition of the detrusor may contribute to the bladder overactivity observed after DMSO treatment. Surprisingly urothelial contractile responses to carbachol were enhanced following DMSO pretreatment. It has been suggested that this activity is responsible for correct folding of the urothelium on bladder emptying [27] or contractile activity of this layer may drive detrusor contraction [28] but the clinical relevance of this urothelial activity is currently unknown.

Detrusor responses to carbachol were not affected by DMSO pretreatment suggesting that only lower concentrations of DMSO permeate to these deeper tissues. However detrusor responses to EFS were altered by pretreatment which indicates that the parasympathetic nerves in this tissue are more sensitive to the actions of DMSO than the detrusor muscle itself. Unexpectedly, responses to EFS were enhanced by DMSO pretreatment. Since detrusor responses to exogenous carbachol were unchanged, this suggests that DMSO increases neurotransmitter release to EFS. The enhanced responses may be the result of DMSO causing damage to the nerve terminals which may ease when depletion of these stores has been completed. This is consistent with previous research which also noted acute reflex firing of pelvic nerve efferent axons in response to DMSO [13]. Atropine inhibited responses to EFS similarly in control and DMSO pretreated tissues, suggesting that the cholinergic contribution to neurotransmission did not change following treatment.

The acute administration of DMSO has been found to cause irritation [29] and the associated pain is reportedly caused by mast cell degranulation, in response to chemical

injury which eases on depletion [30]. The increase in contractile responses and high levels of ATP are consistent with initial flare up in IC/PBS symptoms after DMSO treatment.

In conclusion, this study demonstrates a physical and functional disruption of the bladder urothelium/lamina propria following luminal exposure to DMSO. During treatment there was a large release of mediators from the urothelium/lamina propria which are known to trigger micturition and initiate sensations of pain. These sensations would be greatly enhanced by the increases in muscarinic and purinergic receptors previously reported for patients with IC/PBS. The detection of LDH in the treatment medium suggests that release was due to permeabilisation of the urothelial membranes rather than stimulated physiological release. These effects on mediators and also the reduced inhibitory role of the urothelium/lamina propria on detrusor contraction following DMSO pretreatment may contribute to the initial flare up in symptoms experienced by most patients following intravesical DMSO treatment. Thus antagonists of sensory nerve purinergic receptors currently in development may potentially offer relief during this initial period after DMSO treatment.

Abbreviations

Ach: Acetylcholine
ATP: Adenosine triphosphate
DMSO: Dimethyl sulfoxide
EFS: Electric field stimulation
IC/PBS: Interstitial cystitis/painful bladder syndrome
LDH: Lactate dehydrogenase
PGE2: Prostaglandin E2
UDIF: Urothelial-derived inhibitory factor.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Evidence for Bladder Urothelial Pathophysiology in Functional Bladder Disorders

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Understanding of the role of urothelium in regulating bladder function is continuing to evolve. While the urothelium is thought to function primarily as a barrier for preventing injurious substances and microorganisms from gaining access to bladder stroma and upper urinary tract, studies indicate it may also function in cell signaling events relating to voiding function. This review highlights urothelial abnormalities in bladder pain syndrome/interstitial cystitis (BPS/IC), feline interstitial cystitis (FIC), and nonneurogenic idiopathic overactive bladder (OAB). These bladder conditions are typified by lower urinary tract symptoms including urinary frequency, urgency, urgency incontinence, nocturia, and bladder discomfort or pain. Urothelial tissues and cells from affected clinical subjects and asymptomatic controls have been compared for expression of proteins and mRNA. Animal models have also been used to probe urothelial responses to injuries of the urothelium, urethra, or central nervous system, and transgenic techniques are being used to test specific urothelial abnormalities on bladder function. BPS/IC, FIC, and OAB appear to share some common pathophysiology including increased purinergic, TRPV1, and muscarinic signaling, increased urothelial permeability, and aberrant urothelial differentiation. One challenge is to determine which of several abnormally regulated signaling pathways is most important for mediating bladder dysfunction in these syndromes, with a goal of treating these conditions by targeting specific pathophysiology.

1. Introduction

Although the cause(s) of urinary bladder functional syndromes is (are) largely unknown, there is an emerging body of evidence to support the involvement of bladder urothelial abnormalities in these illnesses. In this review, we will present published information concerning bladder urothelial abnormalities for two of these syndromes in humans (bladder pain syndrome/interstitial cystitis or BPS/IC, and overactive bladder syndrome or OAB), as well as one that occurs in cats (feline interstitial cystitis or FIC). To aid in understanding the possible relationship of these disorders to each other, we will also summarize those bladder urothelial abnormalities that these disorders have in common, as well as list possible future studies that could help us to understand whether additional similarities may exist for these three syndromes.

2. Bladder Urothelial Cell Gene Expression and Function in BPS/IC

BPS/IC is a debilitating chronic painful bladder syndrome that may affect as many as one million people in the USA, 80–90% of whom are women [1, 2]. BPS/IC often has a rapid onset with pain, urgency, and increased frequency of urination and cystoscopic abnormalities including petechial hemorrhages (glomerulations) in approximately 90% of patients or ulcers that extend into the lamina propria (Hunner's ulcers) in approximately 10% [2, 3], though current incidence of Hunner's ulcers is believed to be much lower. While no definite etiology for this syndrome has been identified, bladder urothelial abnormalities are a cardinal finding in biopsies from BPS/IC patients, with the predominant and most consistent histologic findings in the Interstitial Cystitis

Data Base Study and other reports including denudation and tears in the bladder urothelium (Hunner's ulcers or glomerulations) [3–5] and/or thinning of the bladder urothelium to 1-2 cell layers thick [5]. Electron microscopic studies confirmed bladder urothelial abnormalities in BPS/IC patients with disruption of the asymmetric unit membrane and bladder urothelial junctions of the surface umbrella cells [6]. The bladder urothelial mucin layer has also been shown to be abnormal in BPS/IC [5], and increased bladder urothelial permeability also has been suggested by some studies [7, 8].

Certain evidence suggests that these findings may result from intrinsic bladder urothelial abnormalities rather than extrinsic damage to the bladder urothelial cells. For example, although tissue from patients with Hunner's ulcers typically contains inflammatory cell infiltrates in the lamina propria (often consisting of T lymphocytes with or without mast cells) [3, 5] little inflammation is usually seen in tissue from the much larger number of patients without ulcers [5], suggesting that inflammation might occur in response to, rather than being a cause of, bladder urothelial damage. In addition, urothelial gene expression also consistently appears to be abnormal in bladder tissue from BPS/IC patients [9–13], with altered levels of specific cell proteins and proteoglycans, providing additional evidence for an intrinsic bladder urothelial cell defect related to an abnormal program of gene expression and/or differentiation. Finally, studies with isolated, explanted bladder urothelial cells from BPS/IC patients have displayed abnormalities consistent with the histopathologic findings, including decreased cell proliferation, abnormal gene expression, and abnormal function as compared to bladder urothelial cell explants from normal controls.

This section will review in more detail published findings regarding each of the bladder urothelial cell abnormalities described in tissue biopsies and/or bladder urothelial cell explants from BPS/IC patients, concentrating on those with initial descriptions of bladder urothelial abnormalities, large studies, and studies in which BPS/IC tissue and cell explants were compared to controls; abnormalities in urine components are beyond the scope of this review. In addition, we will review correlations between urothelial abnormalities in bladder biopsy specimens and abnormalities in explanted bladder urothelial cells when available, as well as published correlations between bladder urothelial abnormalities and the voiding abnormalities found in this illness.

2.1. Histopathologic Evidence for Bladder Urothelial Abnormalities in BPS/IC Patients. The earliest published description of histopathologic abnormalities in BPS/IC bladders from patients with Hunner's ulcers described areas where the bladder urothelium was absent and replaced with granulation tissue, as well as underlying increases in inflammatory cells, fibrosis, and capillaries [14]. Since that time, many other studies have also described urothelial abnormalities as one of the predominant histopathologic findings in BPS/IC bladders, including thinning and/or ulceration on the microscopic level in tissue from both patients with Hunner's ulcers as well as nonulcer BPS/IC patients. For example, the first description of electron microscopic findings in bladder tissue from 3 nonulcer BPS/IC patients as compared to findings in tissue from controls with urinary retention and infection or bladder tumors showed consistent evidence for abnormal tight junctions in the BPS/IC but not control groups [15]; this finding was then confirmed by showing significantly greater leakage of radioactive substances across the bladder wall and into the bloodstream following intravesical instillation of either ^{24}Na or ^{14}C-urea in the BPS/IC patients. However, it appears that only the BPS/IC patients in this study were hydrodistended prior to biopsy. Similar electron microscopic findings were also noted in a subsequent larger study of over 300 BPS/IC patients, in which the bladder tissue had abnormal widening of the space between cells in all layers of the bladder urothelium [16]. Increased absorption of intravesically instilled urea was confirmed in 56 (both nonulcer and ulcer) BPS/IC patients as compared to 31 controls; notably, a subset of the BPS/IC (8 patients) and normal controls (26 patients) that did not undergo hydrodistention under anesthesia prior to urea instillation showed similar results, with 22.1% of the BPS/IC patients having urea absorption versus 4.7% of controls [7]. The same investigators later documented increased pain from intravesical instillation of potassium ions in BPS/IC versus control patients [8], providing additional evidence for abnormal bladder urothelial leakiness in this illness.

Findings from subsequent histologic studies also confirmed the presence of bladder urothelial abnormalities in patients with nonulcer as well as classical (ulcer) BPS/IC. While a comparison of 64 patients with classical BPS/IC to 44 nonulcer patients (including both males and females in both groups) and 20 control women showed evidence for true ulceration only in bladders from the classical BPS/IC group, it also noted that the predominant histopathologic feature in the nonulcer group was multiple mucosal ruptures [3]; apparently with few exceptions both BPS/IC and control biopsies were obtained after hydrodistention. This finding was confirmed in a subsequent larger study by the same group, in which biopsies from nonulcer BPS/IC patients were compared to biopsies from ulcer BPS/IC patients. While 96% of 146 ulcer BPS/IC patients had ulceration, 83% of 64 nonulcer BPS/IC patients were found to have only mucosal ruptures [17]. However, other histopathologic studies, including an earlier study by the same group in which they compared BPS/IC biopsies to controls, have described actual microscopic ulcerations in patients with nonulcer BPS/IC. For example, comparison of 41 nonulcer BPS/IC patients to 16 controls that did not fulfill symptom criteria for BPS/IC revealed primarily mucosal ulceration, as well as degenerative changes to the bladder urothelial cells with vacuolization and detachment in the nonulcer BPS/IC patient group [18]; this study is similarly important for implicating epithelial abnormalities in nonulcer as well as classic BPS/IC because both the nonulcer BPS/IC patients and the controls were hydrodistended prior to biopsy. These latter findings were also described by other groups, including one study that compared bladder tissue from 22 nonulcer BPS/IC patients to tissue from 10 controls (all specimens obtained after hydrodistention) and found evidence for a significantly greater incidence of microscopic mucosal ulceration in nonulcer BPS/IC patients (7 of 22) versus controls (0 of 10) [19]. Another study that compared tissue

from 34 BPS/IC patients to tissue from 35 controls also found a significant increase in bladder urothelial denudation in the BPS/IC biopsies. The investigators noted that while 0% of the control specimens had completely denuded epithelium, this finding was noted in approximately 20% of biopsies from BPS/IC patients (with 50% of patients with severe BPS/IC having this finding); however, whether either or both patient groups underwent hydrodistention prior to biopsy is not noted (described in [20]). Finally, the Interstitial Cystitis Database Study by the National Institutes of Health (which studied over 200 both ulcer and nonulcer BPS/IC patients, hydrodistended prior to biopsy) indicated that complete loss of bladder urothelium was highly significantly correlated with nighttime voiding frequency, and the percentage of mucosa denuded of urothelium was highly associated with pain by multivariate predictive modeling [5]. Cluster analysis of the same biopsy specimens indicated that only a small percentage (3.4%) of the BPS/IC patients had complete erosion of the bladder urothelium with cellular inflammation, mastocytosis, and vascular damage, while 96.6% of specimens showed either complete or partial erosion of the bladder urothelium with very limited evidence for any other pathologic features [21].

2.2. Evidence for Abnormal Bladder Urothelial Cell Proliferation. Based on these and other reports that (with few exceptions) described an abnormal or absent bladder urothelium in BPS/IC patients, investigators then sought to determine whether these cells had intrinsic abnormalities in their cell proliferation and/or gene expression compared to controls. The first evidence that they may have altered proliferative ability indicated that a subpopulation of basal progenitor cells from BPS/IC patients had an increased proliferative ability as compared to the same basal cells from normal controls when cultured with complete F12 medium containing bovine pituitary extract [22]. However, subsequent studies in which bladder urothelial cells were grown from BPS/IC patients and controls in completely defined cell culture medium (without any growth factors) indicated that bladder urothelial cells explanted from BPS/IC biopsies proliferated at a significantly slower rate than control cells under these conditions [23]. This finding was determined to result from the production of a small Frizzled-8 protein-related glycoprotein (called antiproliferative factor, or APF) that appears to be made uniquely by urothelial cells from BPS/IC (but not control) bladders, and which inhibits human bladder urothelial cell proliferation [24]. This APF was shown to block primarily G2/M in the cell cycle [25, 26] as well as upregulate expression of certain transcription factors associated with decreased cell proliferation (p53 and $p21^{cip1/waf1}$) [26–28].

2.3. Evidence for Abnormal Bladder Urothelial Gene Expression. In addition to producing APF, BPS/IC cells have been shown to have abnormal expression or activation of many other gene products as compared to normal controls both in bladder biopsy specimens and in explanted bladder urothelial cells, shown by using a variety of techniques including primarily immunofluorescence staining, Western blotting, polymerase chain reaction, and microarray technology. Unlike the histologic studies described above in which tissue morphology could easily be influenced by a procedure such as hydrodistention (and which therefore generally included hydrodistention of both controls and BPS/IC patients), findings of abnormal protein expression are less likely to be affected during the time between that procedure and tissue fixation; therefore, more of these studies were performed without hydrodistention of the control group(s). Two early papers reported evidence for abnormal HLA antigen expression in BPS/IC bladder urothelial cells, including increased HLA-DR expression both in biopsy specimens as well as in explanted cells (although the latter study used ureteral urothelial tissue and cells for controls and demonstrated findings in cultured cells following treatment with gamma interferon and TNFα: unclear whether either group included specimens obtained after hydrodistention) [11, 29]. These reports have been followed by many others that showed significantly abnormal expression of additional urothelial proteins and proteoglycans in bladder biopsies from BPS/IC patients as compared to controls, including *decreases* in chondroitin sulfate proteoglycans (both groups hydrodistended prior to biopsy) [9, 30]; uroplakin (both groups hydrodistended prior to biopsy) [9]; neurokinin receptor 1 (only BPS/IC patients underwent uroflowmetry, postvoid residual and urodynamic investigations) [10]; and the urothelial tight junction proteins zonula occludens 1 [9, 10], junctional adhesion molecule 1, occludin, and claudin 1 [10]. BPS/IC bladder biopsies have also been shown to have *increased* expression of many other bladder urothelial cell genes including nerve growth factor (all control groups also hydrodistended prior to biopsy in [31]; only BPS/IC group hydrodistended prior to biopsy in [32]), E-cadherin (both BPS/IC and control specimens obtained after hydrodistention) [9], inducible nitric oxide synthase (unclear whether some BPS/IC patients and controls did not undergo hydrodistention prior to biopsy) [33, 34], caveolin 1 (no mention of hydrodistention for BPS/IC patients or controls) [35], vascular endothelial growth factor (only BPS/IC patients hydrodistended prior to biopsy) [36], human chorionic gonadotropin beta (no mention of hydrodistention for BPS/IC patients or controls) [37], claudin 2, and a variety of cell signaling receptors including bradykinin B(1) receptor, cannabinoid receptor CB1, muscarinic receptors M3–M5 [10], and transient receptor potential vanilloid 2 (TRPV2) [32]. Increased activated (nuclear) NFκB was also evident in BPS/IC bladder urothelial cells [38]. In addition, qRT-PCR of bladder biopsies showed increased mRNA expression for acid-sensing ion channels (ASIC) 2 and 3, but decreased mRNA expression for transient receptor potential vanilloid 1 (TRPV1) (no mention of hydrodistention for BPS/IC patients or controls) [39]. However, as the biopsies used in the latter two referenced microarray studies [32, 39] included both urothelial and other bladder cells, as protein expression was not evaluated for the same genes, and as functional differences related to ASIC or TRPV expression were not evaluated, the significance of these findings for the bladder urothelium in BPS/IC patients is currently unclear. (Please note that because bladder biopsies from patients with ulcerative BPS/IC may be largely or completely denuded of urothelium even in nonulcerative areas, results from microarray analysis

on biopsy specimens exclusively from patients with ulcerative BPS/IC are not included in this review).

Of those gene expression abnormalities identified in BPS/IC tissue specimens, those also noted to be stably abnormally expressed by BPS/IC bladder urothelial cell explants include changes in tight junction and adherens protein expression with decreases in occludin and zonula occludens 1 [40, 41] and claudin 1, 4, and 8 [42], with increases in E-cadherin expression [40, 41]. The decreased tight junction protein expression was also shown to correlate with increased paracellular permeability of radiolabeled tracers *in vitro* [41]. In addition, microarray analysis of BPS/IC cell explants also revealed significant decreases in vimentin, putative tRNA synthetase-like protein, neutral amino acid transporter B, alpha 1 catenin, alpha 2 integrin, and ribosomal protein L27a, and significant increases in arylsulfatase A, phosphoribosyl-pyrophosphate synthetase associated protein 39, and SWI/SNF BAF 170 [40]. The fact that promoters for all these genes with abnormal mRNA expression by microarray analysis have AP-1 and/or Sp-1 binding sites suggests that transcription factors in addition to p53, $p21^{cip1/waf1}$ and NFκB may be abnormal in bladder urothelial cells from BPS/IC patients. In addition to intracellular proteins, BPS/IC cell explants were also shown to secrete increased levels of certain proteins including IL-1, TNFα, class I and class II HLA molecules, and certain epithelial cell growth factors (including EGF and IGF1) but decreased levels of other proteins such as HB-EGF [11, 23, 29, 43].

Notably, all abnormalities in gene expression, paracellular permeability, and the secretion of specific epithelial cell growth factors found in BPS/IC cell explants *in vitro* have also been induced in normal bladder urothelial cells by treatment with a synthetic active derivative of APF [24, 40], and the expression of uroplakin and zonula occludens 1 was specifically attenuated by the same APF derivative in a mouse model during bladder urothelial repair [44]. APF was also shown to decrease cell secretion of heparin-binding epithelial growth factor-like growth factor (HB-EGF), as well as an enzyme that cleaves pro-HB-EGF with release of active HB-EGF-matrix metalloproteinase-2 (MMP-2) [28]. Finally, several of these abnormalities (including tight junction protein expression and paracellular permeability) were also normalized in BPS/IC cells by treatment with functional antagonists of APF [45]. Therefore, it is possible that many of the bladder urothelial abnormalities identified in BPS/IC patients result from the production of this toxic factor (APF), and it is also possible that APF antagonists may be helpful for normalizing bladder urothelial gene expression in BPS/IC.

The large number and types of genes whose expression was significantly abnormal in BPS/IC bladder urothelial cells (both in tissue specimens and in culture of explanted cells), along with abnormal levels of specific transcription factors, suggested a pattern of abnormal bladder urothelial cell differentiation in BPS/IC [9, 12, 24, 30]. Additional evidence for abnormal bladder urothelial differentiation in this illness was provided by demonstration of abnormal cytokeratin expression in BPS/IC biopsies (as compared to historical controls), indicating a change from transitional cell to squamous urothelial cytokeratin expression [13]. In addition, in one model to determine the capacity of bladder urothelial cells to differentiate into transitional cells *in vitro*, the absence of a normal differentiation response was noted in cells from 4 of 7 BPS/IC patients [46].

2.4. Abnormal Cell Signaling in BPS/IC Bladder Tissue and Explanted Bladder Urothelial Cells. In addition to abnormal expression of those gene products mentioned above, abnormal expression in BPS/IC bladder cells also appears to extend to the production of various cell signaling molecules and signaling receptors, as well as ultimate downstream modification of certain target proteins/enzymes. For example, BPS/IC biopsies displayed increased purinergic (P2X2 and P2X3) receptor protein [47], as did bladder urothelial cell explants (P2X3) following stretch *in vitro* [48]. While the latter study compared cells explanted from biopsies of BPS/IC patients following hydrodistention to those explanted from nonhydrodistended controls, the former study obtained all biopsy specimens from patients and controls at the time of cystectomy or radical prostatectomy (no mention of hydrodistention being performed at the same time). Urothelial biopsies from patients with BPS/IC were also shown to release significantly more ATP in response to stretch or electrical field stimulation than normal bladder urothelial biopsies [49], and BPS/IC bladder urothelial explants showed increased ATP release *in vitro* following either stretch [50] or exposure to exogenous ATP [51] as compared to controls (however, all of these studies used tissue obtained following hydrodistention in BPS/IC patients as compared to nonhydrodistended control tissue). Both P2X3 expression and ATP-stimulated ATP release, which suggested an association between cytokine and purinergic signaling in bladder urothelial cells, could also be induced in control cells by treatment with an active synthetic derivative of APF [52]. In addition, in single cell electrophysiologic studies, explanted IC/PBS bladder urothelial cells displayed reduced inward rectifying potassium current with conductance of the Kir2.1 channel as compared to normal control cells (only BPS/IC patients hydrodistended prior to biopsy) [53]. Finally, BPS/IC explants were also shown to have an enhanced sensitivity to carbachol, suggesting that muscarinic signaling may also be abnormally regulated in BPS/IC (only BPS/IC patients hydrodistended prior to biopsy) [54].

APF has also been shown to affect signaling via certain pathways in bladder urothelial cells, indicating the possibility that these pathways may also be abnormally regulated in BPS/IC cells. For example, HPLC-purified native APF was shown to inhibit Erk/MAPK while stimulating p38MAPK signaling in T24 cells [55], and the latter finding was subsequently corroborated by the finding of increased phospho-p38MAPK in urothelial cells of BPS/IC biopsies as compared to controls (both BPS/IC patients and controls hydrodistended prior to biopsy) [56]. This latter study also demonstrated an increase in phospho-p53 and other proapoptotic proteins in these cells, supporting a possible role for APF in abnormal cell signaling in BPS/IC. In addition, recent studies have shown evidence for inhibition of Wnt/Akt signaling in T24 cells by an active synthetic APF derivative including decreased phosphorylation of Akt, GSK3β, and

β-catenin ser45/thr41, with increased phosphorylation of β-catenin ser33,37/thr41 [28]. Quantitative proteomics subsequently confirmed the role of a beta catenin network in HPLC-purified native APF-induced signaling [57]. Whether signaling by APF is mediated directly or indirectly by its known functional receptor CKAP4/p63 [58] is unknown. However, an intriguing recent report indicated that APF induces phosphorylation of CKAP4/p63 ser3, 17, and 19, resulting in nuclear translocation and DNA binding of the APF receptor [59].

Additional evidence for possible abnormal cell signaling in BPS/IC also comes from studies of a similar bladder illness in cats called Feline Interstitial Cystitis, as described in the next section.

2.5. Summary. There now exists a large body of data to indicate extensive abnormalities in bladder urothelial cell gene expression, function, and signaling in BPS/IC, with many of these abnormalities being found both in patient tissue specimens and in explanted bladder urothelial cells. These findings have largely been described and/or confirmed in studies in which both patients and controls underwent hydrodistention, indicating that they are not artifactual (i.e., not caused by the procedure alone). The extent and specific types of differences in expression of intracellular and secreted proteins, in components of the various signaling pathways, and in downstream modification of target proteins, are compatible with the hypothesis of aberrant bladder urothelial cell differentiation in this illness. The resultant abnormalities of bladder urothelial cell proliferation, gene expression, and signaling by these abnormally differentiated bladder urothelial cells could ultimately result in bladder urothelial thinning or ulceration, leakiness, and altered downstream afferent neuronal cell activation, causing increased urinary frequency, urgency, and pain which characterizes BPS/IC.

3. Urothelial Cell Physiology in Feline Interstitial Cystitis (FIC)

A number of animal models have been used for the study of BPS/IC such as administration of an irritant to otherwise healthy rodents [60]. There is a naturally occurring disease in cats that has been termed feline interstitial cystitis or FIC that shares a number of characteristics of the nonulcerative form of BPS/IC in humans [60, 61].

3.1. Alterations in Urothelial Barrier Function. Functional pain syndromes such as BPS/IC have been associated with alterations in the urothelium, which include an alteration or degradation of the barrier function. Similar to patients with BPS/IC, cats diagnosed with FIC exhibit alterations in urothelial ultrastructure and tight junction proteins [62]. Changes in the urothelial barrier can permit water, urea, and noxious substances present in the urine to pass into the underlying tissue (neural and other layers), which may result in symptoms of urgency, frequency, and pain during bladder distension. In addition, disruption of the urothelial barrier may also be due to hormonal and neural mechanisms. Findings of elevated nitric oxide levels have been reported in cats with FIC [63] as well as in patients with BPS/IC, which appear to be similar to those in other epithelia where excess production of NO has been linked to altered epithelial integrity [64].

3.2. Alterations in Urothelial Sensory Function. BPS/IC has often been described as a disease of the urothelium, which is likely to communicate in a multidirectional fashion with underlying bladder nerves, smooth muscle, and cells of the immune and inflammatory systems [65]. For example, the increased neural excitability that has been reported in cats with FIC [66, 67] may be affected by changes in release of various transmitters or trophic factors from the urothelium [66]. Augmented release of ATP from the urothelium can lead to painful sensations via sensitization of sensory fibers [68, 69]. The increase in ATP release (evoked by mechanical stimuli) in FIC urothelium [70] is similar to that described in urothelial cells isolated from patients with BPS/IC [71]. In addition, both FIC and BPS/IC patient urothelium exhibited plasticity in purinergic receptor expression [48, 72], which may be linked with changes in sensory pathways. Also, it is hypothesized that physical and emotional stress, via alterations in stress-mediators (such as corticotrophin releasing factor or CRF, as shown in animal models) may exacerbate a number of lower urinary tract disorders including BPS/IC. In FIC urothelium, the functional activity of CRF receptors is altered compared to urothelium from healthy, unaffected cats [73]. Thus, changes in stress-related peptide signaling may result in alterations in urothelial-cell signaling and bladder function. In addition, increased nerve growth factor (NGF) in urine and tissue has been linked with bladder pathologies including idiopathic sensory urgency as well as BPS/IC [74]. Increased NGF in bladder urothelium has been reported in FIC as compared to urothelium from healthy, unaffected cats [75]. Studies have shown that a major source of NGF comes from smooth muscle of the bladder in addition to the urothelium, and altered NGF levels may contribute to altered neural excitability and emergence of bladder pain [76].

3.3. Comorbidity in FIC. Patients diagnosed with BPS/IC often exhibit a number of comorbid disorders that can also include other pelvic pain problems (irritable bowel syndrome or fibromyalgia) [77–79]. These individuals typically exhibit diffuse hyperalgesia or allodynia, which suggests the possibility of a generalized dysfunction in pain or sensory processing. Though the etiology is unknown, a number of factors have been proposed that may contribute to or modulate the pathophysiology of such overlapping disorders which can include changes in epithelial sensor/barrier functions, inflammation, or autoimmune involvement [80]. Changes in epithelial barrier or sensory functions are not specific to that of the urinary bladder. For example, patients with gastroesophageal reflux disease (GERD) have been reported to exhibit changes in esophageal epithelial structure and function, similar to that of BPS/IC patients [81]. Thus, similar to BPS/IC, a loss of esophageal epithelial integrity may allow access of luminal acid to underlying afferent terminals that can ultimately lead to esophageal pain. There is evidence that nonneuronal acetylcholine plays a role in sensation, cell-cell signaling,

proliferation, and cell growth as well as maintaining barrier function [82]. There is an alteration in expression of components of the nonneuronal acetylcholine machinery in FIC bladder urothelium as well as the esophageal epithelium [83, 84]. It is likely that many of these alterations could contribute to epithelial hypersensitivity and barrier dysfunction that often occur in patients with functional and inflammatory urological as well as gastrointestinal esophageal symptoms.

3.4. Summary. There are number of unanswered questions in terms of research that could be done to better relate the animal model to that of the human condition. Use of both animal and human tissue and explants (from different bladder areas) could be used to explore whether similar types of receptors are expressed and to determine whether there are similarities in abnormal signaling between animal and human interstitial cystitis. In addition, a study of sex/gender differences could even influence research on a number of comorbid conditions that share epithelial abnormalities that influence function. These and other research questions could influence treatment paradigms and also increase our understanding of mechanisms underlying urothelial function in health and disease.

4. Urothelial Abnormalities in Nonneurogenic, Idiopathic OAB

Overactive bladder (OAB) is a highly prevalent clinical syndrome. The International Continence Society has defined OAB as the presence of urinary urgency, usually accompanied by daytime urinary frequency and nocturia, with or without urgency urinary incontinence, *in the absence of infection or other identifiable etiology* [85]. While there are several possible etiologies for OAB, historically, the mechanism receiving the most attention is increased detrusor smooth muscle (DSM) contractility (overactivity) [86]. This mechanism provides the foundation for using oral antimuscarinics to block DSM overactivity. A newer hypothesis for OAB is augmented bladder urothelial-afferent signaling which gives rise to OAB syndrome and, more generally, lower urinary tract symptoms (LUTS) [87, 88].

Use of animal models to study OAB can be problematic because bladder symptoms cannot be ascertained in an animal and surrogate physiologic and behavioral outcomes have to be utilized. There has been a wide range of animal models used to study OAB, most models involving invasive manipulations or injuries to the bladder (i.e., cyclophosphamide treatment), urethra (i.e., urethral obstruction), neural tissues (i.e., spinal cord injury, autoimmunity to nerves), or pancreas (i.e., creation of diabetes model with streptozotocin). Because, by clinical definition, OAB does not arise secondary to these manipulations or injuries (if LUTS arose from these causes, then the term OAB would not apply), these models are less than ideal. Recently, transgenic animals with alterations specifically targeting the urothelium (urothelial restriction or conditional transgenics) have been developed to test the hypothesis that urothelial dysfunction can drive altered bladder function. Therefore, the contribution of specific urothelial mechanisms can be studied in a "natural" *in vivo* state without need for purposeful manipulations and/or tissue damage. A review of different animal models in OAB research has been published [89]. However, the pathophysiologic relationship of all of these animal models to OAB in humans is unknown.

The more direct approach of searching for evidence of epithelial pathophysiology in the human disease using human OAB urothelial tissue has also been utilized to determine whether urothelial pathophysiology contributes to OAB pathogenesis. However, limited numbers of mechanistic experiments can be performed with human tissues and cells. Perhaps then the best approach to studying urothelial contributions to OAB symptoms is first to identify specific abnormalities from human OAB urothelium and then reproduce these abnormalities in an animal model (e.g., by using an urothelial conditional transgenic approach). If the conditional transgenic animals recapitulate the clinical OAB phenotype, then the urothelial abnormalities can be targeted for clinical treatment (and the transgenic animal can also be used to test different treatments).

The focus of this section is to highlight urothelial research performed using both human OAB urothelium and OAB animal models that do not involve tissue injury. A caveat is that studies on urothelial protein expression in animals often use antibodies which may incur a high degree of nonspecificity when applied to the urothelium [90]. The problems of nonspecific antibody binding (adsorption) (which requires the use of appropriate antibody isotype controls), low antibody sensitivity (which can lead to false negative results), and the presence of nonurothelial cells in the preparations can all lead to spurious results. While some studies have adequately addressed these issues, others have not, and therefore new guidelines have been suggested for future research that include use of multiple techniques to verify protein expression (such as immunolocalization, *in situ* hybridization, knockout transgenics, laser capture microdissection, immunogold electron microscopy, and additional controls). In addition, other emerging techniques such as targeted proteomics using liquid chromatography coupled with tandem mass spectroscopy (LC/MS), that can precisely quantitate proteins without need of antibodies [91, 92], have become available since the time many of the published studies were performed, and their application to urothelial biology remains to be implemented.

4.1. Urothelial NGF. The concept of urothelial nerve growth factor (NGF) overexpression (OE) contributing to pathophysiology of OAB is based on the findings from several investigative groups that urine from OAB subjects has increased NGF [93]. It is possible that the increased urinary NGF is from increased secretion by the bladder urothelium, though other tissue types may also be the source of urinary NGF (e.g., nerves, suburothelium, and smooth muscle). A urothelially restricted NGF-OE mouse was therefore created to study what happens to the micturition phenotype [94]. These investigators concluded that the morphological and functional features of the NGF-OE transgenic mice reflected the changes observed in micturition reflex pathways in patients with OAB (and BPS/IC). However, other investigators have found that NGF levels in bladder urothelial tissue from OAB patients were not associated with detrusor

overactivity, bladder contractility, or increased bladder sensation [95], suggesting that urothelial NGF may not be the critical regulator of bladder overactivity in OAB. While phases I and II clinical trials of anti-NGF monoclonal antibody (tanezumab) for treatment of pain symptoms of BPS/IC were conducted [96] which showed some benefits, unfortunately, these trials were closed by the FDA because of concerns over avascular hip necrosis from this agent. This agent has not been tested in OAB.

4.2. Urothelial Purinergic Signaling. Ferguson first proposed the concept that the bladder urothelium can release ATP in response to stretch, with the released ATP serving as a sensory neurotransmitter [97]. The ATP receptor, P2X3, when constitutively knocked out resulted in a hyporeflexive (hyposensitive) bladder [98]. The role of ATP in OAB has been shown in several studies. Investigators found that urinary ATP was a better predictor than urinary NGF for detrusor overactivity in OAB [99]. Urinary ATP levels in OAB patients decreased after treatment with antimuscarinics [100], and a higher pretreatment urinary ATP level predicted a better response to antimuscarinic therapy. However, the sources of the urinary ATP (e.g., whether from the urothelium, suburothelium, nerves, and/or muscles) were not identified in these studies. When urothelial specimens were obtained surgically from OAB and control subjects and then stretched *in vitro*, investigators found that stretched OAB urothelium released significantly more ATP than control urothelium [101]. This finding suggested that urinary ATP levels in OAB patients could reflect urothelial release. In a fructose-fed animal model of metabolic syndrome, which is associated with OAB [102, 103], P2X3 expression was upregulated in the urothelium and intravesically infused ATP induced greater detrusor overactivity in fructose fed animals compared to controls [104]. This suggested increased urothelial purinergic signaling in the fructose fed animals. Taken together, these data suggest that urothelial purinergic signaling is augmented in OAB.

The role of TRP (transient receptor potential) and muscarinic receptors in modulating ATP release by urothelial cells is discussed below in Section 4.7 on urothelial TRPV1.

4.3. Urothelial β1-Integrin. Integrins are transmembrane proteins that connect the intracellular cytoskeleton with extracellular matrix allowing cellular sensing of changes in organ shape or changes in force. Investigators theorized that integrins could be upstream regulators of mechanosensory apparatus in the urothelium. This hypothesis was tested by creating a β1-integrin urothelially restricted knockout mouse [105]. The micturition behavior phenotype showed increased voiding frequency with small volume voids and evidence of "urinary incontinence" based on urinary spot analyses. Urodynamics also showed increased bladder activity (analogous to detrusor overactivity). Interestingly, the urothelium from this transgenic animal also had a 2-fold increase in urinary ATP concentration compared to wild-type animals. These findings suggest that urothelial integrins are important for regulating bladder mechanosensory transduction, and loss of the β1-integrin recapitulates an OAB phenotype.

4.4. Urothelial Tight Junction Expression. The quantities of tight junction proteins including zona occludens 1 (ZO-1), occludin, and claudin-4 were shown to be decreased in cultured OAB compared to cultured control urothelial cells (manuscript in preparation). This difference was demonstrated using three complementary techniques: Western blotting, quantitative RT-PCR, and immunofluorescence of cultured cells. There was also increased secretion of matrix metalloproteinase-2 (MMP-2) and HB-EGF into the supernatant by OAB urothelial cells similar to what was detected in BPS/IC urothelial cells [43]. The transcript levels of these three tight junction proteins were increased by inhibiting ornithine decarboxylase (ODC), the rate limiting synthetic enzyme for intracellular polyamine production (see Section 4.5). This suggested that polyamines can modulate the mRNA levels of these tight junction proteins.

4.5. Urothelial Polyamines. Using cultured urothelial cells from OAB patients, investigators have found significantly increased intracellular polyamine concentrations of spermine, spermidine, and putrescine in OAB urothelial cells [106, 107]. The increased polyamines, associated with an expected increase in expression of the rate limiting synthetic enzyme, ornithine decarboxylase (ODC), blocked the function (measured electrophysiologically) of the large conductance calcium activated potassium channel (BK). The reason that BK function was measured in this urothelial study [106] was because a previous constitutive BK-knockout mouse was shown to have an OAB phenotype [108]. While there is no question that detrusor smooth muscle from the BK-knockout mice had overactivity, the question raised by the 2009 publication is whether there might also be a urothelial contribution to the OAB phenotype in the constitutive BK-knockout mouse, since BK function is also reduced in the urothelium as well as the detrusor smooth muscle.

The increased polyamines also appeared to affect intracellular calcium rise in response to exogenously added muscarinic agonist, oxotremorine (OXO) [107]. It was shown that OAB urothelial cells reacted (as measured by intracellular calcium rise) more strongly to OXO, compared to control cells, based on dose-response curve. This augmented intracellular calcium reactivity to OXO was abrogated when ODC was blocked, suggesting that polyamine can modulate the intracellular calcium raise in response to muscarinic receptor activation.

4.6. Urothelial Muscarinic Signaling. As mentioned above, antimuscarinics are currently the mainstay pharmacologic treatment for OAB. The mechanism of action of these agents is to block overactivity of the detrusor smooth muscle. However, muscarinic blockade might affect urothelial signaling through muscarinic receptors located within the urothelial cells. As presented in the prior section on polyamine signaling, OXO, a muscarinic agonist, caused OAB urothelial cells to have higher maximal response in terms of intracellular calcium rise, compared to control urothelial cells [107]. The half maximal concentration for OXO's effect on intracellular calcium rise was also significantly lower for OAB compared to control urothelial cells. This suggested an increased

sensitivity to muscarinic agonists in OAB urothelial cells. Whether this increased calcium response to OXO results in some altered downstream effects was not tested in this paper and remains unknown.

However, one of the downstream effects of urothelial muscarinic receptor activation is ATP release by the urothelial cells. Investigators have studied this mechanism in guinea pig and human urothelium [109]. The human urothelial tissues were obtained from "male patients. . .undergoing diagnostic endoscopy," but their clinical symptoms were not described. OXO caused the urothelial tissues in both species to release ATP. The ATP release by the urothelium had a paracrine effect and modulated the underlying detrusor smooth muscle.

Other investigators studied ATP release induced by acetylcholine (ACh) in cultured OAB and control urothelial cells [110]. There was no difference in ATP release between the OAB and control cells in response to ACh, which was surprising as it would be expected that there would be increased ATP release by OAB cells. Possible explanations for this unexpected finding include cultured cells not replicating *in vivo* tissue effects and the low number of OAB subjects studied.

Another downstream effect of urothelial muscarinic receptor activation is release of ACh [111]. These investigators concluded that release of ACh has a negative feedback loop on urothelial ACh release and that the urothelial release of ACh is mediated through mechanisms different than neuronal ACh release (which is vesicular exocytosis). Urothelial ACh release induced by muscarinic receptor activation by OXO was studied in cultured OAB cells [107]. OAB cells released significantly more ACh than control cells, but this was only after 6 hours of relatively high dose (10 μM) OXO exposure. The ACh release was abrogated by inhibition of polyamine synthesis.

Whether urothelial muscarinic signaling plays a central role in regulating bladder function (i.e., contributing to OAB symptoms) is not known at this time. While it has been theorized that antimuscarinic therapies for OAB might target the urothelium, this is speculative at this point without much supportive data. Nevertheless, investigators are continuing to investigate the role of muscarinic receptors on the urothelium.

4.7. Urothelial TRPV1. The relevance of TRPV1 (transient receptor potential vanilloid 1) channel in bladder function was studied in the constitutive TRPV1 knockout mouse [114]. This transgenic animal had an OAB bladder phenotype on micturition behavior (significantly higher number of small urine spots/hour) and cystometrogram testing (significantly higher nonvoiding contractions/minute). However, in a different study [115, 116], investigators did not find a difference in frequency of bladder contractions on cystometry between the TRPV1 knockout versus the wild-type animals, though nonvoiding contractions were not calculated. Because this transgenic animal is a constitutive knockout, the contribution of urothelial TRPV1 to bladder functional phenotype is uncertain.

While TRPV1 is typically thought of as expressed in c-fiber bladder afferents, this channel is also found on urothelial cells from both rats [117] and humans [112, 113]. Investigators have studied the role of TRPV1 in OAB by using human urothelial tissues. It was found that TRPV1 mRNA was differentially expressed in the human bladder (different expression levels in trigone versus nontrigone areas). Furthermore, when OAB was separated into "sensory urgency" versus "detrusor overactivity" (two terms which are urodynamic definitions), increased TRPV1 mRNA expression was associated with sensory urgency, but not detrusor overactivity [113].

Cultured OAB urothelial cells exhibited a higher maximal response, as measured by intracellular calcium rise, to capsaicin compared control cells [112]. The dose of capsaicin to elicit a maximal response for both OAB and control cells was 10 μM. There was evidence also for increased TRPV1 channel activity in OAB cells, using electrophysiologic measurements. A result of the increased TRPV1 activity is increased ATP release [110]. However, a recent investigation found that while human bladder urothelium expressed mRNA for TRPV1, cultured human bladder urothelial cells did not respond to capsaicin [118], as measured by intracellular calcium rise, unless a very high dose (10–100 μM) of capsaicin was used.

There is also an interaction between TRPV1 and NGF in bladder function. This was studied in the TRPV1 knockout animal [115, 116]. It was found that NGF-induced bladder overactivity was dependent on TRPV1, as TRPV1 knockout animals did not respond with detrusor overactivity to NGF treatment.

Treatment with resiniferatoxin (RTX), which blocks TRPV1, has been tried for OAB. A single dose (50 nM RTX), placebo-controlled trial was performed in 58 patients with idiopathic detrusor overactivity and urgency incontinence (which would be considered idiopathic OAB) [119]. This trial failed to show a positive benefit of RTX. Another trial, which was open label, using a different subject phenotype including those with neurogenic OAB, found a beneficial effect of 50 nM RTX [120]. It should be noted that trials that showed positive effect for RTX were typically utilizing a neurogenic OAB population (e.g., postspinal cord injury). It appears that the benefits of RTX were more pronounced for neurogenic rather than idiopathic OAB.

The role of urothelial TRPV1 in OAB remains unclear. The controversies with regard to the existence of functional TRPV1 protein within the urothelium was highlighted in a published review [90]. Reasons for differences in findings from the different studies include cell culture techniques which could give rise to differently differentiated cells, presence of nonurothelial cells within cell cultures, lack of sensitivity and specificity of anti-TRPV1 antibodies, and species differences.

4.8. Summary. OAB is a clinical syndrome that is defined purely by LUTS in the absence of other disease-defining abnormalities. In order to better understand the contribution of urothelial pathophysiology to LUTS, different approaches have to be utilized. Studies of urothelial biopsies from human subjects provide relevance; however, sufficient amounts of clinical samples are limited and in depth *in vivo* mechanistic studies cannot be performed in humans. Furthermore, the phenotypes of OAB subjects in these translational studies

Table 1: Evidence for abnormal bladder urothelial cell structure and function in BPS/IC, FIC, and OAB.

Clinical condition	Bladder urothelial abnormality	Source	Reference
BPS/IC	**Structural abnormalities** (e.g., absence of epithelium, mucosal ulceration, mucosal ruptures, abnormal/leaky tight junctions, widening of spaces between cells, cell vacuolization, and urothelial detachment)	Tissue	Hunner 1914 [14]; Eldrup et al. 1983 [15]; Fall et al. 1985 [18]; Said et al. 1989 [16]; Lynes et al. 1990 [19]; Johansson and Fall 1990 [3], Johansson and Fall 1994 [17]; Tomaszewski et al. 2001 [5]; Rosamilia et al. 2003 [20]; Leiby et al. 2007 [21]
	Abnormal protein expression (e.g., abnormal cytokeratin expression; decreased chondroitin sulfate proteoglycans, uroplakin, zonula occludens 1, occludin, Junctional adhesion molecule-1, NK1, NK2, TRPV1, ASIC1, and 2; increased HLA-DR, E-cadherin, NFkB, claudin 2, bradykinin B1 receptor, cannabinoid receptor CB1, muscarinic receptors M3-M5, TRPV2, beta-hCG, VEGF, caveolin-1, inducible nitric oxide synthase, ICAM-1, IL1-α, and TNF-α)	Tissue	Christmas and Bottazzo 1992 [29]; Liebert et al. 1993 [11]; Hurst et al. 1996 [30]; Abdel-Mageed and Ghoniem 1998 [38]; Slobodov et al. 2004 [9]; Laguna et al. 2006 [13]; Koskela et al. 2008 [33]; Freire et al. 2010 [10], Sánchez-Freire et al. 2011 [39]; Lee and Lee 2011 [36]; Lin et al. 2011 [35]; Schwalenberg et al. 2012 [37]; Logadottir et al. 2013 [34]; Homma et al. 2013 [32]
	Abnormal protein expression (e.g., specific production of a frizzled-8-related antiproliferative factor; increased induction of HLD-DR; increased E-cadherin, arylsulfatase A, phosphoribosyl-pyrophosphate synthetase associated protein 39, and SWI/SNF BAF 170; decreased tight junction proteins (including zonula occludens 1, occludin, plus claudins 1, 4, and 8), vimentin, putative tRNA synthetase-like protein, neutral amino acid transporter B, alpha 1 catenin, alpha 2 integrin, and ribosomal protein L27a; decreased secretion of HB-EGF and MMP-2)	Culture	Liebert et al. 1993 [11]; Keay et al. 2003 [23], Keay et al. 2004 [24]; Zhang et al. 2005 [41], Zhang et al. 2007 [42]; Shahjee et al. 2010 [28]
	Abnormal protein expression (e.g., decreased uroplakin and zona occludens 1 in animal model with APF)	*In vivo* (*animal model*)	Keay et al. 2012 [44]
	Functional abnormalities (e.g., increased intravesical urea absorption)	Tissue	Eldrup et al. 1983 [15]
	Functional abnormalities (e.g., increased paracellular permeability)	Culture	Zhang et al. 2005 [41]
	Functional abnormalities (e.g., increased intravesical urea absorption, increased sensitivity to intravesical potassium ions)	*In vivo* (*humans*)	Parsons et al. 1991 [7], Parsons et al. 1998 [8]
	Correlation of urothelial abnormalities with voiding symptoms (including denudation with nighttime frequency and pain)	Tissue	Tomaszewski et al. 2001 [5]
	Abnormal cell proliferation	Culture	Elgavish et al. 1997 [22]; Keay et al. 2003 [23]
	Abnormal cell signaling (e.g., Akt and/or beta catenin signaling, increased purinergic signaling, reduced Kir2.1 channel activity, increased intracellular calcium to carbachol, increased ATP release, increased p38MAPK phosphorylation/signaling, and increased p53 phosphorylation)	Culture/Tissue	Sun et al. 2001 [50], Sun et al. 2004 [48]; Tempest et al. 2004 [47]; Sun and Chai 2006 [51]; Kumar et al. 2007 [49]; Gupta et al. 2009 [54]; Shahjee et al. 2010 [28]; Kim et al. 2009 [55]; Yang et al. 2011 [57]; Shie et al. 2012 [56]
	Physiologic effects of APF (e.g., G2/M cell cycle block; regulation of transcription factor expression, phosphorylation/palmitoylation/nuclear translocation of CKAP4)	Culture	Abdel-Mageed and Ghoniem 1998 [38]; Rashid et al. 2004 [25]; Kim et al. 2007 [26]; Kim et al. 2012 [27]; Shahjee et al. 2010 [28]; Zacharias et al. 2012 [59]

Table 1: Continued.

Clinical condition	Bladder urothelial abnormality	Source	Reference
Feline IC	Increased inducible nitric oxide synthase	Tissue	Birder et al. 2005 [63]
	Urothelial denudation, decreased transepithelial resistance, and increased permeability	Tissue	Lavelle et al. 2000 [62]
	Increased purinergic signaling	Culture	Birder et al. 2003 [70], Birder et al. 2004 [72]
Overactive bladder	Increased purinergic signaling	Tissue	Kumar et al. 2010 [101]
	Increased ornithine decarboxylase	Tissue	Li et al. 2009 [106]
	No difference in NGF (protein ELISA) OAB DO versus OAB non-DO	Tissue	Birder et al. 2007 [95]
	Increased polyamine and block of BK channel	Culture	Li et al. 2013 [107] and Li et al. 2009 [106]
	Increased TRPV1 signaling	Culture	Birder et al. 2013 [110]; Li et al. 2011 [112]; Liu et al. 2007 [113]
	Increased muscarinic signaling (increased intracellular calcium)	Culture	Li et al. 2013 [107]
	Decreased zonula occludens 1, occludin, claudin 4 in cells; decreased secretion of HB-EGF and MMP-2	Culture	Chai et al. 2014 (manuscript in preparation)
Transgenic urothelial restricted models	Decreased β1-integrin	Tissue	Kanasaki et al. 2013 [105]
	Increased NGF	Tissue	Schnegelsberg et al. 2010 [94]

vary greatly (or are not described in detail) thus limiting generalizability. Animal models, specifically urothelially restricted transgenics, are valuable tools to test the hypothesis that perturbations in urothelial function results in altered bladder function, but the relevance of animal models to the human illness must be established. As this field progresses, the research community ought to create animal models justified from findings derived from human OAB urothelial tissues. This would result in creating the most relevant animal models, which then can be used to test treatments targeting the urothelium for OAB. The published literature suggests that urothelial pathophysiology in OAB includes altered purinergic, muscarinic, polyamine, NGF, and TRPV1 signaling and preliminary data shows decreased tight junction protein expression.

5. Concluding Remarks

This review highlights investigations that showed urothelial abnormalities in three functional bladder conditions: BPS/IC, FIC, and OAB. A broad sweeping unified, single urothelial etiologic mechanism to explain entirely these 3 conditions is currently not possible given the published data and may not exist. However, the literature suggests that certain types of urothelial aberrations might contribute to the development and/or persistence of LUTS in these three conditions, making it possible that the two functional bladder conditions found in humans (BPS/IC and OAB) may be pathophysiologically related to each other as well as to FIC. A summary of these findings are presented in Table 1. Some of the common themes include altered urothelial differentiation, increased urothelial permeability (due to decreased tight junction proteins), and augmented urothelial "transducer-sensor function" (increased ability of the urothelial cells to release and/or respond to putative neurotransmitters). These urothelial abnormalities may be downstream to a more proximal cause or could be the primary defects. For example, in BPS/IC, an antiproliferative factor (APF) has been identified, completely characterized, synthesized, and shown to induce some of the same abnormalities in expression of cellular and secreted protein expression in both normal urothelial cell explants *in vitro* [24, 26–28, 40–43] and in mouse urothelial cells *in vivo* [44]. APF and/or urothelial growth factors whose expression can be regulated by APF (HB-EGF and EGF) have further been shown to induce certain abnormalities in ATP and potassium channel signaling found in BPS/IC cells [52, 53]. Whether the same or similar factors cause the urothelial abnormalities found in OAB and FIC remains to be determined.

Because the urothelium is comprised of three cell layers (apical, intermediate, and basal) with each layer providing different functional roles, studying the way by which the urothelium might regulate bladder function is complex. However, using mouse genetic tools, investigators are now able to study a primary urothelial defect and how this defect alone, as a proximal cause, can affect bladder function. The findings presented herein support the hypothesis that urothelial abnormalities adversely affect bladder function. A focus on targeting urothelial abnormalities may therefore be very beneficial for advancing treatment outcomes for these functional bladder disorders.

Conflict of Interests

The authors have the following potential conflicts to declare. Susan Keay is named as an inventor on patents for

antiproliferative factor (owned by the University of Maryland and the Veterans Administration) and is a consultant for Trillium Pharmaceuticals and Merck and Co., Inc.; Lori Birder is the recipient of research funding from Astellas Pharma, Inc.; Toby Chai is a consultant for Allergan, Inc., Ion Channels, and Taris Pharmaceuticals.

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Review Article

Bladder Cancer and Urothelial Impairment: The Role of TRPV1 as Potential Drug Target

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Urothelium, in addition to its primary function of barrier, is now understood to act as a complex system of cell communication that exhibits specialized sensory properties in the regulation of physiological or pathological stimuli. Furthermore, it has been hypothesized that bladder inflammation and neoplastic cell growth, the two most representative pathological conditions of the lower urinary tract, may arise from a primary defective urothelial lining. Transient receptor potential vanilloid channel 1 (TRPV1), a receptor widely distributed in lower urinary tract structures and involved in the physiological micturition reflex, was described to have a pathophysiological role in inflammatory conditions and in the genesis and development of urothelial cancer. In our opinion new compounds, such as curcumin, the major component of turmeric *Curcuma longa*, reported to potentiate the effects of the chemotherapeutic agents used in the management of recurrent urothelial cancer in vitro and also identified as one of several compounds to own the vanillyl structure required to work like a TRPV1 agonist, could be thought as complementary in the clinical management of both the recurrences and the inflammatory effects caused by the endoscopic resection or intravesical chemotherapy administration or could be combined with adjuvant agents to potentiate their antitumoral effect.

1. Introduction

Inflammation and neoplastic cell growth represent the main two pathologies of the bladder and they have a huge impact on patient's quantity and life-span.

The urothelium has been recognized to be more than a simple barrier separating the luminal contents from the inner layers of the urinary tract. It works with suburothelium as a functional unit, which responds to external stresses by the release of modulator agents that regulate the activity of not only the nearby afferent nerves but also of the underlying smooth muscle and urothelial stem cells. In particular, it may act as an efficient chemomechanosensor, the "afferent function," and at the same time, it is able to synthesize and release, into suburothelium layer, molecules involved in the bladder storage/voiding activity, the "efferent function." Furthermore, urothelium may protect the basal cells from toxins or other substances capable of activating a pathological cell growth.

Since the early 90s, investigators focused their basic science and clinical research on the expression, function, and clinical application of a subset of capsaicin-sensitive primary sensory afferents of the lower urinary tract (LUT) [1–7].

Both the upper and LUT are densely innervated by capsaicin-sensitive primary afferent neurons in a number of species including humans [8]. Early pharmacological studies revealed that capsaicin-sensitive, C type, bladder fibers play a role in micturition reflex and it was shown that capsaicin sensitive nerves exhibit both a sensory (afferent) and an "efferent" function, which is determined by the release of peptides including tachykinins, substance P (SP) and calcitonin gene-related peptide (CGRP) [9]. The sensory function includes the regulation of the micturition threshold and the perception of pain from the urinary bladder, while the efferent

function controls nervous tissue excitability, smooth muscle contractility and plasma protein extravasation (neurogenic inflammation). The discovery of specific binding sites for capsaicin in several tissues and organs, including the rat urinary bladder [9], initiated a rush that ended up with the cloning of the vanilloid receptor [10], presently known as TRPV1 (transient receptor potential vanilloid subfamily 1).

In the lower urinary tract, TRPV1 expression is now firmly documented not only in a large subpopulation of nerve fibers but also in nonneuronal tissues. Knowledge about the presumable function of TRPV1 also evolved rapidly. From a receptor initially considered as an integrator of thermal and chemical noxious stimuli, TRPV1 is emerging as a possible regulator of bladder reflex activity and cell differentiation. These findings, together with the promising clinical applications of TRPV1 targeting in the LUT, justified our interest in the distribution and function of capsaicinoids and their receptors in normal and pathological conditions. Recently it has been demonstrated that capsaicin and other vanilloids, which are linked to TRP receptors, may promote cellular death [11] and inhibit the growth of normal and neoplastic cells by apoptosis induction [12–15].

In this paper, we report the documented role of TRPV1 in the transitional cell carcinoma (TCC) of human bladder in presence of urothelium impairment and explore the opportunity of considering TRPV1 as a drug target.

2. Bladder Cancer

Bladder cancer is the second most common genitourinary cancer in the United States, overall the fourth most frequent cancer in men and the ninth in women, with 72,570 new cases (54,610 men and 17,960 women) and 15,210 deaths both in men and women predicted to occur in 2013 [16].

Usually, bladder cancer is distinguished in muscle invasive and non-muscle-invasive urothelial carcinoma of the bladder (NMIBC). Due to its pathophysiological characteristics and the worldwide assessed management workup, NMIBC has a high probability to recur with a low progression and metastasis rate. For the management of the noninvasive bladder cancer EAU, FICBT, NCCN, and AUA guidelines agree about the importance of endoscopic resection in all patients and the benefit of adjuvant intravesical therapy [17].

The benefits of a single, early, intravesical instillation of mitomycin C after transurethral bladder resection in patients with low risk NMIBC have been investigated. In 1999, Solsona et al. showed how mitomycin C instillation at 24-month follow-up significantly increased the recurrence free interval, but the result could not be reproduced at long-term follow-up. More recently, a single center study described a significant reduction in risk of both early and late recurrences [18]. In 2013, an updated meta-analysis confirmed that intravesical administration of chemotherapy after transurethral of NMIBC prolongs recurrence free interval and reduces early recurrences [19].

However, mitomycin C was seen to own several side effects and in particular after transurethral resection it has been described to delay the healing of the mucosa at resection sites both in animal and human [20, 21]. Urologists remained unconvinced that the benefits of MMC (immediate + maintenance), including a 30% relative reduction in the recurrence of a nonlethal disease, outweigh the potential harms, for example, cystitis, which can occasionally be severe and irreversible [22, 23].

In our opinion, to delay the recurrence time, from the endoscopic treatment procedure, of patients affected by NMIBC is of outstanding importance. In 2004, Sylvester et al. demonstrated, with a meta-analysis of seven randomized clinical trials, that single intravesical chemotherapy (IVC) following transurethral resection reduces recurrences [23]. Despite this, the adoption of this practice has been modest and a recent analysis described the rate of perioperatively intravesical administration of chemotherapy in as few as 0.3% to 3.2% cases [24].

Recently, the consideration that an urothelial disfunction could be the basis of benign bladder pathology: barrier disfunction in pathological bacteria urinary infections, increased permeability, decreased E-cadherin content, and an increased number of apoptotic urothelial cells in interstitial cystitis (painful bladder syndrome), has grown up. Sensory function impairment due to stones or neoplasms can cause changes to the urothelium resulting in bladder overactivity and urge incontinence, or in underactive bladder in case of outlet obstruction [25]. Similarly, an urothelial disfunction could be supposed to be linked with the development of bladder cancer. Based on such observation, some compounds that are able to repair the urothelium defect are now entering the clinical practice for the treatment of urothelial dysfunction. We could point out hyaluronic acid for the therapy of not malignant bladder conditions, such as bladder pain syndrome, interstitial cystitis, or recurrent urinary tract infections [26], or in combination with paclitaxel for the treatment of bladder bacillus Calmette-Guérin refractory carcinoma in situ [27].

3. Urothelium Impairment

The urothelium is the most superficial layer of the urinary tract that separates the lumen from underlying tissues of the wall. Formed by three layers, a basal, an intermediate, and a superficial or apical layer was composed of large hexagonal cells known as "umbrella cells" [28]; there are now strong evidences that the urinary bladder urothelium exhibits specialized sensory properties and plays a role in the detection and transmission of both physiological and mechanical stimuli, such as luminal pressure, urine composition, and nociceptive stimuli, beyond acting as an effective barrier [29]. Bladder's barrier function is conferred by a mucin layer formed by sulphated polysaccharide glycosaminoglycan (GAG), which covers the cellular apical surface. The mucin layer acts as a nonspecific antiadherence factor and as a defence mechanism against infection and irritants [30], but several agents, such as chronic bacterial infections, autoimmune diseases, chemotherapeutic agents, or external sources

(e.g., radiation exposure), can lead to urothelial damage and loss of the GAG function [31].

There is a wide consensus that many clinical conditions may arise from a primary defective urothelial lining [32] and in particular from a GAG injury. This injury induces a loss of the watertight function and leads to an infiltration of normal and abnormal constituents of urine through the lesion causing a failure in the healing process and producing chronic bladder epithelial damage and neurogenic inflammation [33]. In a randomised placebo-controlled trial, it has been shown that, restoring the GAG layer with intravesical administration of a combination of hyaluronic acid and chondroitin sulphate, in women with a recurrent urinary tract infection (UTI), the UTIs rate could be reduced without causing severe side effects while improving quality of life over a period of a year [34].

As mentioned previously, bladder urothelium acts as a specialized sensory tissue mediating both afferent and efferent signals through a flourishing subset of receptors and mediators. Receptors for purines [35], noradrenaline [36], bradykinin [37], and acetylcholine [38, 39] and several transient receptor potential (TRP) channels (TRPV1, TRPV2, TRPV4, TRPM8, TRPA1) [40–43] are expressed on the membranes of urothelial cells. From a neural point of view, an urothelial damage and the loss of the GAG function lead, in the suburothelium, to the activation of a subset of unmyelinated C fibres selectively sensitive to capsaicin. These unmyelinated C fibres serve as primary afferents in the regulation of micturition reflex and pain sensation and activation of visceral reflex but are even involved, through their efferent function, in the regulation of the lower urinary tract influencing the smooth muscle contraction [44], immune cell migration, mast cells degranulation, and neurogenic inflammation, thus playing a role in bladder inflammation [45].

These notions, added to the description of a decrease in both rate of contraction and bladder hyperreflexia in cyclophosphamide-inflamed rat urinary bladders after administration of Capsazepine, a selective antagonist for TRPV1 [46], lead to speculation about a role of this family of sensory receptor in the treatment of cystitis-induced hyperalgesia, through targeting their activity on C fibres.

Furthermore, the prolonged GAG defect persistence leads to a chronic stimulation of suburothelial tissues, which results in the allodynia caused by a visceral hypersensitivity of bladder C-fibre nociceptors, and in molecular changes, such as altered expression of transcription factors or activation of cellular inflammatory pathways (e.g., NF-κB related), producing an increase in frequency, urgency, nocturia, and chronic pain, characteristic symptoms of cystitis [47, 48].

According to these basics and to the more recent studies [26, 49], the early repair of the mucin layer by intravesical administration of mucopolysaccarides should be obtained to avoid the chronic evolution of the bladder inflammatory pathologies. A chronic defect of GAGs might allow irritants to reach the basal cells and to determine an anomalous cell growth.

4. Transient Receptor Potential Vanilloid 1

Transient receptor potential (TRP) superfamily of ion channels was first cloned from the visual system of *Drosophila* at the end of the 1980s [50].

In mammalian, the 28 TRP superfamily members are expressed in the plasma membrane of several cell types, principally mediating calcium and sodium inward membrane currents [51].

TRP proteins were first divided into three main subfamilies: the canonical or classical TRP proteins (TRPC), the vanilloid receptor related TRP proteins (TRPV), and the melastatin-related TRP proteins (TRPM). Later, the mucolipins (TRPML), the olycystins (TRPP), and the ankyrin transmembrane protein 1 (TRPA1) subfamilies have been included [40].

Numerous members of TRP superfamily of ion channels are expressed in the LUT. TRP expression in the mouse bladder was described showing the presence of 27 TRP family members mRNA in whole bladder tissue [52]. A pivotal role of the thermosensitive TRPs TRPV1, TRPM8, and TRPA1 and of TRPV2 and TRPV4, in normal and pathological LUT function, has been established, mainly as sensors of stretch or chemical irritation [53, 54], but information regarding the functional significance of others TRP proteins in the LUT still remains limited.

In 1993, Szallasi et al. characterized binding sites for capsaicin in rat urinary bladder [55], while in 1997 Caterina et al. cloned the vanilloid receptor presently known as TRPV1 [10], a nonselective, highly Ca^{2+}-permeable cationic channel.

This channel is widely distributed in LUT structures [15] and, although there are few solid data, it has been reported that in some species, for example, mouse, the TRPV1 channel is expressed not only in bladder neurones, but also in urothelial cells' membranes [56, 57].

Firstly, TRPV1 used to be considered as an integrator of thermal and chemical noxious stimuli, but evidences led it to be thought as a possible regulator of bladder reflex activity and cell differentiation [58].

The role of TRPV1 in bladder function is well established and basic scientific evidence supports TRPV1 action in the regulation of the frequency of bladder reflex contractions, even in chronically inflamed rat urinary bladders [46].

TRPV1 channels in the suburothelium are needed for normal excitability of low-threshold bladder fibres. In TRPV1-knockout mice low-threshold response has been seen attenuated compared with their wild type TRPV1 littermates, whereas high-threshold sensitivity was unchanged [59]. Furthermore, the knockout of TRPV1 in mice led to intermicturition spotting, suggested by an increase in the frequency of nonvoiding contractions in cystometrograms, but it does not affect normal micturition [53].

Maggi et al. described in humans a functional modulation of capsaicin sensitive C-fibers on the micturition reflex. Moreover, after the intravesical administration of capsaicin, authors observed a reduction of the first desire to void, bladder capacity and pressure threshold for micturition, and an attenuation of symptoms of hypersensitive LUT disorders. Moreover, TRPV1 activation generates a burning

pain sensation, similar to that reported by CP/CPPS patients during micturition and ejaculation [60].

Although both capsaicin and resiniferatoxin (RTX) have been tested by intravesical administration to reduce pain in patients with painful bladder syndromes, including interstitial cystitis [6, 60, 61], the first large randomized clinical trial testing several concentrations of RTX, compared against placebo, did not detect any advantage in the use of the compound [62].

Moreover, the expression of TRPV1 in terminal nerves ending near to the mucosa and to the smooth muscle cells could indicate its involvement in maintaining the tone of the muscular layer [51], and the relationship between the capsaicin selective activation of bladder TRPV1 and the increase of dieresis and natriuresis supported the hypothesis of a TRPV1 involvement in fluid homeostasis [2].

It is well described how malignant cell transformation is often accompanied by changes in ion channel expression [63] and among them several members of the TRP family Ca^{2+} and Na^{+}-permeable channels show altered expression in cancer cells.

To date, most changes involving TRP proteins do not involve mutations in the TRP gene but rather an increase or a decrease in the wild type TRP protein levels of expression, depending on the stage of the cancer. It is not yet possible to say whether these changes in TRP expression are central steps in the progression of the cancer or are secondary to other cellular modifications. Irrespective of the answer to this question, several TRP proteins have been shown in the last years to be valuable markers in predicting the progress of urological cancers and have been considered potential targets for pharmaceutical treatment [64–66].

Recently, the expression of TRPV1 has been described associated with urothelial cancers of human bladder [67].

Capsaicin, the major TRPV1 agonist, has been shown to inhibit in vivo and in vitro cancer growth and progression and to induce apoptosis of different cancer cells [68–70].

An antimigration activity of capsaicin on highly metastatic B16-F10 melanoma cells was investigated. A significant inhibition in the migration of melanoma neoplastic cells, effect correlated with the downregulation of phosphatidylinositol 3-kinase (PI3-K) and of Akt, PI3-K downstream target were described. Moreover in the same study, capsaicin was found to significantly inhibit Rac1 activity in a pull-down assay [71].

The genetic deficiency of TRPV1 was described to induce a higher incidence and number of tumors in the distal colon and an accentuated development of colonic adenomas, in mice affected by colitis-associated cancer and in APCMin−/+ model of spontaneous colon cancer, respectively [72].

In bladder urothelium, Lazzeri et al. firstly demonstrated how the expression of TRPV1 decreases from normal urothelium to transitional cell carcinoma (TCC) of human bladder. From specimens, obtained by multiple cold cup and full-thickness biopsy during open surgery, they showed a reduced labelling intensity after immunohistochemistry in superficial TCC a very light labelling was occasionally detected in the muscle invasive TCC from scattered superficial cells, and no labeling was present in the basal cells and in those that had invaded the adventitia. Similarly, in the Western Blot, which in the controls recognized two thick stained bands, in all superficial TCCs the two bands were similar to control ones, whereas they were very thin in muscle invasive and no band was detected in the patients staged as pT4 [73].

More recently, Amantini et al. displayed a marked decrease or absence of TRPV1 labelling in urothelial cancer specimens proportionally to differentiation levels decrease after a quantitative real-time PCR and that TRPV1 mRNA level was highly expressed in low-grade cancers, whereas its expression, confirming the previous results, was reduced in high-grade tumors or in advanced stage invasive pathologies. In the same study, the treatment of low-grade RT4 human urothelial cell carcinoma with capsaicin at 100 μM dose induced a TRPV1-dependent G0/G1 cell cycle arrest and apoptosis, effect that was seen associated with the transcription of proapoptotic genes including Fas/CD95, Bcl-2, and caspases, and the activation of the DNA damage response pathway [74].

On the other hand, attention has to be paid to the Capsaicin property to exhibit tumor-promoting effects, in a receptor-dependent manner, in particular in cancer strain cells lacking TRPV1 receptor, where the transfection with the TRPV1 cDNA leads to an increase in capsaicin-mediated calcium level, growth inhibition, apoptosis, and capsaicin-induced migration regression, suggesting that the TRPV1 plays an inhibitory role in urothelial cancer invasion and metastasis [75].

However, it is necessary to recognize that the mechanism of action of agonists such as capsaicin may be independent by TRPV1 activation. An example is the aforementioned work of Shin et al. on B16-F10 melanoma cells, where the authors described how capsaicin could have a role in the regulation of intracellular pathways independently from TRPV1 activity [71]. Other studies suggested an inhibition of migration induced by capsaicin without an involvement of TRPV1.

In 2002, Surh indicated that capsaicin could mediate apoptosis in human skin cancer cells through the inhibition of mitochondrial and plasma membrane electron transport systems inducing an excessive generation of reactive oxygen species [76]. In the same way, an increase in the reactive oxygen species after capsaicin administration was confirmed in 2005 by Qiao et al. [77].

Recently, Gonzales et al. demonstrated that, in vitro and in mouse xenografts, the local delivery of capsazepine decreases cellular duplication rate and reverses the growth of oral squamous carcinoma cells, inducing the production of reactive oxygen species and apoptosis, and mediating these actions independently from TRPV1 activation. This data was confirmed by calcium imaging technique, which showed how TRPV1, even if present, did not respond to capsaicin (alone or in combination with capsazepine) activation at noncytotoxic concentrations in all cancer cell lines, whereas a significant calcium influx was described, in positive controls, after ionomycin (nonselective cation channel agonist) administration. Moreover, they described that at equal concentration capsazepine is more effective at inhibiting cell viability than capsaicin, without adverse effects on nonmalignant tissues,

after in vitro and in vivo administration of the TRPV1 antagonist [78].

All the data showed lead to speculation about a possible clinical involvement for the TRPV1, not only for the treatment of bladder urothelial inflammatory condition or in pain reduction in patients with painful bladder syndromes, but even in the management of tumoral conditions.

The in vivo and in vitro cancer growth and progression inhibition, the induction of apoptosis, and the antimigration activity of capsaicin in tumor of other tissues, such as gastric cancer and glioma, and the studies of Lazzeri and Amantini's groups suggest a theoretical role of targeting the TRPV1 for clinical management of urothelial cancer.

In particular, a modulation of TRPV1 activity could be interestingly investigated in the management of NMIBC after a transurethral resection for both the anti-inflammatory and cancer recurrence rate decreasing possible features.

5. Curcumin

Curcumin [diferuloylmethane, 1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is an active principle held inside in the rhizome of *Curcuma* species widely used as a yellow coloring and flavoring agent in food [79].

Its pharmacological benefits have been appreciated since ancient times in particular for the antioxidant and anti-inflammatory properties.

Curcumin's metabolism is explicated by the endogenous reductase system that reduces it to dihydrocurcumin, tetrahydrocurcumin (THC). and trace of hexahydrocurcumin, in a stepwise manner, to be lately glucuronidated by the uridine 5′-diphospho (UDP)-glucuronosyltransferase [80]. Furthermore, some studies have described a higher antioxidant activity [81, 82], and in some models a higher preventive effect on carcinogenesis [83] of tetrahydrocurcumin was compared to curcumin.

Several studies have shown that curcumin induces apoptosis, via deactivation of nuclear factor-kappa B (NF-κB), and its regulated gene products, in addition to suppression of cell proliferation, invasion, and angiogenesis. Curcumin was also found to suppress several inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukins (IL-1, -1b, -6, and -8), and cyclooxygenase- 2 (COX-2) [84].

Shehzad et al. described the main roles that curcumin may perform in inflammatory pathways (the most important of them are summarized in Table 1) and in the management of the related chronic inflammatory diseases [85].

Cheng et al. demonstrated, in urinary bladder isolated from Wistar rats, that curcumin has an ability to activate M1-mAChR for increase of glucose uptake in muscle. Believed as the most common subtype of muscarinic receptor in skeletal muscle, mAChR has been introduced as an important factor in the regulation of muscle tone.

In the same study, Cheng's group found that curcumin caused a concentration-dependent increase of muscle tone in urinary bladder isolated from Wistar rats. This action was inhibited by pirenzepine at concentration high enough to block M1-mAChR [86].

Table 1: Main inflammatory pathways influenced by curcumin activity.

Chronic disease	Major mediators involved
Arthritis	COX, MMPs, STAT3, NF-κB
Scleroderma	MAP kinase, NF-κB, TGIF
Psoriasis	STAT3, NF-κB, Bcl-xL, IAPs
Allergies and asthma	MAP kinase, NF-κB
Diabetes	PPAR-γ, NOS
Obesity	TNF-α, IL-6, Wint/β-catenin
Neuropathies	IL-1β, VEGF, NF-κB, TNF-α, NO, LRRK2
Cardiopathies	Bcl-2, IL-6, Caspase, NF-κB, TNF-α
Renal ischemia	HSP-70, MAP kinase, NF-κB, TNF-α

Due to the pleiotropic beneficial effects of curcumin in inflammatory disease, its role in urothelial pathologies has been speculated.

Though not well established, the main field investigated is the curcumin preventive effect in the development of hemorrhagic cystitis after cyclophosphamide administration.

The prior administration of curcumin before cyclophosphamide challenge, possibly through modulating the release of inflammatory endocoids, was shown to improve all the biochemical and histologic alterations induced by the cytotoxicity, ameliorates the energy status, and restores the oxidant/antioxidant balance [87].

Feasibility and curative effects of an intravesical treatment for cystitis glandularis, a metaplastic alteration of the urothelium in the urinary bladder due to persistent infection, calculi, bladder exstrophy, outlet obstruction, or even tumor, were, respectively, explored and displayed administrating curcumin in 14 patients, diagnosed with the pathology, which remained symptomatic after the state-of-the-art designed primary treatments [88].

Curcumin controls cell proliferation and cycle progression via the modulation of enzymes, growth factors and their receptors, cytokines and various kinase proteins activities. A potential therapeutic involvement has been discussed for pulmonary, digestive system, reproductive system, breast, hematological, thymic, bone, and brain tumors.

Furthermore, studies have shown how not only curcumin, but also its analogues 3,5-Bis(2-fluorobenzylidene)-4-piperidone (EF24) and 3,5-Bis(2-pyridinyl-methylidene)-4-piperidone (EF31), a more potent inhibitor of NF-κB activity than either EF24 or curcumin, exhibit both anti-inflammatory and anticancer activities [89].

In the urological field, curcumin seems to have a role in the management of prostate, kidney, and urothelial bladder cancer regulating cell survival, proliferation, invasion, and angiogenesis (Table 2).

In prostate, curcumin induces apoptosis in androgen-dependent (LNCap) and androgen-independent (DU15) prostate cancer cell lines [90], downregulating antiapoptotic genes, such as Bcl2 and Bcl-xL, and inducing procaspase-3 and 8. Curcumin also inhibits the prostate specific antigen and decreases the expression of AP-1, cyclin D1, NF-κB, cAMP response element-binding (CREB), EGFR tyrosine

Table 2: Cancer regulator factors influenced by curcumin activity in urological neoplasia.

Urological cancers	Major mediators involved
Prostate cancer	EGFR, AP-1, cyclin D1, NF-κB, CREB
Kidney cancer	Bcl_2, Bcl-xL, ROS, Akt, TRAIL, IAP
Bladder cancer	Bcl_2, AP-1, cyclin D1, VEGF, NF-κB

kinase, and the activities of androgen receptor-dependent NKX3.1 [91]. Furthermore, a decrease of cell proliferation, colony formation, and cell motility and an enhancement of cell aggregation through the activation of protein kinase D1 have been described, which in turn inhibits nuclear b-catenin transcription activity [92]. Herbal preparations based upon curcumin extracts were given to the HGPIN patients three times per day for 18 months. The 18-month biopsy revealed no markers of HGPIN and a reduction in NF-κB and C-reactive protein [93].

In human, bladder cancer cells studies have shown that curcumin induces apoptosis downregulating Bcl2 and increasing the levels of Bax and p53, and moreover it inhibits the development of urothelial tumors in a rat bladder carcinogenesis model [94]. Other effects described are the downregulation of VEGF and VEGF receptor 1 (VEGFR1) and the inhibition of NF-κB and cyclin D1 [95]. Furthermore, it has been described that intravesical injection of curcumin can inhibit bladder cancer in female C57BL/6 mice implanted with MB49 bladder cancer cells [96].

Tharakan et al. described that curcumin potentiates the apoptotic effects of gemcitabine against human bladder cancer, where curcumin also suppresses the cell survival transcription factor NF-κB activated by gemcitabine. Moreover, in orthotopic mouse model curcumin alone significantly reduced the bladder tumor volume and decreased the proliferation marker Ki-67 and microvessel density, but maximum reduction was observed when curcumin was used in combination with gemcitabine. At least, as just described in other studies, they confirmed how curcumin abolishes the constitutive activation of NF-κB in the tumor tissue; decreases cyclin D1, VEGF, COX-2, c-myc, and Bcl-2 expression in the bladder cancer tissue; and induces apoptosis [97].

At least, curcumin has the same vanilloid ring as capsaicin, a particular structure considered necessary for the activation of the TRPV1 receptor [98], suggesting that TRPV1 is a plausible target for the pharmacological action of curcumin.

Several studies have investigated the role of curcumin in the thermal and pain regulatory patterns involving the TRPV1. It was shown that curcumin attenuates thermal hyperalgesia in diabetic neuropathic pain [99], produces an antihyperalgesic effect in a formalin-induced orofacial pain model in rats [100], and ameliorates the severity of damage in dinitrobenzene sulphonic-acid-induced colitis, possibly by acting as a TRPV1 agonist [98]. At least, curcumin reduced capsaicin-induced currents in a dose-dependent manner in both trigeminal ganglion neurons; thus, the antagonism of TRPV1 may provide a potential mechanism underlying the antihyperalgesic effect of curcumin [101].

6. Clinical Perspectives

The genesis of neoplastic lesions of urothelial epithelium, in particular of bladder urothelium, recognizes different causes and the major risk factors could be divided into inherited or acquired. The most important risk factor is undoubtedly the habit of smoking, but even the work-related and environmental conditions play an important role.

Among medical conditions, chronic inflammation, chronic urinary retention, and upper tract dilation, which cause the increase of urothelial exposure to carcinogens, are the most pathological features involved in the carcinogenesis.

It has been hypothesized that the inflammatory condition linked to the disruption of the urothelial layer could be involved in the processes of cancer development as seen in other tumoral conditions (e.g., colorectal cancer [102]).

In this context, agents like TRP channel ligands, involved in functional and pathological pathways, could play an important role.

The vanilloid receptor TRPV1, owning a role in the modulation of urothelial inflammatory condition, could be thought as an interesting factor in the management or in the prevention of neoplastic pathologies.

In particular, its targeting could be conjectured in case of NMIBC after a transurethral resection where its characteristic of anti-inflammatory agent could be helpful for the epithelium healing or in the management of patients during the postoperative time.

Moreover, the property of inducing apoptosis and the antimigration activity makes TRPV1 an interesting target in the handling of recurrences.

Curcumin, the major component of turmeric *Curcuma longa*, has been described to own benefic effects in pathological pathways common of both inflammation and carcinogenesis.

Its applications for pathologies involving urothelium disruption such as cystitis glandularis or hemorrhagic cystitis cyclophosphamide-induced have been successfully investigated. In a like manner, its intrinsic property in cell survival and angiogenesis regulation has been shown in several tumor tissues including bladder cancer, where in particular an increase of apoptotic effect has been described after its association with Gemcitabine.

Moreover, owning a vanilloid ring, curcumin might be able to activate the TRPV1 receptor. The combination of the intrinsic properties of curcumin in association with the capacity of acting on TRPV1 could make this compound a very interesting agent in the management of urothelial dysfunctions. However, this hypothesis needs further studies to be confirmed.

In this context new compounds, such as curcumin, could be complementarily used in the clinical practice to manage the recurrences and soothe the inflammatory effect of transurethral resection or intravesical chemotherapy administration, or in combination with the chemotherapies to potentiate the antitumor effect.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Cystitis: From Urothelial Cell Biology to Clinical Applications

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Cystitis is a urinary bladder disease with many causes and symptoms. The severity of cystitis ranges from mild lower abdominal discomfort to life-threatening haemorrhagic cystitis. The course of disease is often chronic or recurrent. Although cystitis represents huge economical and medical burden throughout the world and in many cases treatments are ineffective, the mechanisms of its origin and development as well as measures for effective treatment are still poorly understood. However, many studies have demonstrated that urothelial dysfunction plays a crucial role. In the present review we first discuss fundamental issues of urothelial cell biology, which is the core for comprehension of cystitis. Then we focus on many forms of cystitis, its current treatments, and advances in its research. Additionally we review haemorrhagic cystitis with one of the leading causative agents being chemotherapeutic drug cyclophosphamide and summarise its management strategies. At the end we describe an excellent and widely used animal model of cyclophosphamide induced cystitis, which gives researches the opportunity to get a better insight into the mechanisms involved and possibility to develop new therapy approaches.

1. Introduction

Cystitis is inflammation of the urinary bladder with diverse and often unknown etiology. Our understanding of cystitis rests in the comprehension of currently insufficient but rapidly growing knowledge about structure-function relationships within urinary bladder and its interaction with other organ systems, especially with nervous system. Urinary bladder wall is composed of three layers: (i) the mucosa, (ii) the muscularis propria, and (iii) the adventitia/serosa. The mucosa contains the urothelium, the epithelium which faces the urine, basal lamina, which separates urothelium from underlying connective tissue, and lamina propria. Lamina propria is composed of an extracellular matrix containing several types of cells, including fibroblasts, myofibroblasts/interstitial cells, immune cells, and afferent and efferent neurons. In addition, lamina propria contains blood and lymphatic vessels, elastic fibres, and smooth muscle fascicles (muscularis mucosae). Muscularis mucosa is not very well defined in the human bladder and sometimes seems to be absent [1]. Muscularis propria is formed by the detrusor muscle, which is organised into three layers of smooth muscle fibres running in different directions. Urothelium lines not only the inner surface of the urinary bladder, but also the renal pelvis, ureters, and proximal urethra [2]. The urothelium of the urinary bladder is composed of three distinctive cell layers. Functionally, it forms a high-resistance permeability barrier (blood-urine barrier) to molecules and ions as well as pathogens in the urine and can accommodate to large changes in urine volume during micturition cycles [3]. Terminally differentiated superficial cells, called umbrella cells, are responsible for maintaining the blood-urine barrier, which depends on two structures: tight junctions with the highest resistance in the mammalian body [4] and the apical plasma membrane with unique specializations named urothelial plaques [5]. Furthermore, these cells are resistant to large mechanically deforming forces such as stretch (during filling and storage) and sudden compression (during voiding), which is accomplished by their high foldability and capacity to alter their apical surface area by exocytosis and endocytosis [6]. Moreover, the urothelium acts as an integral part of the urinary bladder sensory web, which receives,

amplifies, and transmits information to the underlying tissues including sensory nerve fibres, myofibroblasts, and smooth muscle cells [7]. In this respect, urothelium releases various mediators and neurotransmitters to reflect its degree of physical distension, so that both sympathetic and parasympathetic nerves can coordinate normal bladder function during filling and voiding [8]. The permeability barrier and sensory function of the urothelium are compromised in various diseases that affect the urinary bladder. For example, it is proposed that in patients with neurogenic detrusor overactivity lower permeability barrier of the urothelium might lead to enhanced signalling responsible for urinary frequency and bladder pain [9]. Similar events are observed in cystitis, where lower permeability barrier of the urothelium could be directly correlated with defective differentiation of urothelial cells [4].

2. Urothelial Differentiation and Formation of Blood-Urine Permeability Barrier

The function of the urothelium as an effective blood-urine barrier is accomplished by its normal differentiation process. Differentiation runs from basal cell layer, facing the basal lamina, across intermediate to the superficial cell layer, which is in contact with urine. Basal cells are small and they can divide mitotically. Some of the basal cells are urothelial stem cells but their identification remains controversial because of the lack of specific markers [10]. It has been proposed that 9% of basal cells represent putative urothelial stem cells in rat urothelium [11]. Above basal cell layer towards the lumen of the urinary bladder there are intermediate cells. The thickness of intermediate cell layer differs between mammalian species; in rodents it is one cell layer thick, while in human it is up to six cell layers thick. Intermediate cells in rodents start to express urothelium-specific proteins, uroplakins (UPs; Figure 1(a)) [12, 13]. However, UPs are detected primarily in the superficial umbrella cells in human urothelium [14]. UPs belong to a group of evolutionary conserved integral membrane proteins that comprises four major members, UPIa (27 kDa), UPIb (28 kDa), UPII (15 kDa), and UPIIIa (47 kDa) [15, 16]. UPIa and UPIb belong to the tetraspanin family, while UPII and UPIIIa have a single transmembrane domain. All four UPs have large extracellular domains, which gives the urothelial membranes a thickened (12 nm) asymmetric appearance, readily seen with transmission electron microscope [17]. UPs appear in dimers, namely, UPIa/UPII and UPIb/UPIIIa heterodimers. These heterodimers associate to form heterotetramers and such six heterotetramers are assembled into a 16 nm intramembrane particle [18]. Hexagonally packed 16 nm particles form two-dimensional crystals known as urothelial plaques, which are interconnected by thinner membranes known as hinges [19]. In intermediate cells of rodents, UPs are present in the membranes of cytoplasmic vesicles, called fusiform vesicles, but not in the plasma membrane [20]. In superficial umbrella cells, terminal urothelial differentiation is achieved. Umbrella cells have high levels of UPs expression (Figure 1(a)), which is reflected in the formation of large urothelial plaques in post-Golgi compartments [21]. Two urothelial plaques form each fusiform vesicle, which is therefore flattened in shape (Figure 1(b)). Usually 4–15 fusiform vesicles are joined together into stacks [20]. Such shape and organisation of fusiform vesicles make them a perfect storage compartment, which can transport large amounts of urothelial plaques to the apical plasma membrane of umbrella cells. It is believed that fusiform vesicles fuse with the apical plasma membrane during distension of the urinary bladder. This exocytotic event is not completely understood in umbrella cells but it was proposed that cytokeratins, Rab27b, and MAL protein play important roles [22, 23]. Urothelial plaques cover 70–90% of the urothelial apical surface, which can be demonstrated by scanning electron microscopy (Figure 1(c)), and they represent structural basis for blood-urine barrier.

The expression of UPs and the presence of urothelial plaques are therefore two main characteristics for establishing urothelial differentiation and also for predicting functional, high-resistance permeability barrier [24, 25]. Moreover, UPs are also suggested as useful markers for diagnosis, detection, and prognostic prediction of urothelial carcinomas [26].

3. Cystitis and Advances in Its Research and Patients Care

Cystitis can be clinically described as a syndrome of dysuria, urgency, frequency, and lower abdominal pain. Although cystitis is usually caused by bacterial infection, it can also be caused by noninfectious conditions such as carcinoma in situ, bladder cancer, and bladder stone or it can even emerge from unknown origin as in interstitial cystitis [27]. Urologists usually distinguish cystitis of infectious origins and of noninfectious origins. The category of infectious cystitis can further be classified into uncomplicated cystitis and complicated cystitis (Table 1). Uncomplicated cystitis can be described as an infection in women with a structurally and functionally normal urinary bladder. However, complicated cystitis is associated with structurally or functionally abnormal urinary bladder where the host is compromised and pathogens develop antimicrobial resistance. After careful differential diagnosis, appropriate treatment must be used, which results in successful management in most cystitis instances [28, 29].

Most cases of cystitis occur in women. In addition, each year approximately 10% of all women report a urinary tract infection and more than 50% of all women have at least one such urinary bladder infection in their lifetime [27, 30]. The symptoms of cystitis are very variable but usually painful urination, urgency, frequency, lower abdominal pain, and haematuria can develop (Table 1). Presence of clinical symptoms or signs is sufficient to diagnose uncomplicated cystitis in addition to simple urine analysis with microscopic findings and gram staining. Urine culture in every patient with the infection is usually recommended [27, 31]. Some of the patients may experience recurrent cystitis [32]. The definition of recurrent cystitis is two or more symptomatic cystitis episodes over a 6-month period or three or more cystitis episodes within a one-year period (Table 1) [27,

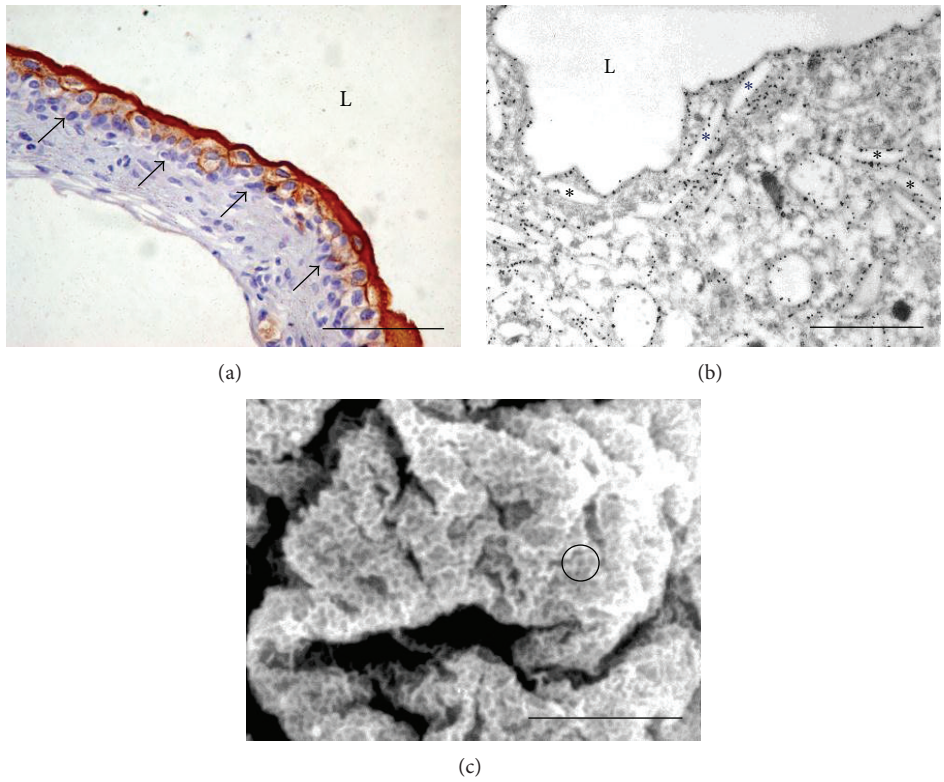

Figure 1: Normal mouse urinary bladder with terminally differentiated umbrella cells in the superficial layer of the urothelium. (a) Immunolabelling on paraffin section shows strong expression of uroplakins in umbrella cells (dark brown), weaker expression in intermediate cells (light brown), and negative basal cells that lie on the basal lamina (arrows). (b) Immunolabelling on ultrathin section demonstrates uroplakins (12 nm colloidal gold particles, black) in the membranes of fusiform vesicles (asterisks) and in the apical plasma membrane facing bladder lumen (L). (c) Scanning electron microscopy of the umbrella cell shows that its apical plasma membrane is covered with urothelial plaques (grey) interconnected by hinges (white). One such plaque and hinge region is encircled. Scale bars (a) = 50 μm, (b) = 1 μm, and (c) = 10 μm.

Table 1: Classification and clinical features of cystitis.

Category	Clinical features
Cystitis	Dysuria, urgency, frequency, lower abdominal pain, and haematuria
Uncomplicated cystitis	Cystitis in women with a structurally and functionally normal bladder
Isolated or sporadic	No cystitis symptoms in 4 weeks before this episode
Reinfection	At least 3 episodes of uncomplicated infection in past 12 months or at least 2 episodes of uncomplicated infection in past 6 months
Complicated cystitis	Cystitis in men or compromised host; cystitis with a structurally and functionally abnormal bladder

33]. Reinfection and bacterial persistence are two typical phenotypes in recurrent cystitis. In healthy women recurrent cystitis is usually caused by reinfection with new pathogens or different pathogens from outside the urinary tract and is classified as the category of uncomplicated cystitis [34]. Nevertheless, recurrent cystitis in compromised men is caused by the same pathogens from the same site within the urinary bladder due to bacterial persistence. This kind of recurrent cystitis is classified as chronic infection and usually occurs in structurally or functionally abnormal urinary bladder and can therefore be classified into the category of complicated cystitis [27, 34]. It is commonly caused by various pathogens with antimicrobial resistance [27] and the patients with chronic cystitis usually have various additional complicating factors, which contribute to the infection [29]. Uncomplicated cystitis usually occurs at variable intervals by different species, while chronic infection is due to the same organism at very close time intervals.

The most common pathogen in uncomplicated and complicated cystitis is uropathogenic *E. coli* (UPEC) strain, followed by *Staphylococcus saprophyticus*, enterococci, coagulase-negative staphylococci, and other species of Enterobacteriaceae [27, 35]. The pathogenesis of UPEC in host cells has been relatively well documented [35, 36]. UPEC strains

originate from host's large intestine. However, in contrast to intestinal *E. coli* strains, UPEC strains have a number of virulence factors that enable them to invade into urothelium and survive against host defences [35, 37]. UPEC strains from intestine can adhere to and colonize the perineum and vagina and subsequently migrate to the urinary tract where they cause an inflammatory response in the urothelium [38, 39]. In addition, the increased epithelial receptivity for *E. coli* on the genitourinary organs can be associated with recurrent cystitis [40]. Almost all of UPEC strains express type 1 fimbriae and its adhesin, FimH, enables them to attach to urothelial surface receptor and invade into urothelium of the urinary bladder [37, 41]. Furthermore, UPEC strains typically express an array of toxins such as siderophores for iron acquisition systems and hemolysin and cytotoxic necrotizing factors for exploiting host nutrients and facilitating bacterial dissemination [35, 37]. UPEC strains gain a foothold in the urinary tract by binding FimH to uroplakin UPIa [36, 38]. Seeking intracellular refuge within urothelial cells is the only way that UPEC can avoid elimination by the voiding of urine from the urinary bladder or by the host's innate immunity [36, 38, 42]. Upon ligation of the UPEC to UPIa, widespread conformational changes within the apical plasma membrane of umbrella cells are induced, followed by engulfment of the UPEC into sanctuary [6, 38]. This bacterial invasion is mediated by localized host actin rearrangement and phagocytosis of the bound UPEC by zippering of the membrane around the microorganism [6, 43, 44].

Blocking the binding between UPIa of umbrella cells and FimH of bacteria is an ideal target for infectious cystitis treatment. Specific targeting of the FimH adhesion could be achieved by using the soluble receptor analogues or mannosides that act as antiadhesives. These molecules bind FimH and prevent it from interacting with host receptors [45]. Moreover, it has been reported that surfactant protein D (SP-D) inhibits bacterium-induced cytotoxicity by preventing adherence of UPEC to the umbrella cells and dampen UPEC-induced inflammation in mice [46]. However, we must point out a concern regarding the systemic administration of either mannosides or pilicides that are potentially adversely affecting commensal *E. coli* and other members of the intestinal microbiota, many of which also express type 1 pili [47].

Invasion into the umbrella cells allows UPEC to establish a new niche in an effort to protect itself from the host innate immune response [39, 43]. The intracellular UPEC can multiply within umbrella cells intracellular compartment to form the so-called intracellular bacterial communities, some of which can then switch into a quiescent phase to persist in the cells indefinitely. Intracellular quiescent nature of these bacteria provides their resistance to antibiotics and protects them from host neutrophils and other host surveillance systems [39, 43, 48, 49]. Intracellular signals, such as the reorganization of the actin filaments, can trigger the resurgent growth of UPEC, prompting the development and dispersal of intracellular bacterial communities leading to the recurrence of clinical symptoms [32, 50]. Recently, it has been proposed that the resurrection of these quiescent forms of UPEC is coincident with recurrent cystitis or bacterial persistence [51]. The urothelial cells must therefore prevent the UPEC attacks to survive. Upon contact with UPEC or their products, the host immune surveillance molecules evoke a variety of immune responses aimed at the early elimination of the invading uropathogens. There are many evidences that toll-like receptors (TLRs) are the major contributing factors to the immunogenic resistance of the urinary tract to this microbial attack. Mutant mice with inactive TLR4 are defective in their ability to clear urinary tract infections [52, 53]. This defect is attributed to the inability of urothelial cells to evoke an appropriate cytokine response to uropathogens, which results in limited recruitment of neutrophils to sites of infection in the urinary tract [53].

Even though the natural course of uncomplicated cystitis is usually self-limited and is spontaneously healed, the oral antibiotic agents are the first choice for its treatment [27, 32]. Empirical antibiotics that reveal less than 20% drug resistance among *E. coli* strains are usually recommended. Trimethoprim or trimethoprim and sulfamethoxazole have been widely used as effective and inexpensive agents for empirical therapy in the most areas of the world [27]. In some areas where high resistance to trimethoprim or trimethoprim and sulfamethoxazole has been observed, fluoroquinolone antibiotics are recommended as an alternative drug. Women with recurrent cystitis usually require careful consideration of medical history for the risk factors of reinfection and must consider long-term medical suppressive management [33, 54]. Spermicide use for birth control or for prevention of sexually transmitted infections can be associated with an increased risk of cystitis and vaginal colonization with *E. coli* [55]. Because spermicides with nonoxynol-9 may lead to reduction of vaginal lactobacilli, the preventive mechanisms against bacterial interference can become weak and therefore enhancement of the adherence of *E. coli* strains to vaginal epithelial cells occurs [55, 56]. In addition, the lack of estrogen in menopause women also causes marked changes in the vaginal microflora, including a loss of lactobacilli and increased bacterial colonization [57]. Sexual intercourse in women is also one of the risk factors for cystitis. Women with recurrent cystitis usually require low dose of continuous prophylaxis, self-start intermittent therapy, or postintercourse prophylaxis [27, 54, 55].

Complicated cystitis is the one that occurs in a patient with a compromised urinary tract or that is caused by a very resistant pathogen [29]. These infections are usually caused by an atypical and broad range of bacteria with resistance to multiple antibiotics. Urine cultures, therefore, are mandatory to identify the bacteria and decide for appropriated antimicrobial agents. Patients with chronic cystitis can usually be cured of the recurrent infections by identification and surgical removal or correction of the focus of infection [27]. In addition, functional or structural abnormalities should be corrected, and urinary tract function must be restored by medical, pharmacologic, or surgical management.

4. Haemorrhagic Cystitis and Its Treatment

Haemorrhagic cystitis (HC) is defined by urinary bladder irritation signs and haematuria. The disease can be triggered

by many circumstances including going through chemotherapy, receiving radiation therapy, and experiencing various bacterial and viral infections [59, 60]. The severity of HC has been reported to range from asymptomatic microscopic haematuria to life-threatening haematuria [61]. The clinical courses of HC are variable depending on the causes. The HC induced by infection is usually self-limited and resolves spontaneously or with appropriate antibiotic therapy. In some patients, however, the removal of the urinary bladder is necessary to save their lives, since life-threatening haematuria from anticancer agents sometimes cannot be controlled by conventional medical methods [62]. Additionally, physicians do not prescribe full therapeutic doses of anticancer agents in the treatment of cancers because severe urologic side effects of these agents have frequently been reported [63].

Cyclophosphamide (2-[bis(2-chloroethyl)amino]tetrahydro-2H-1,2,3-oxazaphosphorine 2-oxide) was first introduced as an antineoplastic agent in 1958 [64] and since then numerous reports have been published concerning haemorrhagic cystitis, a side effect not observed with other alkylating agents. Currently cyclophosphamide is still widely used in chemotherapy of B cell malignant diseases and some solid tumours, conditioning before bone marrow transplantation, and in the treatment of certain immunoinflammatory conditions, for example, Wegener's granulomatosis, rheumatoid arthritis, and systemic lupus erythematosus [65, 66]. Cyclophosphamide side effects depend on the dosage of cyclophosphamide used and can affect up to 75% of the patients receiving a high intravenous dose. The frequent side effects of cyclophosphamide in the urinary bladder range from irritative voiding symptoms, urinary frequency, dysuria, urgency, suprapubic discomfort, and strangury, with microhematuria, to the potentially life-threatening complication of haemorrhagic cystitis [63, 67]. Cyclophosphamide is metabolized in the liver and possibly in the kidney to 4-hydroxy metabolites (e.g., phosphoramide mustard, PAM, and acrolein) which are renally excreted and stored in the urinary bladder until voiding [68, 69]. PAM is the primary chemotherapeutic metabolite but it has minimal effects on the bladder, while acrolein was recognised as the causative agent in cyclophosphamide induced haemorrhagic cystitis [70]. Acrolein is a highly reactive aldehyde and the mechanism by which acrolein reaches the bladder is unclear, although it is suggested that it might be formed in the lumen of the bladder. Effects of acrolein on the bladder wall are contributed to its contact with umbrella cells and include necrosis, desquamation, oedema, ulceration, neovascularization, and haemorrhage [71]. The therapeutic targets in cyclophosphamide induced haemorrhagic cystitis are dysuria or micturition symptoms and massive haematuria. Dysuria, frequent voiding, and urgency may be controlled with medications, but massive haematuria is a life-threatening symptom and should be immediately controlled. Hyperhydration, bladder irrigation, and agents that can detoxify cyclophosphamide such as Mesna (2-mercaptoethane sodium sulphonate) have been the most frequently used prophylactic measures to prevent treatment-related cystitis but are not always effective [72]. In the search for new prevention and treatment approaches hyperbaric oxygen therapy, flavonoids or polyphenols, and melatonin are suggested as supportive treatment, but further studies are required for their translation into clinic [59, 73, 74]. Another promising clinical prophylactic agent is the epinephrine, which is a very important medicine for controlling vascular bleeding and the function of the sympathetic action. Interestingly, epinephrine also decreases the incidence and severity of cyclophosphamide induced cystitis in rats and has even a greater protective effect than Mesna [75]. The research team of Lee has recently reported that intravesical application of epinephrine has an attenuating effect on uroplakin expression, submucosal edema, and hemorrhage in cyclophosphamide induced rat cystitis [58, 76] (Figure 2). Concurrently, intravesical epinephrine preserved both subtypes of alpha1A- and alpha1B-adrenergic receptor expressions in urinary bladder [58]. Before the clinical application of intravesical epinephrine therapy for cyclophosphamide induced haemorrhagic cystitis, one must consider some hypothetical weak points. First, since α-adrenergic stimulation produces relaxation in the bladder body and contraction in bladder neck or prostatic urethra [77], delayed voiding or acute urinary retention can occur. Second, to expect optimal therapeutic effects through intravesical instillation therapy, it is very important to hold the intravesically injected epinephrine within the bladder for maximal absorption. However, exposure to prolonged stagnant urine also poses a risk of longer contact with toxic metabolites of cyclophosphamide. Third, vigorous diuresis or continuous urinary bladder irrigation and frequent urination cannot sustain the therapeutic dosage of intravesically instilled epinephrine and can therefore weaken the effect of the treatment [58].

The first experimental study of cyclophosphamide induced bladder toxicity was that of Philips et al. [78]. In this study it was concluded that urotoxicity is due to contact between the urothelium and cyclophosphamide metabolites in the urine. In rats approximately 70% of the metabolites of the drug are excreted in the urine within 4 h after administration of a single intraperitoneal dose [69]. Although single intraperitoneal injection of cyclophosphamide causes reversible urothelial hyperplasia with gradual restoration of normal three-layered urothelium [69, 79, 80], repeated doses can lead to premalignant and ultimately to malignant transformation [81, 82]. Moreover, it is known that patients treated with cyclophosphamide have up to a ninefold increased risk of developing bladder cancer [83, 84].

5. Experimental Models of Haemorrhagic Cystitis

An animal model of cyclophosphamide induced haemorrhagic cystitis is one of the best described and methodically developed models. With minor modifications it is currently widely used experimental tool for investigation of pathogenesis, prevention, and treatment of haemorrhagic cystitis as well as urothelial injury, bladder inflammation,

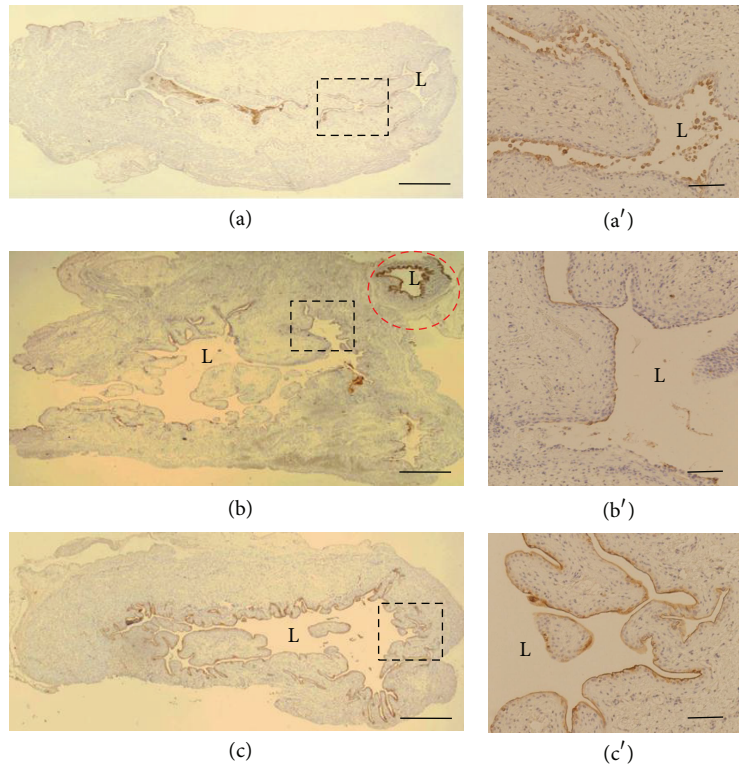

Figure 2: Epinephrine treatment preserves UPII expression in rat urinary bladder 24 hours after cyclophosphamide injection. UPII expression (brown) is a well-established transitional urothelial marker that is strongly expressed along the mucosal area in dilated ureter (red circle). (a) Cyclophosphamide injected rats showed a decrease or loss of UPII expression. (b) Urethral obstructed and null-treated rats at 24 hours after cyclophosphamide injection revealed a significant decrease or loss of UPII expression. (c) Intravesical epinephrine treated rats after cyclophosphamide injection showed much better expression pattern of UPII along the bladder mucosa. L: lumen of the urinary bladder or of the ureter. Scale bars (a–c) = 1000 μm and (a′–c′) = 200 μm. Reprinted from Kyung et al., 2012 [58], with permission of Springer-Verlag.

bladder-related pain, and acute and chronic overactive bladder [58, 59, 76, 81, 85, 86]. An immediate effect of cyclophosphamide metabolites is seen as widespread destruction of the urothelium, which is accompanied not only by necrosis but also by apoptosis of urothelial cells, with only a few surviving cells remaining after 24 hours (Figure 3) [78, 87]. The surviving cells retain their ability to proliferate and reepithelialize denuded areas [78, 79, 86]. It seems that EGF initiates cell proliferation by binding to EGFR and rapid proliferation of remaining urothelial cells leads to hyperplastic urothelium formation [80]. It should be noted that the normal bladder urothelium is unresponsive to EGF from urine because of the absence of epidermal growth factor receptors (EGFRs) from the superficial layer. Cyclophosphamide exposes partially differentiated urothelial cells that express EGFR in their plasma membranes, which enables urinary EGF to stimulate proliferation. Reversible hyperplasia develops already by days 2 and 3 after cyclophosphamide injection (Figure 3), while gradual restoration of a normal three-layered urothelium is achieved within 2 to 3 weeks [69, 79, 80]. Hyperplastic urothelium enables fast resealing of the injury and represents the key mechanism for the maintenance of functional permeability barrier of the urothelium lacking umbrella cells [88]. The main mechanisms for restoration of a normal three-layer urothelium and its regeneration are reduced proliferation and increased apoptosis of urothelial cells, which is accompanied by *de novo* differentiation of umbrella cells [25, 80, 89], which restore efficient blood-urine barrier [69, 79].

6. Conclusion

Our understanding of basic urothelial cell biology is essential for comprehension not only of normal urinary bladder functioning but also, and more importantly, of mechanisms underlying different urinary bladder disorders, including cystitis. Unique differentiation of urothelial cells with expression of specific proteins' uroplakins and their organisation into urothelial plaques ensures proper functioning of the urinary bladder as urine-blood permeability barrier in healthy individuals. In cystitis, the barrier is disrupted leading to different symptoms. Treatment of cystitis is usually restricted to symptom management, but unfortunately it is often ineffective or insufficient. New experimental tools and promising therapeutic targets represent challenging options for future research. In this respect, cyclophosphamide induced cystitis has been proven as an excellent research model. Currently, fundamental research of urothelial biology, cystitis origin, and development as well as its prevention and treatment is

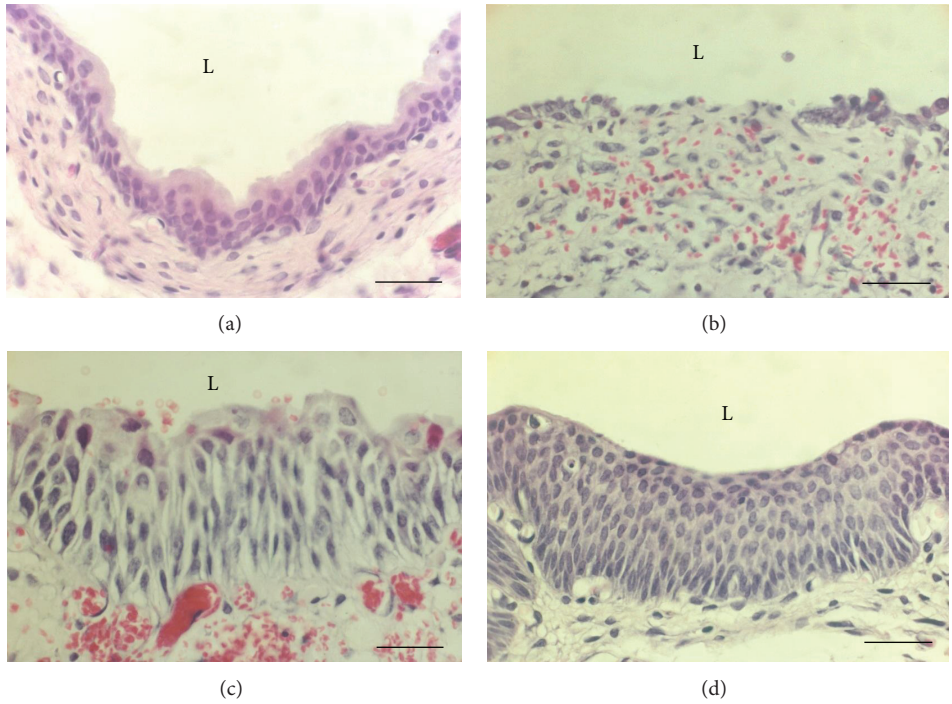

Figure 3: Cyclophosphamide induced changes of the rat urothelium. (a) Normal three-layered urothelium. (b) Urothelium on day 1 after cyclophosphamide injection with some remaining urothelial cells and with denuded areas. Haemorrhage is evident. (c) Urothelium on day 5 after cyclophosphamide injection is hyperplastic, with enlarged intercellular spaces and marked haemorrhage. (d) Urothelium on day 10 after cyclophosphamide injection is hyperplastic and no haemorrhage is seen. L: lumen of the urinary bladder. Scale bars = 50 μm.

a rapidly expanding research field with exciting possibilities and, hopefully, considerable progress in clinical applications will soon be achieved.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Correlation between Urothelial Differentiation and Sensory Proteins P2X3, P2X5, TRPV1, and TRPV4 in Normal Urothelium and Papillary Carcinoma of Human Bladder

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Terminal differentiation of urothelium is a prerequisite for blood-urine barrier formation and enables normal sensory function of the urinary bladder. In this study, urothelial differentiation of normal human urothelium and of low and high grade papillary urothelial carcinomas was correlated with the expression and localization of purinergic receptors (P2X3, and P2X5) and transient receptor potential vanilloid channels (TRPV1, and TRPV4). Western blotting and immunofluorescence of uroplakins together with scanning electron microscopy of urothelial apical surface demonstrated terminal differentiation of normal urothelium, partial differentiation of low grade carcinoma, and poor differentiation of high grade carcinoma. P2X3 was expressed in normal urothelium as well as in low grade carcinoma and in both cases immunolabeling was stronger in the superficial cells. P2X3 expression decreased in high grade carcinoma. P2X5 expression was detected in normal urothelium and in high grade carcinoma, while in low grade carcinoma its expression was diminished. The expression of TRPV1 decreased in low grade and even more in high grade carcinoma when compared with normal urothelium, while TRPV4 expression was unchanged in all samples. Our results suggest that sensory proteins P2X3 and TRPV1 are in correlation with urothelial differentiation, while P2X5 and TRPV4 have unique expression patterns.

1. Introduction

The urothelium, which lines the urinary bladder, performs two major functions. The first one is a well-characterized high resistance permeability barrier, and the second, not so well understood, is a sensory function. Permeability barrier is formed and maintained during urothelial differentiation, which reaches the terminal stage in superficial umbrella cells. Umbrella cells synthesize four major transmembrane proteins, uroplakins (UPIa, UPIb, UPII, and UPIIIa), which form unique membrane specialization, that is, urothelial plaques [1]. It was shown that uroplakins directly contribute to the urothelial barrier function [2]. After synthesis and modifications of uroplakins in the endoplasmic reticulum and the Golgi apparatus, respectively, urothelial plaques are gradually assembled in post-Golgi compartments. They are transported to the apical plasma membrane of umbrella cells by fusiform vesicles [3–5]. Urothelial plaques are encircled by so-called hinge regions, which form microridges at the urothelial apical surface [6].

Urothelium, together with lamina propria, acts also as a sensory web, which is able to receive, amplify, and transmit information about its environment [7]. Numerous receptors and ion channels, including purinergic P2X receptors and transient receptor potential vanilloid (TRPV) channels, have been identified in urothelial cells. They respond to bladder filling, changes of urine composition, or autocrine and paracrine mediators [8]. P2X receptors and TRPV channels are relatively nonselective cation channels [9, 10]. Stretching triggers chemically mediated activation of purinergic P2X receptors and exocytosis of fusiform vesicles [11]. Moreover, stretching stimulates afferent nerve processes and may signal the degree of bladder filling to the central nervous system [12, 13]. TRPV channels may also be involved in response

to mechanical and chemical stimuli [14, 15]. It has been proposed that TRPV1 and TRPV4 are involved in bladder filling sensation and regulation of the voiding reflex [16–18].

We have shown previously that uroplakin expression decreases during bladder carcinogenesis [19–21], which is reflected in partial urothelial differentiation and barrier disruption [22, 23]. Compromised permeability barrier results in lower urinary tract symptoms (LUTS), which are divided into three categories: storage, voiding, and postmicturition symptoms. Storage symptoms include increased micturition frequency, nocturia, urinary urgency, and urinary incontinence [24]. Common voiding symptoms contain slow or weak stream, hesitancy, and terminal dribble. Postmicturition symptoms include the sensation of incomplete emptying and postmicturition dribble [24, 25]. Although the aetiology of LUTS is multifactorial, bladder carcinomas represent one of the possible causes [26]. Since P2X receptors are implicated in the bladder sensation mediated by afferent nerves, it is likely that sensory web plays an important role in some bladder diseases accompanied by LUTS [27]. Currently, very little is known about the roles of P2X receptors and TRPV channels in human bladder tumours. It is assumed that P2X receptors, activated by ATP, have a significant antineoplastic action and might be involved in urothelial differentiation in high grade superficial bladder cancer [28]. Regarding TRPV1, its downregulation was reported in superficial and muscle invasive urothelial cancers [17, 29].

Here we report on the expression and localization of P2X3, P2X5, TRPV1, and TRPV4 in normal human urothelium and in low and high grade papillary urothelial carcinomas. The results are compared with uroplakin expression and urothelial apical surface ultrastructure. Our results suggest correlation between sensory function of the urothelium and urothelial differentiation.

2. Material and Methods

2.1. Patients and Sampling. The study was conducted in accordance with the Helsinki Declaration and approved by the Slovenian National Medical Ethics Committee number 76/10/10. Eighteen patients with papillary urothelial carcinoma who underwent transurethral resection of the bladder were included in the study. Informed consent was obtained from all patients. Two samples were acquired by cold-cup biopsies from each patient: (i) the urothelial tumour and (ii) the normal urothelium 1 cm posterior from the interureteric ridge. Biopsies captured urothelium and lamina propria. For pathological staging and grading, EAU Guidelines on Non-muscle-invasive Bladder Cancer [30] were used. Urothelial tumours were diagnosed as low grade papillary urothelial carcinoma with no lamina propria invasion: pTa (12 patients; 60 to 88 years old; mean age 73.2 years), high grade papillary urothelial carcinoma with lamina propria invasion: pT1 or with muscularis propria invasion: pT2 (6 patients; 57 to 70 years old; mean age 65.5 years). Regarding normal samples, only those showing no signs of hyperplasia or dysplasia were further processed (13 samples). Each sample was processed for Western blotting, immunofluorescence, and scanning electron microscopy.

2.2. Western Blotting. Samples were homogenized in ice-cold buffer (0.8 M Tris-HCl, 7.5% SDS, and 1 mM phenylmethylsulfonyl fluoride). The lysates were centrifuged and the protein concentration in the supernatant was determined by using a BCA protein assay kit (Pierce, Rockford, IL). From each patient, the protein sample (50 μg/lane) from the normal urothelium was loaded next to the protein sample (50 μg/lane) from the urothelial tumour. Proteins were size fractionated on 7.5%, 10%, or 12% SDS-polyacrylamide gels and then transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK) by electroblotting. After blocking overnight at 4°C in 5% skim milk in phosphate buffer saline with 0.1% Tween (PBS-Tween), membranes were incubated for 2 hours at room temperature with rabbit polyclonal anti-uroplakin (1 : 10.000; kindly provided by Tung-Tien Sun, New York University Medical School, USA), guinea pig polyclonal anti-P2X3 (1 : 1000; cat. number NB100-1658, Novus Biologicals, Littleton, USA), goat polyclonal anti-P2X5 (1 : 200; cat. number sc-15192, Santa Cruz Biotechnology, Dallas, USA), rabbit polyclonal anti-TRPV1 (1 : 200; cat. number ACC-030, Alomone Labs, Jerusalem, Israel), or rabbit polyclonal anti-TRPV4 (1 : 500; cat. number ab39260, Abcam, Cambridge, UK). After washing in PBS-Tween, membranes were incubated for 1 hour, depending on primary antibody either with horseradish peroxidase-conjugated goat anti-rabbit (1 : 5000; Santa Cruz Biotechnology), goat anti-guinea pig (1 : 5000; Santa Cruz Biotechnology), or donkey anti-goat (1 : 7000; Jackson ImmunoResearch Laboratories, West Baltimore Pike, USA). Membranes were finally probed with enhanced chemiluminescence reagent (ECL; Amersham Biosciences, Buckinghamshire, UK) and exposed to X-ray films. To confirm equal protein loading, the blots were stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and reprobed with anti-actin antibody (diluted 1 : 2000; Sigma, Taufkirchen, Germany).

2.3. Immunofluorescence. Samples were fixed with 4% formaldehyde in PBS for 2.5 hours at 4°C. They were washed and impregnated with 30% sucrose, embedded in OCT mounting medium (Tissue Tek, Sakura Finetek Europe B.V., The Netherlands), and frozen in liquid nitrogen. Frozen sections were cut in cryostat at −25°C, collected on glass slides, and air dried. Sections were incubated in 1% BSA in PBS for 1 hour. Immunolabeling was performed using the same antibodies as for Western blotting: antibodies against uroplakins (1 : 10.000), P2X3 (1 : 1000), P2X5 (1 : 200), TRPV1 (1 : 200), and TRPV4 (1 : 500). After washing with PBS, sections were incubated for 90 minutes either with rabbit anti-goat AlexaFluor555 for uroplakins (Molecular Probes), donkey anti-guinea pig TRITC or FITC for P2X3 (Jackson ImmunoResearch Europe Ltd.), rabbit anti-goat AlexaFluor555 for P2X5 (Invitrogen), or goat anti-rabbit AlexaFluor488 for TRPV1 and TRPV4 (Molecular Probes). All secondary antibodies were diluted 1 : 400 in 0.1% bovine serum albumin (BSA) in PBS. A series of negative controls were performed, omitting the primary antibody or incubating sections with nonrelevant antibodies. Sections were washed, stained with DAPI, and immersed in

Vectashield embedding medium. Slides were examined with a fluorescence microscope Eclipse TE300 (Nikon).

2.4. Scanning Electron Microscopy. Samples were fixed in 4.5% paraformaldehyde and 2% glutaraldehyde for 3 hours. The samples were postfixed in osmium tetroxide, dehydrated in ethanol, and critical-point dried. After sputter-coating with gold, they were examined at 15 kV with a Jeol JSM 840A scanning electron microscope (Jeol Ltd., Tokyo, Japan).

3. Results

3.1. Normal Urothelium. Uroplakins, differentiation dependent and urothelium-specific transmembrane proteins, were detected by polyclonal anti-uroplakin antibody, which reacts strongly with UPIIIa (47 kDa). The expression of UPIIIa in all samples of normal human urothelium was positive (Figure 1). To localize uroplakins within urothelium we used immunofluorescence. Normal urothelium showed strong uroplakin labelling of the superficial cells (Figure 2(a)). Scanning electron microscopy revealed large, polygonal cells covering urothelial surface (Figure 2(b)). They had scalloped appearance with microridges, demonstrating the presence of urothelial plaques. All features indicated above provided evidence that umbrella cells formed the superficial cell layer of normal urothelium, and they were therefore considered to be terminally differentiated. Confirming this, we further analysed the expression and distribution of four nonselective ion channels.

Western blotting confirmed the expression of P2X3 (approximately 60 kDa) in the normal urothelium (Figure 1). Antibodies against P2X3 labelled all urothelial cell layers in all samples of normal urothelium, with more intense labelling in the superficial layer, where umbrella cells are located (Figure 2(c)). Regarding P2X5, the expression of monomer protein (70 kDa) was confirmed, while dimmer protein (140 kDa) was not detected by Western blotting (Figure 1). An additional band of approximately 50 kDa was also observed with anti-P2X5 antibody. In immunofluorescence, anti-P2X5 antibody labelled all urothelial cell layers in all samples of normal urothelium, with weaker labelling intensity in the basal than in the superficial layer (Figure 2(d)).

Data on TRPV1 molecular weight range from 30 to 150 kDa. Anti-TRPV1 antibody, which was used in this study, revealed the most intense band for TRPV1 at approximately 50 kDa and weak band at approximately 60 kDa (Figure 1). Immunolabeling of TRPV1 was evident in all urothelial cell layers in all samples of normal urothelium (Figure 2(e)). Anti-TRPV4 antibody used in this study detected band at 104 kDa, as predicted. TRPV4 immunolabeling was weak in basal and intermediate cell layers and moderate in umbrella cells in all samples of normal urothelium (Figure 2(f)).

3.2. Low Grade Papillary Urothelial Carcinoma. In low grade papillary urothelial carcinoma, decreased or abolished uroplakin expression was detected (Figure 1) when compared to normal urothelium. In all samples of low grade papillary urothelial carcinoma, immunolabeling of uroplakins

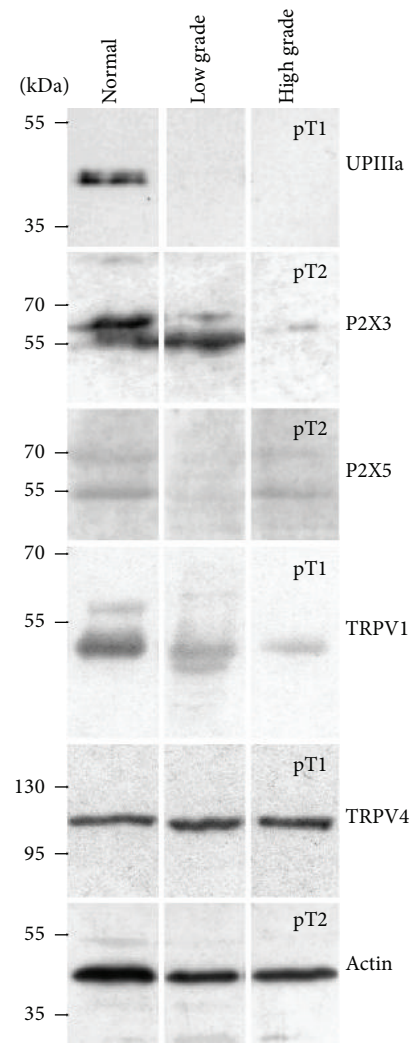

Figure 1: The expression pattern of uroplakins, P2X3, P2X5, TRPV1, and TRPV4 in normal human urothelium and in low and high grade papillary urothelial carcinomas as determined by Western blotting. In the protein samples of normal urothelium, UPIIIa, P2X3, P2X5, TRPV1, and TRPV4 are expressed. In low grade carcinoma, there is no expression of uroplakins. P2X3 and TRPV4 are expressed as in normal urothelium, while P2X5 is greatly diminished. TRPV1 expression is decreased in comparison to normal urothelium. In the protein samples of high grade carcinoma with lamina propria invasion (pT1) and those with muscularis propria invasion (pT2), the expression patterns were similar and therefore three examples of each are presented here. The expression of uroplakins is negative and expressions of P2X3 and TRPV1 are substantially decreased compared to normal urothelium. The expressions of P2X5 and TRPV4 are the same as in normal urothelium. Western blots were done in duplicate. Molecular weights are shown in kilodaltons (kDa).

was heterogeneous and we were able to discriminate (i) regions with uroplakin positive labelling of all superficial cells (Figure 3(a)), (ii) regions with uroplakin positive and uroplakin negative superficial cells (Figure 3(b)), and (iii) regions with only uroplakin negative superficial cells. Urothelial apical surface displayed altered appearance in comparison

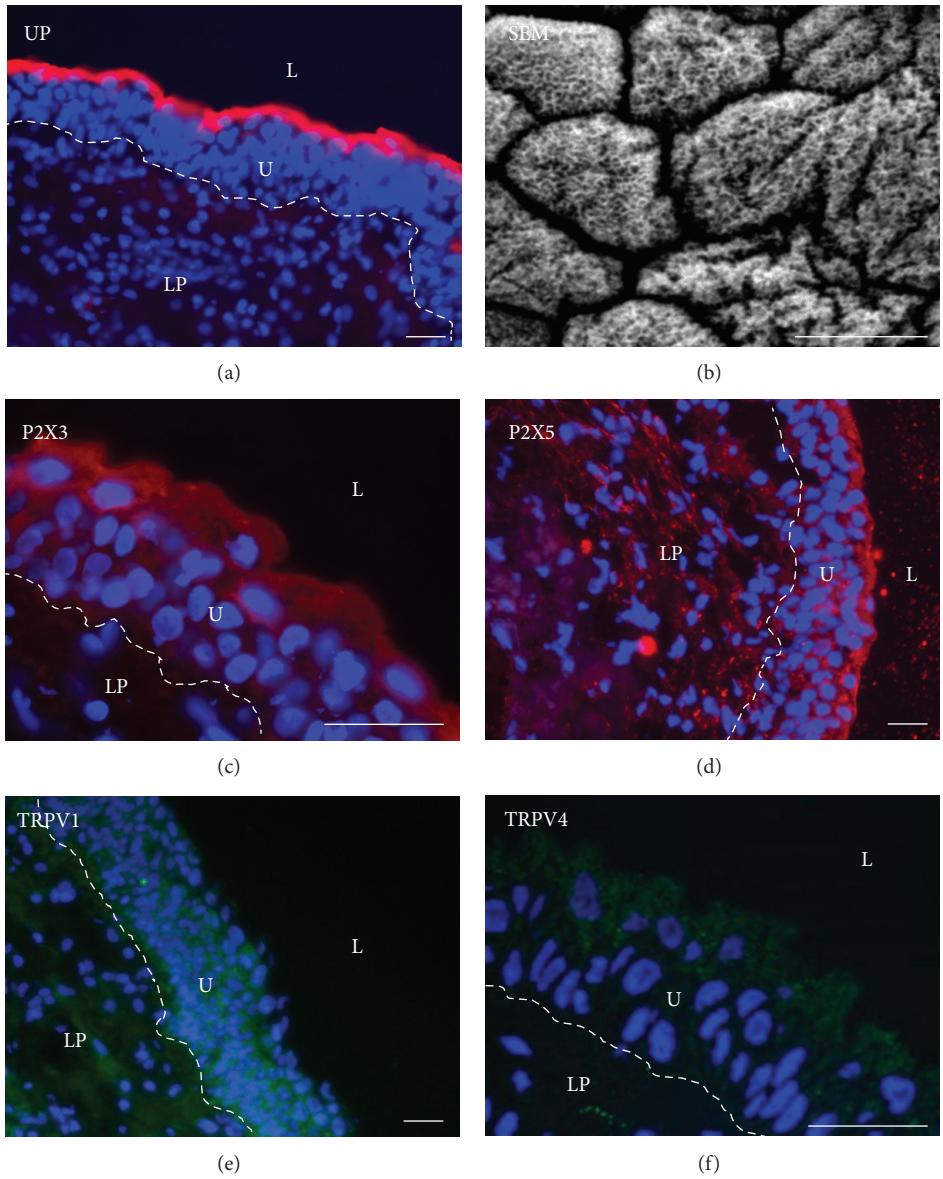

Figure 2: Normal human urothelium (U). (a) Strong uroplakin (UP) immunolabeling (red) is restricted to umbrella cells. (b) Scanning electron microscopy (SEM) shows large polygonal umbrella cells with microridges on the urothelial apical surface. (c) Antibodies against P2X3 and (d) P2X5 label (red) all layers of the normal urothelium. The reaction is stronger in umbrella cells than in basal cells. (e) Anti-TRPV1 antibody labels (green) all urothelial cell layers. (f) Anti-TRPV4 labelling (green) is weak in basal and intermediate cell layers and moderate in umbrella cells. In (a), and (c)–(f) nuclei are labelled blue with DAPI. L = lumen, LP = lamina propria. Scale bars = 50 μm.

to normal urothelium with disrupted superficial cell layer and gaps between adjacent superficial cells (Figure 2(c)). Superficial cells were not covered with microridges and they were smaller in comparison to umbrella cells. Taken together, these results indicated partial differentiation of superficial cells in low grade carcinoma.

In the protein samples of low grade carcinoma strong expression of P2X3 was detected (Figure 1). In all samples of low grade papillary urothelial carcinoma, P2X3 immunolabeling was similar as in normal urothelium; that is, the labelling of the superficial cell layer was the strongest (Figure 3(d)). Western blotting revealed that P2X5 expression was much weaker in the low grade carcinoma than in normal urothelium (Figure 1). By P2X5 immunolabeling, two types of regions were observed in all samples: (i) regions with immunolabeling of superficial cells and individual intermediate cells (Figure 3(e)) and (ii) regions with negative immunolabeling in all cell layers, except weak labelling in individual superficial cells (Figure 3(f)).

TRPV1 expression determined by Western blotting was weaker in the low grade carcinoma than in normal urothelium (Figure 1). Immunofluorescence of TRPV1 revealed stronger immunolabeling in the basal and in the intermediate cell layers than in the superficial cell layer (Figure 3(g)). In the low grade carcinoma, TRPV4 expression was stronger or equal to the expression of TRPV4 in the normal urothelium

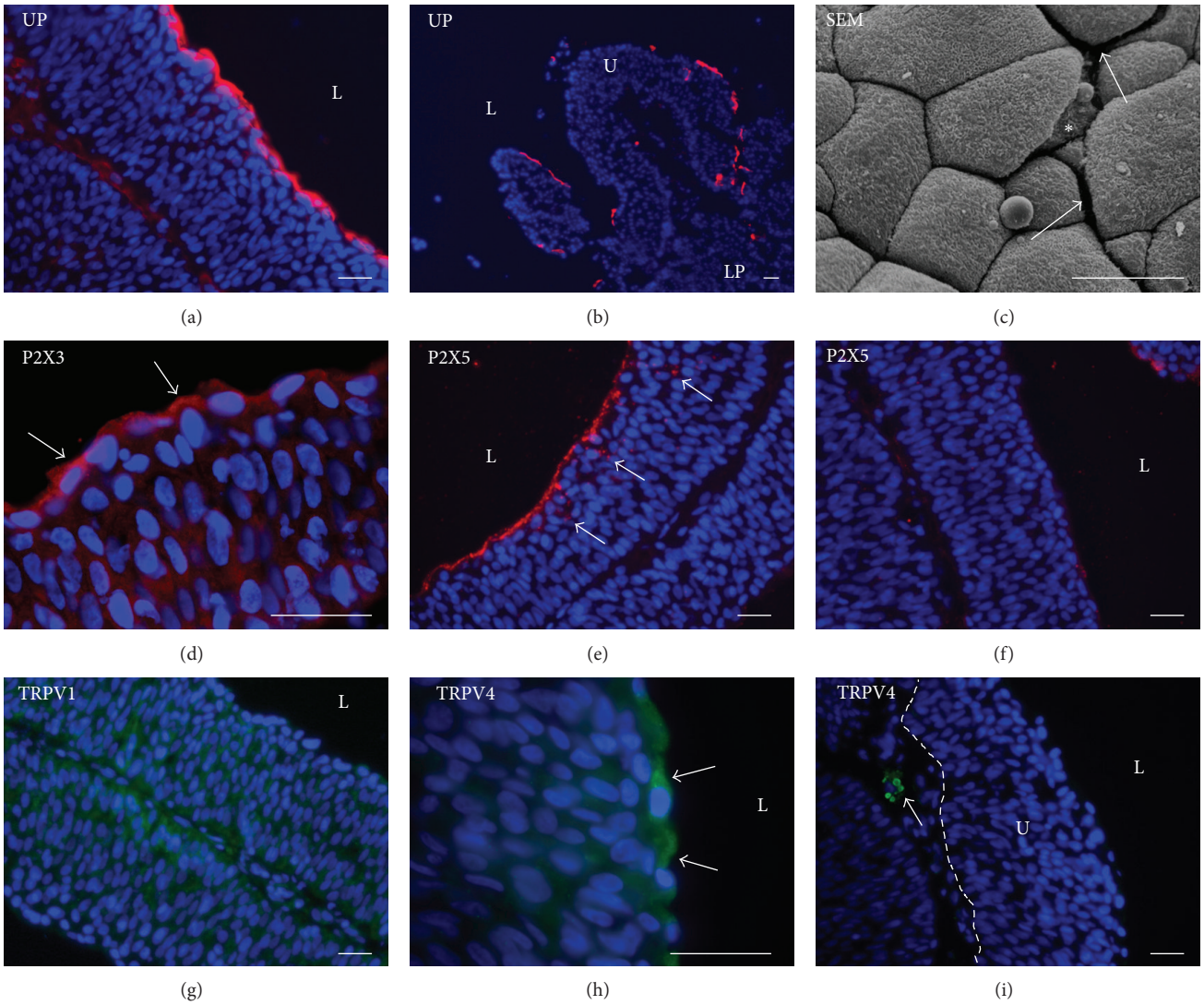

Figure 3: Low grade papillary urothelial carcinoma (pTa). (a) Uroplakin (UP) immunolabeling (red) is detected either continuously throughout the urothelial (U) superficial cell layer or (b) as regions where some superficial cells are uroplakin positive (red) and some uroplakin negative. (c) Scanning electron microscopy (SEM) reveals altered appearance of the urothelial apical surface in comparison to normal urothelium. Some neighbouring superficial cells are separated from one another (arrows) and underlying intermediate cell can be seen (asterisk). (d) P2X3 immunolabeling (red) is positive in all urothelial cell layers with the strongest immunolabeling in the superficial cells (arrows). (e) Some regions are intensely immunolabeled with anti-P2X5 antibody (red) in the urothelial superficial cell layer and in individual intermediate cells (arrows), (f) while other regions are P2X5 negative. (g) TRPV1 immunolabeling (green) is seen in basal and intermediate cells, but not in superficial cells. (h) In some regions, superficial cells (arrows) are TRPV4 positive (green), while (i) in other regions all urothelial cells are TRPV4 negative. TRPV4 positive immunolabeling is seen in the compartments of the lamina propria (arrow). In images (a)-(b) and (d)–(I), nuclei are labelled blue with DAPI. L = lumen, LP = lamina propria. Scale bars = 50 μm.

(Figure 1). Regarding TRPV4 immunolabeling, two types of regions were discriminated in all samples: (i) regions with positive immunolabeling of superficial and intermediate cell layers (Figure 3(h)) and (ii) regions with negative reaction in all urothelial cell layers (Figure 3(i)). Positive TRPV4 immunolabeling was seen in the lamina propria.

3.3. High Grade Papillary Urothelial Carcinoma. We did not observe any difference between pT1 and pT2 high grade papillary urothelial carcinoma with respect to the protein expression and localization studied here. Western blotting showed that there was no uroplakin expression in these samples (Figure 1) and uroplakin immunolabeling was also negative in all samples of pT1 and pT2 (Figure 4(a)). Scanning electron microscopy revealed superficial cells of different sizes, but prevailing ones were smaller than in normal urothelium (Figure 4(b)). They were covered with microvilli (Figure 4(b)), which are found only on poorly differentiated superficial urothelial cells [6].

The expression of P2X3 was greatly decreased in all samples of the high grade carcinoma when compared to

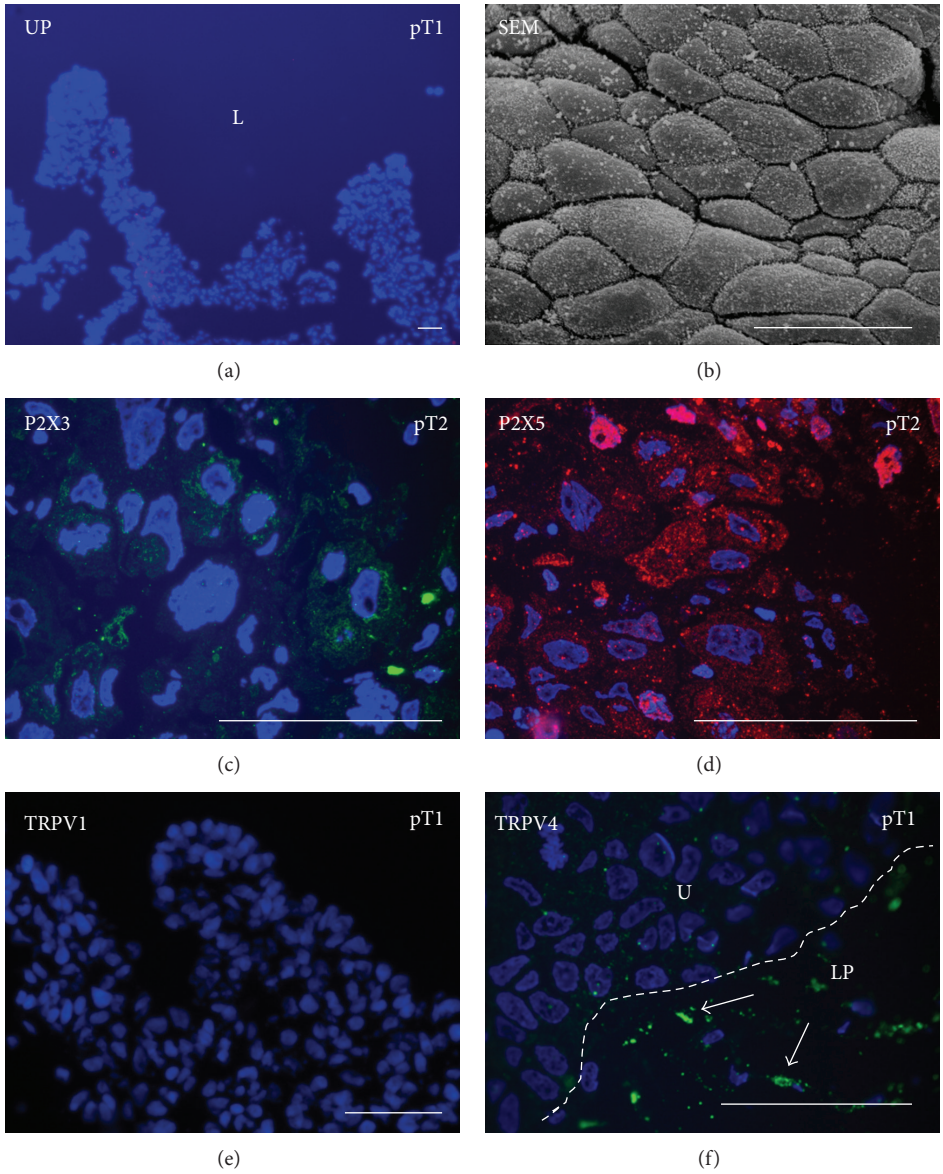

Figure 4: High grade papillary urothelial carcinomas (pT1 or pT2). (a) Uroplakin (UP) labelling (red) is negative. (b) Scanning electron microscopy (SEM) shows that superficial urothelial cells are small and polymorphic. They have microvilli on their apical surface. (c) Antibody against P2X3 weakly labels (green) urothelial cells. (d) P2X5 labelling (red) is present in all urothelial cells. Nuclei of some cells are also labelled. (e) TRPV1 labelling is negative. (f) Weak labelling of TRPV4 in urothelial cells (U) is seen, but strong TRPV4 labelling (arrows) is seen in the compartments of the lamina propria (LP). In images (a) and (c)–(f), nuclei are labelled blue with DAPI. L = lumen. Scale bars = 50 μm.

normal urothelium or to low grade carcinoma (Figure 1). In all samples of high grade carcinoma, antibodies against P2X3 weakly labelled all cell layers (Figure 4(c)). Western blotting confirmed the expression of monomer form of P2X5, which was similar in pT1 and pT2 protein samples (Figure 1). As observed by Western blotting, the expression of P2X5 was higher in high grade than in low grade carcinoma. P2X5 was labelled in all cell layers of all pT1 and pT2 samples (Figure 4(d)). By Western blotting, the expression of TRPV1 was the lowest in high grade carcinoma in comparison to normal urothelium and to low grade carcinoma (Figure 1). By immunofluorescence, all samples of high grade carcinoma were TRPV1 negative (Figure 4(e)). TRPV4 expression in all samples of high grade carcinoma was similar to TRPV4 expression in normal urothelium as well as in low grade carcinoma (Figure 1). Immunolabeling was negative or weak in all cell layers of all high grade carcinoma samples (Figure 4(f)). Positive TRPV4 immunolabeling was detected in the lamina propria.

4. Discussion

Unique differentiation of normal urothelium has been investigated since the 1970s, while its sensory role has been discovered only recently [7, 8]. Among sensory proteins, members

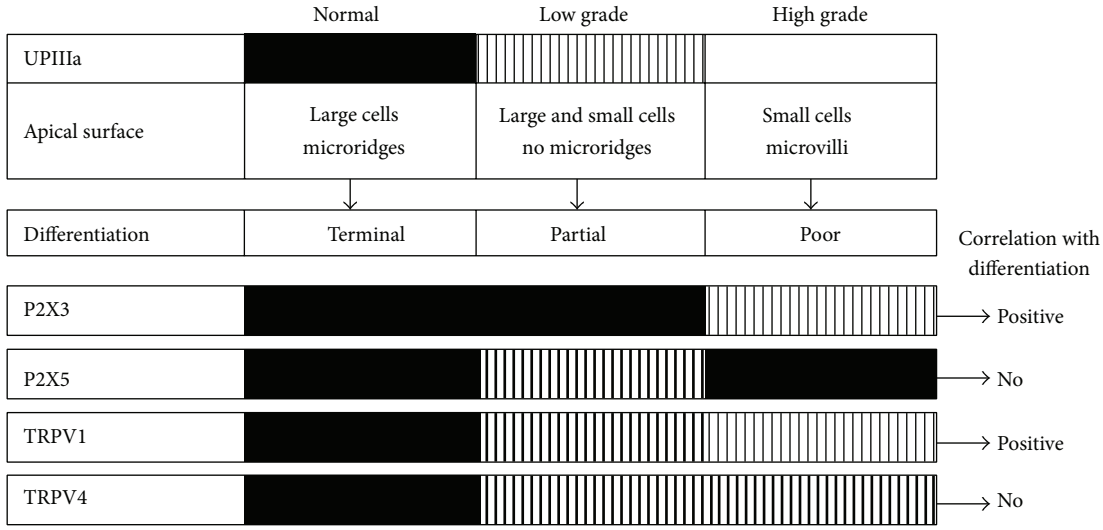

Figure 5: Summarised results of immunofluorescence, Western blotting, and scanning electron microscopy in normal human urothelium and in low and high grade papillary urothelial carcinomas. Uroplakin UPIIIa expression and apical surface appearance indicate differentiation stages of urothelial cells. P2X3, P2X5, TRPV1, and TRPV4 expressions are illustrated and their correlations with differentiation stage of urothelial cells are presented. Black squares indicate high protein expression, dashed squares denote moderate protein expression, and white squares represent no protein expression.

of purinergic P2X receptors and transient receptor potential vanilloid (TRPV) channels are under intense investigation. The majority of studies have been conducted on animal tissues or cell culture models, while very little is known about their distribution and function in normal human urothelium or in urothelial tumours. We investigated the correlation between urothelial differentiation and sensory function-related proteins P2X3, P2X5, TRPV1, and TRPV4 in normal human urothelium and in low and high grade papillary urothelial carcinomas. All our results are summarised in Figure 5.

First we evaluated the differentiation of normal urothelium and that of low and high grade carcinomas. Two well-established criteria were used to determine urothelial cell differentiation. One is the expression and localization of uroplakins and the second criterion is the appearance of urothelial apical surface. In normal urothelium, there was strong expression of uroplakins, which were localized in superficial urothelial cell layer. Apical surface was scalloped, with microridges covering the umbrella cells. These characteristics demonstrate terminal differentiation of the urothelium, as proposed previously [21]. In low grade carcinoma decrease in uroplakin expression was detected and altered urothelial apical surface appearance was observed. Both criteria provide evidence for partial differentiation of low grade carcinoma [19, 21]. In high grade carcinoma there was no expression of uroplakins and superficial cells, which were covered with microvilli, were small and polymorphic. All these features point to poor differentiation of high grade carcinoma. Decreased urothelial differentiation is usually associated with incomplete barrier formation and bladder dysfunction. Since urothelial carcinoma may cause LUTS, we hypothesised that this involves also changes of some sensory proteins expression and localization.

Since the first demonstration of the P2X3 receptor in the human urothelium [31], only few studies confirmed the presence of P2X3 protein [32], while expression analyses revealed no P2X3 mRNA in the human urothelium [33]. These could be assigned to factors such as translational regulation, mRNA stability, and half-life of a protein [34]. In the present study, P2X3 immunoreactivity was observed throughout all urothelial cell layers of normal urothelium. Umbrella cells, which were terminally differentiated, were labelled stronger than cells in other layers, which is in coincidence with previous results [31]. Since differentiation of the urothelium progresses from basal cells to umbrella cells, it seems that P2X3 expression is related to cell differentiation stage. In low grade carcinoma we confirmed the expression of P2X3. Moreover, the expression and localization of P2X3 in partially differentiated low grade carcinoma, where some terminally differentiated superficial cells were preserved, were similar as in normal urothelium. In high grade carcinoma P2X3 expression was decreased when compared to normal urothelium and low grade carcinoma. Since high grade carcinoma was poorly differentiated, these results confirmed our abovementioned assumption that positive correlation exists between the expression of P2X3 and urothelial differentiation.

The expression of P2X5 in normal urothelium was confirmed, which is in coincidence with other reports [34]. The majority of low grade carcinoma samples exhibited no P2X5 expression, while only few showed positive immunolabeling in the superficial urothelial cells. In poorly differentiated high grade carcinoma strong expression of P2X5 in all cell layers was detected. Western blotting revealed similar level of P2X5 expression in the protein samples of highly differentiated normal urothelium and of poorly differentiated high grade carcinoma. It was shown that ATP significantly reduced cell proliferation in high grade bladder cancer and

pharmacological profiling implicated P2X5 receptor in this antineoplastic response [28]. To our knowledge we showed for the first time that P2X5 receptors are expressed by the high grade papillary urothelial carcinoma.

It is known that bladder distension causes urothelial ATP release, which can directly depolarize and initiate firing in sensory nerves by activating P2X receptors [27]. Sensation and micturition are complex and not yet fully understood processes, which are altered during progression of diverse bladder diseases and related to various LUTS. Therefore, we suppose that decreased expression of P2X3 and increased expression of P2X5 detected in high grade carcinoma might be involved in pathogenesis and LUTS manifestations of this kind of carcinoma.

The remarkable finding that TRPV1 is not only expressed by afferent nerves but also in the urothelium [35, 36] gave rise to intensive studies of its expression and localization. Our results showed TRPV1 expression in normal urothelium with terminally differentiated umbrella cells. TRPV1 was not restricted to umbrella cells, as reported previously [29]. Moreover, downregulation of TRPV1 in urothelial cancers of human bladder was determined [17, 29] and the hypothesis that TRPV1 is involved in differentiation was postulated [16]. Our results support this hypothesis, since in partially differentiated low grade and in poorly differentiated high grade carcinoma its expression was decreased and abolished, respectively.

Several studies localized TRPV4 throughout all urothelial cell layers of normal urothelium [18, 37, 38]. Our results confirmed this data and showed variability among the levels of TRPV4 expression in the normal urothelium. We could not find any pattern in these findings that would point to any correlation between urothelial differentiation and TRPV4 channels. To our knowledge, there are no data about TRPV4 expression in urinary bladder tumours. Although Western blotting showed no variations in the expression of TRPV4 in normal urothelium and in low and high grade carcinomas, immunofluorescence revealed great diversity among different parts of the carcinomas. Some parts exhibited strong TRPV4 immunolabeling, while in others immunolabeling was weak and even parts with negative reaction were detected. It seems that TRPV4 was not correlated with urothelial differentiation and further research is necessary to clarify its role in bladder carcinogenesis.

5. Conclusions

Our results show that P2X3 is in correlation with urothelial differentiation and might be involved in high grade papillary carcinoma pathogenesis. We also confirm the correlation of TRPV1 with urothelial differentiation stage. Moreover, our study supports previous proposal that TRPV1 receptor should be accepted as a negative prognostic factor in patients with urothelial carcinoma. Regarding P2X5 and TRPV4 no direct correlation between their expression and urothelial differentiation is demonstrated. Nevertheless, new aspects concerning their localization variability in urothelial papillary carcinoma emerged indicating that they have a role in bladder functioning during pathogenesis.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Effect of Inflammatory Mediators on ATP Release of Human Urothelial RT4 Cells

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Inflammation is an important contributor to the aetiology of a number of bladder dysfunctions including interstitial cystitis, painful bladder syndrome, and overactive bladder. The aim of this study was to examine the effects of inflammatory mediators on urothelial ATP release. Human urothelial RT4 cells were exposed to normal buffer or varying concentrations of inflammatory mediators (bradykinin, histamine, and serotonin) in the presence or absence of hypotonic stretch stimuli (1 : 2 dilution of Krebs-Henseleit buffer). Others have demonstrated that bradykinin increased stretch-induced ATP release; however, we observed no change in control or stretch-induced ATP release with bradykinin. Pretreatment of RT4 cells with histamine or serotonin decreased stretch-induced ATP release ($P = 0.037$, $P = 0.040$, resp.). Previous studies have demonstrated increased ATP release in response to inflammation utilising whole bladder preparations in contrast to our simple model of cultured urothelial cells. The current study suggests that it is unlikely that there is a direct interaction between the release of inflammatory mediators and increased ATP release, but rather more complex interactions occurring in response to inflammation that lead to increased bladder sensation.

1. Introduction

Inflammation is an important contributor to the aetiology of a number of bladder dysfunctions. Pyuria (the presence of white blood cells in the urine) has been associated with lower urinary tract symptoms [1] and biopsy specimens from patients with interstitial cystitis (IC)/painful bladder syndrome (PBS) are commonly characterised by the presence of inflammatory cells in the lamina propria, especially infiltration with mast cells [2–7]. Recently, histological evidence for chronic inflammatory infiltrate has been demonstrated in patients with refractory overactive bladder (OAB) [8] together with pyuria in these patients [8, 9]. Proinflammatory cytokines are increased in the urine from patients with OAB [10–12].

These conditions (IC/PBS/OAB) are all characterised by urinary urgency, together with frequency and nocturia [13]. It is believed that bladder sensation is associated with the interaction of ATP with purinergic receptors located on suburothelial afferent nerves [14, 15] and myofibroblasts [15]. ATP is released from the urothelium in response to stretch of the urothelium triggered by bladder filling [16]. An increase in stretch-induced ATP release has been demonstrated in tissue strips and biopsies from patients with IC/PBS [17–19] and OAB [20]. In addition, the concentration of ATP in intravesical fluid has been shown to correlate with urinary urgency as indicated by the volume at first desire to void in patients with OAB [21, 22].

Similar to the changes in bladder histological structure described with PBS/IC, chemical-induced cystitis causes histological changes in the bladder wall including infiltration of inflammatory cells (e.g., mast cells and macrophages) into the submucosa [4–7], together with increased bladder weight and oedema. In addition, chemical cystitis is associated with activation of previously silent C-fibre afferents and sensitisation of mechanosensitive Aδ-fibres within the submucosa [23–26].

The effect of inflammation causing an increase in urothelial ATP release has been examined in animal models of inflammation, including feline interstitial cystitis [27],

cyclophosphamide cystitis [28], and ketamine cystitis [2]. However, the agents responsible for the increased ATP release are unknown. The aim of this study was to examine the effects of bradykinin, histamine, and serotonin, mediators commonly associated with bladder inflammation, on urothelial cell ATP release.

2. Materials and Methods

2.1. Cell Culture. Human urothelial RT4 cells (obtained from the ECACC) were grown at 37°C with 5% CO_2 in McCoy's 5A culture medium supplemented with 10% foetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of fungizone. At confluence, cells were passaged and then replated onto T75 flasks for continuous passage or onto 24 well plates for use in ATP release when confluent (approximately 3 to 5 days after passage).

2.2. ATP Release. ATP release was determined as previously described [29]. Urothelial cells were washed (three times) with carbogenated Krebs-Henseleit solution (containing mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-glucose 11.7). The basal level of ATP release was then determined by 10-minute incubation in 500 μL Krebs-Henseleit solution. Cells were then exposed to normal Krebs-Henseleit (control) or the indicated concentration of mediator (bradykinin, histamine, and serotonin) in the presence or absence of hypotonic Krebs-Henseleit (1 : 2 dilution of Krebs-Henseleit in distilled water). Hypotonic Krebs-Henseleit was used as a stretch stimulus to examine the effect of the mediators on stretch-induced ATP release. Cells were treated for 10 min before the supernatant (200 μL) was collected and used for ATP determinations.

ATP concentration in the supernatant was measured using the bioluminescence assay. Equal volumes of the cellular supernatant or ATP standard solutions (10^{-6} to 10^{-10} M) were mixed with the bioluminescence assay mix and the luminescence generated was measured immediately using a plate reader (BMG Labtech Polarstar). The ATP concentration in the cell supernatant was calculated relative to the standard curve. Treatments were carried out in triplicate and the mean ATP concentration (in nM) per treatment was determined.

2.3. Statistics. As results are nonnormally distributed and as such are expressed as median with interquartile range (IQR); two different treatments were compared using a Wilcoxon matched-pairs t-test. Dose response relationships were examined using a sigmoidal dose response curve. All statistics were performed using Graphpad Prims (version 6) (San Diego, CA).

2.4. Materials. All cell culture reagents were purchased from Invitrogen (Mount Waverley, Australia). Bioluminescence ATP assay kit and mediators (bradykinin, histamine, and serotonin) were from Sigma-Aldrich (Sydney, Australia). All other reagents were of high analytical grade.

3. Results and Discussion

3.1. Effect of Bradykinin on Urothelial Cell ATP Release. Bradykinin is a peptide neurotransmitter released from sensory afferent nerves. Bradykinin release is closely associated with inflammatory responses. Distension of the bladder has been shown to stimulate release of bradykinin in patients with IC indicating a potential role for this peptide in the pathophysiology of this disorder [30]. Bradykinin exerts its physiological actions via activation of B1 and B2 receptors. Bradykinin B2 receptors are believed to be important in inflammatory pain with animal models showing that block of this receptor reduces inflammatory hyperalgesia [31, 32]. In contrast, bradykinin B1 receptors are expressed at low levels under normal circumstances with their expression upregulated following tissue damage or inflammation [33]. This includes upregulation of bradykinin B1 receptors following cystitis induced by intravesical injection of the detergent Triton X100 [34]. Expression of bradykinin B1 receptors is increased in biopsies obtained from patients with interstitial cystitis [35] and, in cyclophosphamide-induced cystitis, B1 receptor mediated bladder responses are significantly increased [36, 37]. In addition, cyclophosphamide cystitis induced a bladder hyperactivity that was dependent on bradykinin B2 receptor activation and was inhibited by the P2 receptor antagonist PPADS [38]. This indicates a role for both bradykinin and ATP in cyclophosphamide-induced cystitis [38].

Other studies have observed that activation of bradykinin B2 receptors by bradykinin increases stretch-induced ATP release in UROtsa urothelial cells [39] and in primary cultures of rat urothelial cells [38]. However, in the current study, using urothelial RT4 cells, we saw no change in either control or stretch-induced ATP release in the presence of bradykinin (Figure 1). Hypotonic Krebs which was used as a positive control was seen to induce an approximate threefold increase in ATP release (baseline ATP release 34.06 (23.8–99.1) Nm; stretch-induced ATP release 146.7 (72.4–217.6) nM, $P = 0.002$). It is possible that the differences in these findings relate to the individual cell lines used and that the bradykinin receptors usually present on urothelial cells are not functional on RT4 urothelial cells.

3.2. Effect of Mast Cell Mediators on Urothelial Cell ATP Release. Infiltration of inflammatory cells such as mast cells and macrophages into the bladder submucosa has been demonstrated in cyclophosphamide-induced cystitis [3] and ketamine-induced cystitis [2]. In addition, it is well known that there is an increased density of mast cells in the bladder wall in patients with IC [4–7]. Mast cells lie in close proximity to both urothelial cells and afferent fibres in the submucosa of the urinary bladder, and degranulation of these cells has been shown to release a wide range of neurotransmitters and cytokines [38].

Mast cells are granulated immune system cells which make up the major sensory arm of the innate immune system [40]. Mast cells respond to allergens as well as non-immunologic stimuli such as bacteria, chemicals, kinins, and neuropeptides [41] to release mediators such as histamine and

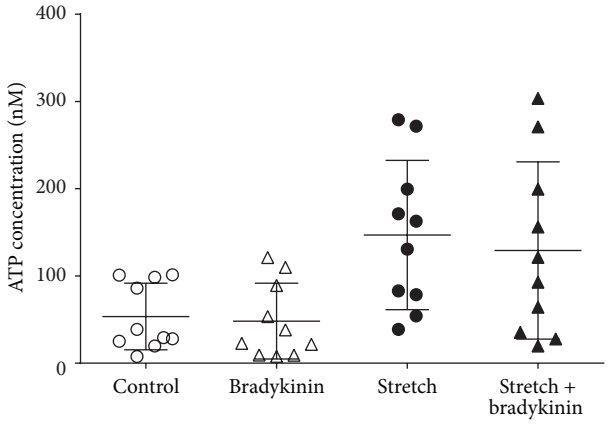

Figure 1: Effect of bradykinin (1 μM, $n = 10$) on baseline level of ATP release and release induced by hypotonic media. Symbols are representative of individual data points for the four groups. Data are shown as median with interquartile range.

serotonin [42]. There are reports of increased concentrations of histamine in urine from IC patients [41] and it is believed that the pain associated with bladder filling in IC is related to release of histamine from mast cells in the bladder wall [43].

In this study, the effect of mast cell mediators, histamine and serotonin, on urothelial cell ATP release was examined. Incubation of the RT4 cells with mast cell mediators, histamine and serotonin, for 10 minutes had no effect on control ATP release (Figures 2(a) and 2(c)). However, pretreatment of urothelial cells with histamine or serotonin for a 10-minute period prior to the addition of the hypotonic stretch stimulus resulted in a decrease in ATP release in response to stretch (Figure 2(a), $P = 0.037$ for histamine; Figure 2(c), $P = 0.040$ for serotonin). When the concentration response effect of histamine on stretch-induced ATP release was determined, it was seen to occur with an IC50 of 0.24 μM (0.19–2.8 μM) (Figure 2(b), $n = 6$). This concentration is within the histamine concentration range that has been reported in urine from IC patients (3 to 31 ng/mL, equating to 24 to 280 nM) [43]. The slope of the concentration response curve was fairly shallow with a Hill slope of -0.74 ± 0.19. A similar concentration dependent inhibition of ATP release was also elucidated for serotonin. In this instance, an IC50 of 1.7 nM (<0.001 nM–50.0 μM) was determined with a Hill slope of -0.37 ± 0.24 (Figure 2(d), $n = 6$).

While the effect of the mast cell mediators on urothelial cell ATP release has never previously been examined, the results obtained were somewhat unexpected. Previous studies have shown an increase in ATP release from the bladder in response to inflammation [17–19, 27, 28]. However, all of these studies were conducted in whole tissue models of inflammation. In these models, determining the causative molecule or cell is complex as numerous interactions between different cell types and mediators are occurring in the one system which could change receptor expression in addition to mediator release in response to the inflammatory stimulus. The model used in the current study of cultured urothelial cells is a much simpler model containing only a single cell type and a single exogenously supplied mediator. It is possible that in the previous studies it was not the inflammatory mediators themselves that were inducing the increase in ATP release, but rather secondary changes that occurred in response to the inflammation. Decreasing importance of ATP in inflamed bladder is supported by findings that the P2 antagonist (PPADS) decreased voiding frequency in control animals but not in cyclophosphamide treated animals [44].

Inflammation causes a number of changes in the bladder. For example, cyclophosphamide induced an increase in COX2 protein expression in the urothelium, which was dependent on release of mediators from mast cells [45]; in addition, cyclophosphamide also increases prostaglandin E_2 production [46]. Prostaglandin E_2 has been shown to increase urothelial cell ATP release [47]. It is also possible that in response to inflammation there are changes in receptor expression that would alter the response of urothelial cells to these mast cell mediators. As mentioned previously, bradykinin B1 receptors are expressed at very low levels under normal conditions but are upregulated following inflammation [38]. It is possible that a similar phenomenon occurs for histamine and serotonin receptors on the urothelium although this has not been examined to date.

In addition, there are further changes that occur in the inflamed bladder including defects in urothelial junction proteins, increased suburothelial inflammation, and increased urothelial cell apoptosis [2]. Bladder inflammation is associated with disruption of the glycosaminoglycan layer that covers the urothelium [48–50]. Disruption of this layer may allow urine contents to evoke irritation and inflammation within the bladder wall. There is also increased density of submucosal afferent nerves in the inflamed bladder [43, 51, 52]. Urinary nerve growth factor (NGF) levels are increased in patients with IC [53] and OAB [54]. In addition, cyclophosphamide-induced cystitis is associated with increased NGF mRNA levels in the bladder [55], increases the density of afferent nerves, and increases expression of $P2X_2$ and $P2X_{2/3}$ receptors in afferent nerves [56]. It is likely that the increase in NGF expression in patients with bladder dysfunction is associated with the increased density of suburothelial nerves in these patients. NGF is also thought to alter expression of neurotransmitters, modulate ion channels, and increase excitability of afferent nerves [57]. In rats, exogenously applied intravesical NGF can induce bladder nociceptive responses and bladder overactivity [58]. In addition to NGF, inflammatory mediators themselves may sensitise the afferent nerves to the effects of ATP [56, 59], which could increase the bladder sensations in response to bladder filling in the inflamed bladder.

4. Conclusions

It is well accepted that bladder inflammation is associated with increased signalling from the bladder and increased sensation. Studies using whole bladder models of inflammation have shown that this is due to increased release of ATP from the urothelium. However, based on the results of the current study, it appears unlikely that there is a simple

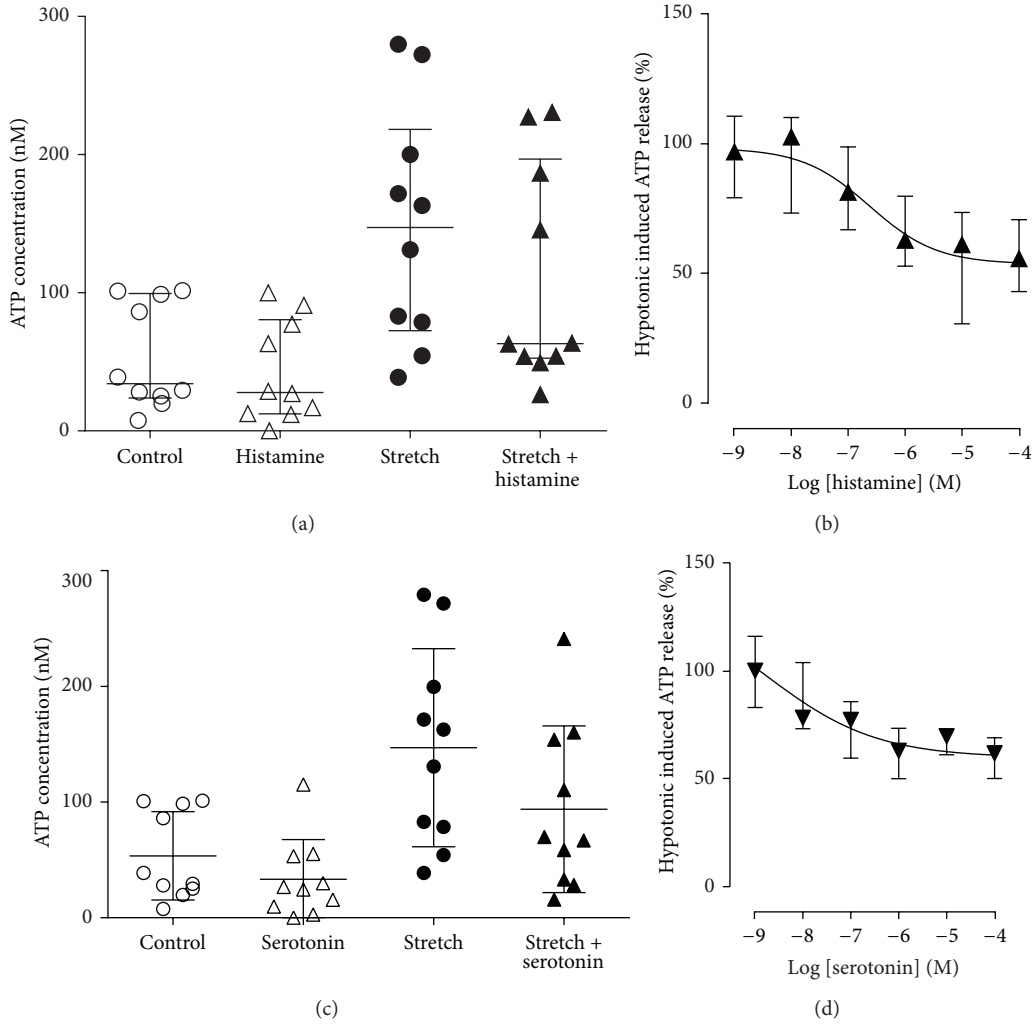

Figure 2: Effect of histamine (1 μM) ((a), $n = 10$) and serotonin (1 μM) ((c), $n = 10$) on baseline level of ATP release and release induced by hypotonic media. Data are shown as median with interquartile range. Concentration response relationships for the inhibition of ATP release induced by hypotonic media in the presence of histamine ((b), $n = 10$) and serotonin ((d), $n = 10$).

interaction between the release of the inflammatory mediators (bradykinin, histamine, and serotonin) and increased ATP release leading to increased bladder sensation. It is more likely that there are numerous factors involved in the interaction between bladder inflammation and increased sensation. These factors include alterations to receptor expression on the urothelium and afferent nerves, increased density of afferent nerves, and hyperresponsiveness of the afferent nerves, altering the neuronal component of afferent signalling.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Alterations of the Myovesical Plexus of the Human Overactive Detrusor

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Objectives. The human bladder shows spontaneous autonomous activity. Detrusor overactivity could be seen as a consequence of exaggerated autonomous activity. Interstitial cells (ICs) play a potential role in coordination of autonomous activity. As it is suggested that changes in ICs coexist with detrusor overactivity (DO), we investigated possible alterations to human bladder ICs. *Methods.* Biopsies were obtained from 23 patients and were categorized into four groups: genuine stress incontinence (without DO) ($n = 5$), neurogenic disease with DO ($n = 6$), bladder outlet obstruction with DO ($n = 6$), or idiopathic DO ($n = 6$). Specimens were processed to investigate expression of N-cadherin and PGP9.5. N-cadherin expression was semiquantitatively analyzed and correlated to PG9.5 expression and bladder wall morphology. *Results.* The population of cells expressing N-cadherin is altered in the overactive detrusor, making no difference between the sources of DO. Punctate distribution of morphological changes was found and downregulation of PGP9.5 expression seemed to coexist with upregulation of N-cadherin expression in the detrusor layer. *Conclusions.* The population of N-cadherin+ cells of the interstitial compartment of the human bladder has the ability to proliferate. As this proliferation seems to coexist with denervation, it could be possible that a highly developed network of interstitial cells replaces the loss of innervation in overactive detrusor.

1. Introduction

The overactive bladder (OAB) is a symptomatic diagnosis based on the presence of urgency, with or without incontinence, and is usually accompanied by frequency and nocturia [1]. Its presence imposes a huge burden on the healthcare system, society, and affected individuals [2]. Patients with OAB symptoms and detrusor overactivity (DO) can be divided into three groups; those with neuropathic laesions, those with bladder outlet obstruction (BOO), and those with neither (idiopathic DO) [3].

Although relatively little is known about the aetiology of DO, it is now clear that the human urinary bladder cannot merely be seen as a passive "black box," solely controlled by neuronal input. Recently, it was found that the isolated bladder shows spontaneous nonneuronal contraction during the filling phase, also known as autonomous activity of the bladder [4]. As frequency and urgency occur during this filling phase, DO can be seen as a consequence of exaggerated autonomous activity during the storage of urine [5].

Two cell types play a potential role in coordination of autonomous activity: interstitial cells (ICs) and intramural neurons of the bladder wall. In the human gastrointestinal tract, specialized ICs of Cajal (ICCs) interconnect through gap junctions and function as pacemakers and conductors of electrical activity between enteric neurons and smooth muscle cells [6]. They hereby coordinate gut peristalsis. ICs are also found in the human bladder, albeit of a different subtypes than the ICs of Cajal [7, 8]. In the bladder, ICs are immunoreactive for the stem cell receptor C-kit, the cytoskeletal filament vimentin, the gap junctional protein connexin-43, the second messenger cyclic guanosine monophosphate (cGMP), and N-cadherin [9–12]. They form a network in the suburothelial area and between the detrusor smooth muscle fascicles. Double labelling confocal microscopy experiments revealed that the ICs are positioned in proximity to nerves [9]. Recent reports have shown that the bladder ICs respond to application of neurotransmitters, firing calcium waves when stimulated by carbachol or ATP [9, 13]. It can therefore be hypothesized that by consecutive interaction of ICs with

smooth muscle cells, neuronal firing could consequently result in detrusor muscle activation. Although the exact role for ICs in bladder function has not yet been elucidated, it is highly likely that either quantitative or qualitative changes in bladder ICs coexist with DO. We therefore investigated possible alterations to the network of human bladder ICs in the overactive detrusor using N-cadherin. Additionally, we used PGP9.5 (Protein Gene Product 9.5) as a pan-neuronal marker as it is generally accepted that this protein is expressed by all neuronal structures of the bladder wall [14].

2. Methods

2.1. Patients. This study was conducted on biopsies from 12 female and 11 male patients, aged 47 to 68 years (mean 60 years), suffering from OAB complaints or genuine stress incontinence. Two cold cup bladder biopsies were obtained from each patient from the posterior bladder wall during cystoscopic procedures. The local ethics committee approved the study and informed consent was obtained from all patients. All patients underwent full urodynamic analysis and were categorized into three groups: neurogenic disease with detrusor overactivity (DO) ($n = 6$; 2 male, 4 female), bladder outlet obstruction (BOO) with DO ($n = 6$; 5 male, 1 female), or idiopathic DO ($n = 6$; 1 male, 5 female). Three females and two males aged 53 to 75 years (mean age 65.6 years) with stress urinary incontinence and urodynamically proven nonoveractive detrusor served as controls. These patients did not suffer from neurogenic disease or bladder outlet obstruction and were all undergoing check cystoscopy. Filling cystometry (50 mL/min) in patients with OAB all revealed DO with a median cystometric bladder capacity (CBC) of 203 mL (range from 28 to 450). The median CMC in control bladders was 400 mL (range from 205 to 500). The CMC of 205 mL was caused by major stress incontinence. In all patients suffering from OAB, intravesical pressure rises during filling were due to DO, as compliance was normal. Acontractile detrusors were not included.

2.2. Immunohistochemistry. Bladder specimens were collected and placed in a mould containing Tissue-Tek (Sakura). They were snap-frozen immediately in isopentane at −80°C. Sections of 4 μm were prepared using a cryostat and mounted on Super Frost Plus (Menzel-Gläser) slides. Using haematoxylin-eosin staining techniques, tissue was analyzed for presence of intact urothelium and smooth muscle.

Immunohistochemical staining was performed as previously described by our group [12]. Antibodies against the following markers were used: N-cadherin (rabbit polyclonal antibody); (M142 Takara, mouse monoclonal antibody; C2542 Sigma Clone GC-4), smoothelin [15], and PGP9.5 (mouse monoclonal antibody 7863-0504; AbD Serotec). Antibodies against PGP9.5 were used as it is generally accepted that PGP9.5 is expressed by all neuronal structures of the bladder wall [14, 16, 17]. Negative controls included omission of primary antibodies and incubation with PBS-Extra instead. Positive controls included human prostate cancer specimens [18].

2.3. Morphologic Analysis. Immunohistochemical photomicrographs were analyzed semiquantitatively for PGP9.5+ nerve profiles and N-cadherin+ structures. As cold cup biopsies were used, a limited amount of detrusor smooth muscle was available for analysis compared to transmural biopsies. A maximum number of four smooth muscle fascicles per biopsy were photographed. Three slides per specimen were analyzed in duplo. Each set of ten slides was separated by approximately 1 mm of tissue. According to expression of N-cadherin and PGP9.5, each fascicle was semiquantitatively graded as follows: features not present in any photographs, 0; present 0–1/3, +; present in 1/3–2/3, ++; present in 2/3-entire fascicle, +++.

3. Results and Discussion

3.1. N-Cadherin+ ICs in Control Bladder. All bladder specimens contained the following three layers of tissue: urothelium, suburothelium, and detrusor. Because of the used cold cup biopsy method, the detrusor smooth muscle layer was penetrated to a maximum of two muscle bundles in depth. Full thickness biopsies of human bladder consist of three layers of smooth muscle bundles.

According to our previous study [12], N-cadherin positive structures were found throughout the entire bladder wall. They coexpressed vimentin (Figures 1 and 2), but showed no coexpression of the pan-neuronal marker PGP9.5 (Figure 3) or the smooth muscle specific marker smoothelin (data not shown). No immunoreactivity for N-cadherin was found in the urothelial layer (data not shown). N-Cadherin expression profile highly resembled punctate C-kit expression as found by others [16].

N-Cadherin+ cells were located immediately below the urothelium and extended throughout the suburothelial lamina propria into the detrusor layer. They showed a branched morphology with multiple processes that were closely associated (Figure 1). In the detrusor layer, N-cadherin was expressed by cells housed at the border of smooth muscle bundles, perifascicular, and within smooth muscle fascicles (Figure 2).

PGP9.5+ was expressed in all control specimens. PGP9.5+ nerves were often closely associated with N-cadherin+ cells. In the detrusor layer; muscle fascicles were neighboured by primary nerve trunks housed in planes of connective tissue. Smaller nerve branches were found between detrusor fascicles, continuing as small fibers penetrating the fascicles.

3.2. Changes to the Population of N-Cadherin+ Cells in the OAB. In all specimens, N-cadherin and PGP9.5 expression showed wide variety between smooth muscle fascicles throughout all detrusor muscle layers. In general, N-cadherin expression seemed upregulated in overactive detrusor specimens compared to control specimens, making no difference between the sources of DO (Table 1). In overactive detrusor specimens, changes to the network of N-cadherin+ cells were found at intrafascicular level, perifascicular level, and at the border of smooth muscle bundles. Detrusor smooth muscle bundles with upregulated expression of N-cadherin seemed surrounded by long N-cadherin positive planes (Figure 4).

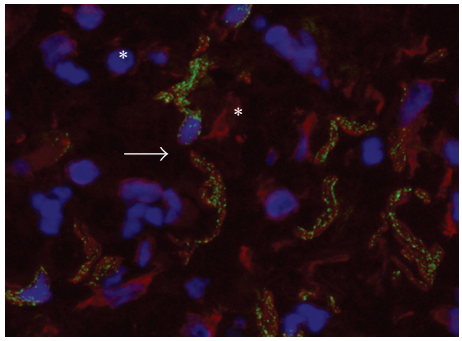


Figure 1: Suburothelial interstitial cells in control bladder. Multiple suburothelial cells with bizarre morphology and multiple processes coexpress a punctate signal for N-cadherin (green) and a filamentous signal for vimentin (red). Nuclei stained with Dapi (blue). Two cells are closely associated with each other (arrow). Cells expressing vimentin but lacking expression of N-cadherin embody fibroblasts (asterisk). Magnification X630. Binocular epifluorescent microscopy.

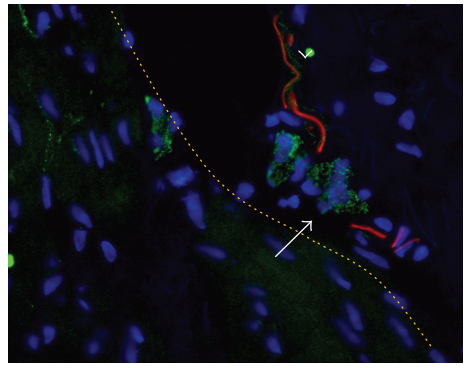


Figure 3: N-Cadherin and PGP9.5. N-Cadherin (green) was double stained with pan-neuronal PGP9.5 (red) and counterstained with Dapi (blue). N-Cadherin positive interstitial cell (arrow) is neighboured by a PGP9.5 positive nerve ending (arrow head). Both cell types do not show coexpression of both markers. Cigar shaped background signal embody collagenous fibres. Enlarged from magnification X400. Binocular epifluorescent microscopy.

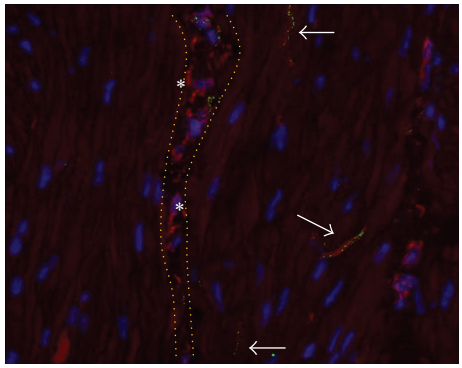


Figure 2: Interstitial cells in the detrusor layer of control bladder. Cells coexpressing N-cadherin (green) and vimentin (red) as found in the detrusor layer. Nuclei stained with Dapi (blue). Note red background signal in smooth muscle cells that was upgraded to facilitate tissue orientation. Arrowheads show slender punctate N-cadherin expression embodying ICs located in the interfascicular clefts (indicated by dotted orange lines). Slender intrafascicular IC-structures (arrows) run in parallel with and between individual smooth muscle cells. Cells expressing vimentin but lacking expression of N-cadherin (asterisk) embody fibroblasts. Magnification X400. Binocular epifluorescent microscopy.

These planes were built up from numerous cells expressing N-cadherin, had a slender morphology, and were housed in a thick layer of connective tissue. At the area in which the interfascicular cleft met the outside of the muscle bundle, the strings were thickened (Figure 5) and seemed to give rise to N-cadherin+ processes running in the interfascicular connective tissue planes. Interfascicular N-cadherin+ processes seemed to junction to nodes (Figure 6). These nodes were not only housed between fascicles, but also between smaller groups of smooth muscle cells. In control specimens, higher expression level of intrafascicular N-cadherin was found in some fascicles as well. However, large strings, interfascicular penetrating structures, and large intrafascicular nodes expressing N-cadherin were never found in control bladders.

The invasion of N-cadherin+ structures did not occur throughout the entire bladder wall but was solely found in smooth muscle bundles that were surrounded by long N-cadherin+ strings. Changes to the IC network were found at each level of depth of the bladder wall and were not restricted to the urothelial side or outer layer of detrusor muscle. However, cold cup biopsies do not enable analysis of full thickness bladder wall morphology. No clear differences between overactive and control specimens were found in the suburothelial N-cadherin+ network.

3.3. Smooth Muscle Denervation Coexists with Upgrade of IC Network. Intrafascicular expression of N-cadherin and PGP9.5 both were heterogeneously expressed and varied from low, to intermediate, and high level of expression (Figure 7). In general, the degree of immunoreactivity for N-cadherin seemed correlated with the level of PGP9.5+ innervation profile. Low expression level of N-cadherin was found to coexist with a high level of PGP9.5+ innervation grade (Table 1). In other fascicles, intermediate level of N-cadherin+ structures coexisted with an intermediate level of PGP9.5 expression. Smooth muscle fascicles with limited or no expression of PGP9.5+ nerve profiles showed intense expression of N-cadherin.

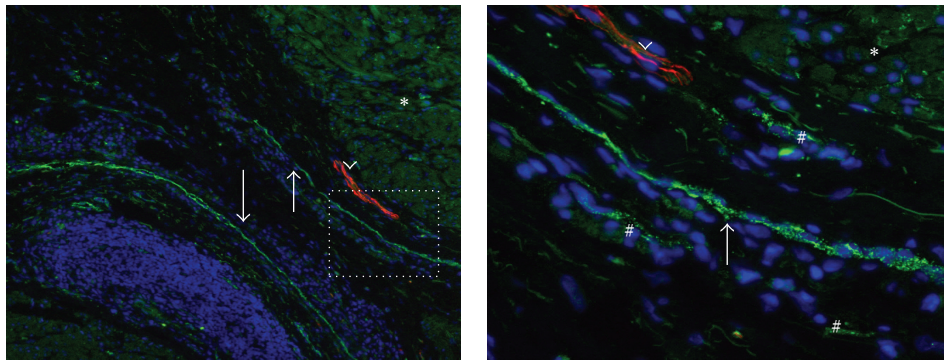
We observed that although PGP9.5+ denervation was found, PGP9.5+ structures were still found in the interfascicular cleft (Figure 7(c)). Large PGP9.5+ nerve trunks located in connective tissue planes outside smooth muscle bundles seemed not downregulated in overactive detrusor specimens.

3.4. Discussion. Understanding the pathophysiological mechanism behind detrusor overactivity (DO) is a challenge, as the bladder wall has a complicated structure. While the molecular basis for cell-cell communication between

Table 1: Semiquantitative analysis of intrafascicular N-cadherin and PGP9.5 expression in control and overactive detrusor.

	n	Fascicle 1		Fascicle 2		Fascicle 3		Fascicle 4	
		N-cad	PGP9.5	N-cad	PGP9.5	N-cad	PGP9.5	N-cad	PGP9.5
GSI	1	+	++	+	+	+	++	+	++
		+	++	+	++	+	++	+	+++
	2	++	++	+	++	++	−	Error	Error
		+	−	+	++	+	+	+	++
	3	+	++	+	++	+	+	+	+
		+	++	+	+	+	++	+	+++
	4	+	+	++	++	++	−	+	+
		++	++	+	++	+	+	+	++
	5	+	++	+	+	+	++	+	++
		+	++	+	++	+	++	+	+
NDO	1	++	+	++	+	++	+	Error	Error
		+	+	++	++	+++	−	++	−
	2	+	+	++	+	+	+	++	+
		+	−	+	−	++	+	++	−
	3	+	+	++	++	+	+	++	+
		++	+	+	+	+	−	++	−
	4	++	+	Error	Error	+	+	Error	Error
		+	−	++	+	++	−	+++	−
	5	++	+	++	+	+	++	+	++
		+	−	+++	−	+	+	++	+
	6	+	+	Error	Error	++	+	+	+
		+	−	++	+	+	−	+++	−
BDO	1	+	+	++	+	+	+	++	+
		++	+	+	−	+++	−	++	−
	2	+	+	+	+++	+	++	++	+
		++	−	++	+	++	+	++	+
	3	++	+	++	+	++	+	Error	Error
		++	−	+	+	++	−	++	−
	4	+	+++	Error	Error	++	+	+	+
		+++	−	+++	−	++	−	++	−
	5	+	+	+	++	+	+	+	++
		+	++	+++	−	+++	−	+	++
	6	+	+	++	++	+	+	+++	+
		++	+	++	++	+	+++	+	++
IDO	1	++	+	+	+	+++	+	+	+
		+++	−	++	−	+++	−	++	++
	2	Error	Error	++	+	Error	Error	++	+
		++	+	+	+	+++	−	++	−
	3	+	++	+	++	++	+	++	+
		+	−	++	+	+	++	++	−
	4	+++	+	++	+	+++	+	Error	Error
		+	+	+++	−	++	−	++	−
	5	++	+	++	+	Error	Error	+	+
		++	++	++	+	++	−	+	++
	6	++	+	+	+++	+	+++	+++	−
		+	++	+++	−	+++	−	+	++

Smooth muscle fascicles were semiquantitatively analysed for intrafascicular expression of N-cadherin and PGP9.5, and graded as follows: features not present in any photographs, − (no expression); present >0–1/3, + (low expression); present in 1/3–2/3, ++ (intermediate expression); present in 2/3–entire fascicle, +++ (high expression). BDO: DO due to bladder outlet obstruction; NDO: neurogenic DO; IDO: idiopathic DO; GSI: genuin stress incontinence = control bladder. Note the heterogeneity of N-cadherin and PGP9.5 expression profile throughout the control and overactive specimens. In some cases, fatty tissue texture complicated specimen processing. Therefore, we were unable to study some fascicles (see: error).



(a) (b)

Figure 4: N-Cadherin positive strings at the border of detrusor bundles in overactive detrusor. N-Cadherin (green) double labeled with PGP9.5 (red) and counterstained with Dapi (blue). (a) Two fascicles are almost entirely surrounded by planes of N-cadherin cells (arrows). A large nerve trunk (arrowhead in (a) and (b)) is found in the perifascicular connective tissue. However, note lack of PGP9.5 expression in the upper fascicle. Instead, this fascicle shows a relatively high expression of N-cadherin (asterisk in (a) and (b)). Note green background signal in smooth muscle cells that was upgraded to facilitate tissue orientation. Magnification X100. (b) Magnification of white rectangle in (a). Slender strings consisting of numerous N-cadherin+ cells (arrow) are accompanied by multiple N-cadherin+ cells (#) in their connective tissue coat. Magnification X400. Binocular epifluorescent microscopy.

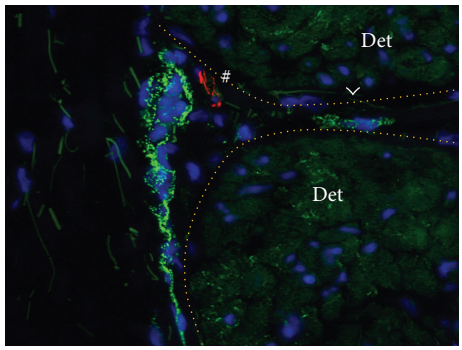


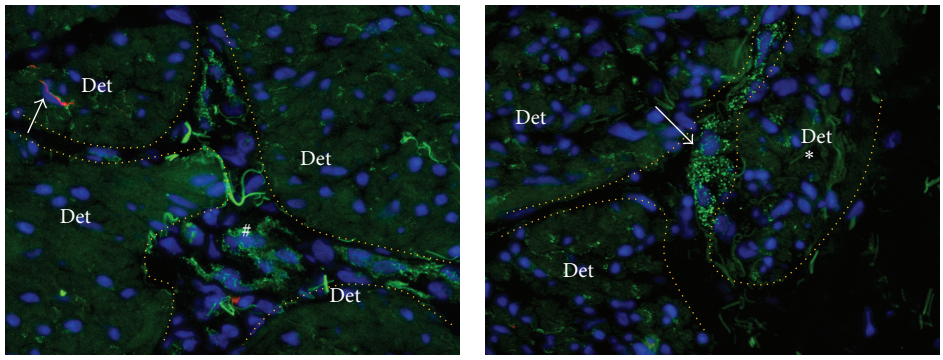
Figure 5: N-Cadherin positive strings and interfascicular cells. N-Cadherin (green) double labeled with PGP9.5 (red) and counterstained with Dapi (blue). Detrusor smooth muscle fascicles (Det) are demarcated by the dotted orange line. Note green background signal in smooth muscle cells that was upgraded to facilitate tissue orientation. N-Cadherin+ string closely associated to an adjacent detrusor smooth muscle fascicle. A cell expressing N-cadherin is housed within the interfascicular connective tissue plane (arrowhead). Note the close association of a PGP9.5+ nerve profile (#) with N-cadherin+ cells. PGP9.5 is not intrafascicularly expressed. Magnification X400. Binocular epifluorescent microscopy.

urothelium, neurons, and detrusor smooth muscle cells is getting more and more clear, the exact function of interstitial cells in the human bladder remains unelucidated.

The human urinary bladder shows spontaneous localized and propagating contractions during the storage phase [5]. The bladder may share characteristics with peristaltic activity in the gastrointestinal tract [19, 20]. In the gut, the myenteric plexus controls peristalsis. This plexus is embodied by the intramural neurons and interstitial cells of Cajal (ICCs) [6]. It has recently been proposed that the human bladder, like the gut, consists of modules that contract independently or synchronously with neighbouring modules [21]. Furthermore, the modules would be abnormally active and better coordinated in DO, compared to the normal bladder.

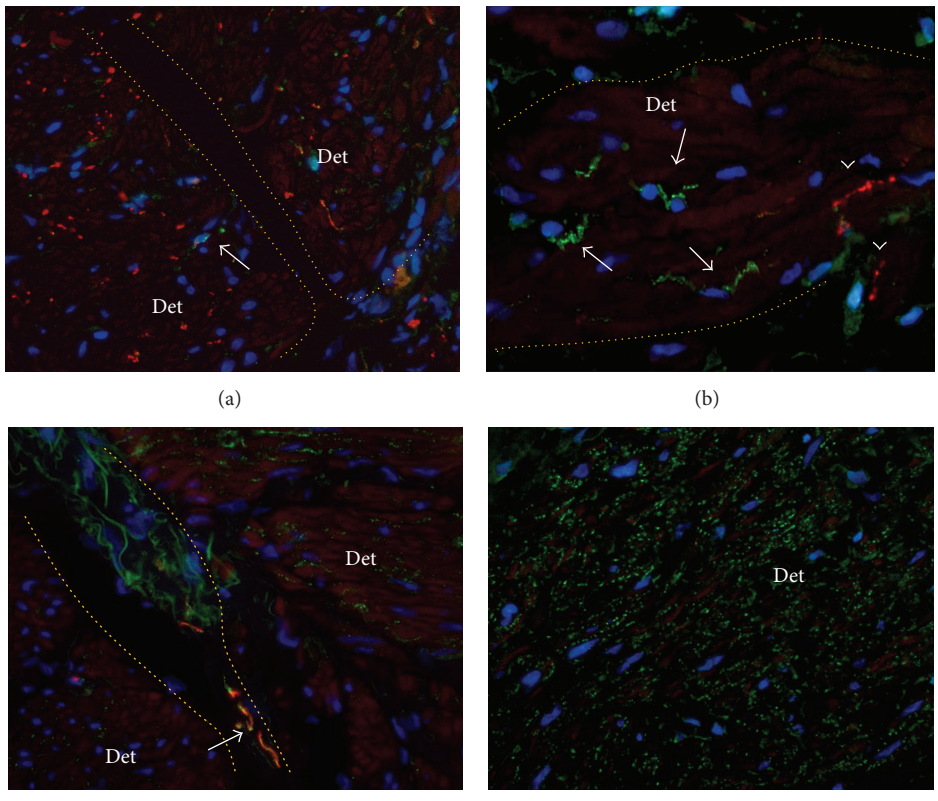
According to this hypothesis, two cell types play a potential role in coordination of autonomous activity in the bladder: interstitial cells (ICs) and intramural neurons. ICs of the bladder share properties of the ICCs [7, 8]. However, they embody a different subtype than the ICCs [7, 8]. In the human bladder, ICs are immunoreactive for the stem cell receptor C-kit, the cytoskeletal filament vimentin, the gap junctional protein connexin-43, the second messenger cyclic guanosine monophosphate (cGMP), and N-cadherin [9–12]. It is highly likely that they form a network in the suburothelial area and between the detrusor smooth muscle fascicles. Although the exact role for ICs in bladder function has not yet been described, it seem that either quantitative or qualitative changes in bladder ICs coexist with increased excitability in the OAB. We therefore investigated possible alterations to the network of human bladder ICs using N-cadherin.

In our study, all biopsies possessed a large population of N-cadherin+ cells. During previous studies we found that N-cadherin most probably can be used as a marker for a subpopulation of bladder ICs [12]. N-Cadherin+ ICs were located in the suburothelial lamina propria and the detrusor layer. In the lamina propria, they showed a bizarre morphology with multiple processes that seemed to form a network. In the detrusor, N-cadherin+ ICs were housed at the border of smooth muscle bundles, perifascicular, and within smooth muscle fascicles. Throughout the bladder wall, ICs expressing N-cadherin were closely associated with PGP9.5 positive neurons. Therefore, these cells could form a myovesical plexus as found in the human gut. Possibly, neuronal information is received in the larger ICs at the border of bundles and passed through the intermediate N-cadherin positive structures to small groups of detrusor smooth muscle cells.



(a) (b)

Figure 6: Intrafascicular N-cadherin positive nodes in the overactive detrusor. N-Cadherin (green) double labeled with PGP9.5 (red) and counterstained with Dapi (blue). Note green background signal in smooth muscle cells that was upgraded to facilitate tissue orientation. Detrusor smooth muscle fascicles (Det) are demarcated by the dotted orange line. (a) At the region where interfascicular connective planes meet, N-cadherin+ cells seem to accumulate and form interfascicular nodes (#). Note high level of intrafascicular N-cadherin in combination with low level of PGP9.5 expression (arrow). Filamentous shaped autofluorescent signal embody collagenous fibres. (b) A small group of muscle cells (asterisk) seems to be separated from the main fascicle by a penetrating N-cadherin positive structure (arrow). Magnification X400. Binocular epifluorescent microscopy.



(a) (b)

(c) (d)

Figure 7: N-Cadherin upregulation and PGP9.5 downregulation in smooth muscle bundles. N-Cadherin (green) was double labeled with PGP9.5 (red) and counterstained with Dapi. Note red background signal in smooth muscle cells that was upgraded to facilitate tissue orientation. Detrusor smooth muscle fascicles (Det) are demarcated by the dotted orange line. (a) Detrusor smooth muscle bundles in normal bladder show a dense PGP9.5 nerve profile in combination with a low level of N-cadherin expression. Arrow shows N-cadherin positive cell. (b) A muscle fascicle in NDO specimen with relatively low PGP9.5 expression (arrowheads) and a relatively high level of N-cadherin+ ICs (arrows). Magnification X630. (c) Cryosection of NDO specimen showing PGP9.5 negative muscle fascicles with high level of punctate N-cadherin expression. Note that PGP9.5+ nerve profiles in the interfascicular cleft closely associate with N-cadherin+ structures (arrow). (d) Smooth muscle fascicle with severely upregulated expression of N-cadherin. Note that the intensity of N-cadherin expressions seems higher than PGP9.5 expression in normal bladder as shown in (a). Magnification X400. Binocular epifluorescent microscopy.

We found changes to the network of N-cadherin+ ICs in most overactive detrusor specimens. These specimens revealed a remarkable variation in N-cadherin expression between and even within detrusor fascicles. This heterogeneous expression profile of N-cadherin is not based on processing artefacts, as in duplo analysis showed exact corresponding pattern of expression.

In the overactive detrusor specimens, upregulation of N-cadherin expression was found at three levels according to anatomical degree, in terms of smooth muscle bundles (level 1), smooth muscle fascicles (level 2), and smooth muscle cells (level 3). It seemed as if large N-cadherin positive structures surrounding smooth muscle bundles were newly formed (level 1), continuing as interfascicular N-cadherin+ processes junctioning to nodes (level 2) and slender intrafascicular N-cadherin+ branches possibly interacting with smooth muscle cells forming small modules.

In fascicles with high expression of N-cadherin, PGP9.5 expression seemed severely downregulated. The intensity of integration of N-cadherin+ structures between smooth muscle cells shows remarkable resemblance with morphological profile of intrafascicular PGP9.5+ innervation in normal bladder, albeit that the N-cadherin+ network seems much more developed. It is generally accepted that PGP9.5 is a nonspecific neuronal marker as it is expressed by all neuronal structures of the human bladder wall [14, 16, 17]. Therefore, it seems that the smooth muscle fascicles lacking PGP9.5 expression are actually denervated. Other investigators also found denervation in overactive detrusor and it is believed to be a general feature of pathological fascicles in the overactive bladder [22–24]. It could therefore be proposed that a highly developed network of ICs replaces the loss of innervation of detrusor smooth muscle fascicles in the overactive detrusor.

Kubota et al. claimed to have found an increased population of suburothelial ICs coexpressing C-kit and vimentin in the BOO-guinea-pig model [25]. However, a network of fibroblasts weakly expressing C-kit and vimentin is also present in the human detrusor layer. These cells do not have IC-like ultrastructure [26]. Therefore, the suburothelial C-kit positive cells as found by Kubota et al. might possibly embody fibroblasts instead of ICs.

De Jongh et al. found differences in the number and distribution of cGMP+ ICs in the bladders of guinea pigs with surgically induced bladder outflow obstruction [17]. Unlike us, they found alterations occurring in the suburothelial area. It is known that suburothelial ICs consist of distinct populations of cells [27]. It might very well be possible that N-cadherin+ ICs embody a subpopulation of ICs as identified by cGMP expression which is not upregulated in the overactive detrusor. Also, as their study was performed in guinea pigs, upregulation of N-cadherin+ ICs in the human bladder could be reserved to the detrusor layer, while the population of suburothelial N-cadherin+ ICs remains unaffected.

Others also found a correlation between upregulation of IC-like cells and exaggerated autonomous activity. Imatinib mesylate (Glivec; a specific C-kit receptor inhibitor) had an inhibitory effect on the overactive detrusor [28]. As C-kit labeling showed significantly more IC-like cells in overactive human detrusor than in normal specimens, it seems likely that this inhibitory effect is due to the upregulation of detrusor ICs. Also, reduction of ICCs is seen in syndromes with reduced autonomous activity of the gut, such as Hirschsprung's disease and functional intestinal obstruction [29].

Normal detrusor smooth muscle cells have poor electrical coupling [30]. Local contractions of groups of smooth muscle cells have been shown to occur in the normal bladder, but these did not lead to intravesical pressure rise [31]. Studies of the overactive detrusor ultrastructure demonstrated the existence of ultraclose abutments and protrusion junctions [32]. It was proposed that these might be the routes of spread of electrical activity in the overactive bladder. However, abnormally wide spread propagation of spontaneous activity could not only result from increased coupling between smooth muscle cells, but also from altered properties of the IC network. If we consider denervated fascicles with an upregulation of N-cadherin expression to be overactive, exceeding the number of overactive fascicles beyond a certain threshold might drive the bladder into behaving overactive. Also, as detrusor smooth muscle bundles house pacemaker cells [9], DO might not need the synchronized nerve-mediated smooth muscle excitation in order to develop.

4. Conclusions

It seems that the network of N-cadherin positive ICs in human urinary bladder has the ability to proliferate. As upregulation of N-cadherin+ ICs was found to coexist with denervation, it could be proposed that a developed network of interstitial cells replaces the loss of innervation of detrusor smooth muscle fascicles in overactive detrusor. However, further study is needed to gain more insight into the role of this cellular mechanism and its possible role in exaggerated autonomous activity in the pathological bladder.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

P2Y Receptor Modulation of ATP Release in the Urothelium

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The release of ATP from the urothelium in response to stretch during filling demonstrates the importance of the purinergic system for the physiological functioning of the bladder. This study examined the effect of P2 receptor agonists on ATP release from two urothelial cell lines (RT4 and UROtsa cells). Hypotonic Krebs was used as a stretch stimulus. Incubation of urothelial cells with high concentrations of the P2Y agonist ADP induced ATP release to a level that was 40-fold greater than hypotonic-stimulated ATP release ($P < 0.0011$, ADP EC50 1.8 μM). Similarly, an increase in ATP release was also observed with the P2Y agonist, UTP, up to a maximum of 70% of the hypotonic response (EC50 0.62 μM). Selective P2 receptor agonists, $\alpha\beta$-methylene-ATP, ATP-γ-S, and 2-methylthio-ADP had minimal effects on ATP release. ADP-stimulated ATP release was significantly inhibited by suramin (100 μM, $P = 0.002$). RT4 urothelial cells break down nucleotides (100 μM) including ATP, ADP, and UTP to liberate phosphate. Phosphate liberation was also demonstrated from endogenous nucleotides with approximately 10% of the released ATP broken down during the incubation. These studies demonstrate a role for P2Y receptor activation in stimulation of ATP release and emphasize the complexity of urothelial P2 receptor signalling.

1. Introduction

The first evidence for P2 receptor signalling from the urothelium was provided in 1997 with the work of Ferguson et al., who demonstrated in tissue strips that ATP is released from the urothelium in response to stretch [1]. Our understanding of the sensory role of the urothelium has since greatly increased, with stretch-induced ATP release from the urothelium also identified in cultured cells [2] and *in vivo* preparations [3].

ATP binds to two families of P2 receptors, P2X ligand-gated ion channels and G protein-coupled P2Y receptors. To date seven P2X receptors have been identified [4], with $P2X_4$ and $P2X_2$ receptors [5] expressed on the urothelium and $P2X_3$ expressed on the urothelium [5, 6] as well as on suburothelial afferent nerves and myofibroblasts [7]. It is hypothesised that ATP released from the urothelium interacts with P2 receptors located on both afferent nerves ($P2X_3$) [8] and myofibroblasts to signal bladder fullness. A role for ATP in bladder dysfunction has been postulated with increased ATP release associated with sensory disorders such as interstitial cystitis [9] and painful bladder syndrome [10]. Furthermore, ATP has been shown to play a role in bladder sensation, with ATP concentration in the intravesical fluid correlating with the first desire to void in patients with both overactive bladder [3] and painful bladder [11] but not in control patients.

P2X receptors are activated preferentially by ATP; thus urothelial derived ATP may have an autocrine action on urothelial P2X receptors. However the urothelium also expresses ectoATPase enzymes [12] and so is able to hydrolyse ATP (and other nucleotides) to breakdown products such as ADP, AMP, and adenosine. The perceived function of these enzymes is to limit the availability of ATP. Recently, the urothelium has been shown to release nucleotides other than ATP with twelve nucleotides quantified by HPLC in patient urine specimens [13]. The levels of ADP, UTP, UDP, and GTP were found to be more than 10 times higher than the level of ATP [13]. Other epithelial cells have also been shown to release nucleotides such as ATP, ADP, AMP, adenosine, UTP, and UDP, in response to hypotonic stimulus [14]. The role of these nucleotides in P2 receptor signalling in the bladder is yet to be determined. It is known that in addition

to ATP, other nucleotides, in particular ADP and UTP, can also be substrates for P2Y receptors; however, their effects on urothelial P2Y receptors have not been explored. Eight P2Y receptors have been identified [15] with $P2Y_1$ [5, 16], $P2Y_2$ [5, 16, 17], $P2Y_4$ [16, 17], and $P2Y_{11}$ [5] expressed on urothelial cells. $P2Y_6$ receptors are expressed on suburothelial myofibroblasts [18] but their expression on urothelial cells remains controversial [5, 19]. The function of these receptors on the urothelium is currently undetermined. In addition, adenosine formed from the breakdown of ATP binds to P1 receptors which are also expressed on the urothelium [20].

It is very clear that the complexity of urothelial P2 receptor signalling and the feedback of ATP breakdown and release have not been adequately explored to date. Therefore the aims of this study were to examine the effect of purine and pyrimidine nucleotides on urothelial cell ATP release. We hypothesise that purines and pyrimidines selective for P2Y receptors will modulate ATP release from the urothelium.

2. Materials and Methods

2.1. Cell Culture. Human urothelial RT4 cells were obtained from the ECACC. Cells were grown at 37°C with 5% CO_2 in McCoy's 5A culture medium supplemented with 10% foetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, and 0.25 μg/mL of fungizone. When confluent, RT4 cells were passaged with 0.05% Trypsin-EDTA for 5 minutes and then plated onto T75 flasks for continuous passage or onto 24 well plates for use in experiments when confluent (approximately 3 to 5 days after passage).

Human urothelial UTOtsa cells were a gift from Dr. Scott Garrett from the University of North Dakota. Cells were grown at 37°C with 5% CO_2 in Dulbecco's modified eagle's medium supplemented with 5% foetal bovine serum, 100 units/mL of penicillin, 100 μg/mL of streptomycin, 0.25 μg/mL of fungizone, and 1% glucose. UROtsa cells were passaged in the manner described above for RT4 cells.

2.2. ATP Release Studies. ATP release was determined as we have described previously [2]. Urothelial cells were washed (three times) with carbogenated Krebs-Henseleit solution (containing mM: NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, and D-glucose 11.7). The basal level of ATP release was then determined by a 10-minute incubation in 500 μL Krebs-Henseleit solution. These media were collected and followed with the experimental treatments. Cells were exposed to normal Krebs-Henseleit (control) or the indicated concentration of nucleotide. Cells were exposed to hypotonic Krebs-Henseleit (1 : 2 dilution of Krebs-Henseleit in distilled water), a stimulus commonly used to mimic stretch in cultured cells [2]. Cells were treated for 10 min before the supernatant (200 μL) was collected and used for ATP determinations.

ATP concentration in the supernatant was measured using the bioluminescence assay according to the manufacturer instructions. Equal volumes of the cellular supernatant or ATP standard solutions (10^{-6} to 10^{-10} M) were mixed with the bioluminescence assay mix and the luminescence generated was measured immediately using a plate reader (BMG labtech Polarstar). The standard concentrations fell within the upper and lower limits of sensitivity of the ATP bioluminescence assay. The ATP concentration in the cell supernatant was calculated relative to the standard curve. Luminescence was also measured for all concentrations of the nucleotides used. The luminescence determined for each concentration of nucleotide was used as a blank for that treatment and was subtracted from the corresponding cellular luminescence before the ATP concentration was calculated. Treatments were carried out in triplicate and the mean ATP concentration (in nM) per treatment determined.

2.3. EctoATPase Studies. Confluent urothelial cells in 24 well plates were washed (three times) in phosphate free media (containing mM: NaCl 120, KCl 5, $CaCl_2$ 2, HEPES 20, and D-glucose 10) [12]. After washing cells were incubated with 100 μM nucleotide. After 30 minutes the supernatant was collected and the phosphate concentration determined. In additional experiments confluent cells in 12 well plates were incubated in control or hypotonic (50% dilution in water) phosphate free media for 10 or 30 minutes and both ATP concentration and phosphate concentration determined.

Phosphate liberated from nucleotides was determined as previously described [21]. Equal volumes of cell supernatant and colour reagent (containing 1% ammonium molybdite, 0.3 M H_2SO_4, 4% $FeSO_4$) were mixed. After 15 minutes absorbance at 750 nm was measured in a plate reader (BMG labtech Polarstar). Phosphate concentration in the cell supernatant was determined relative to a standard curve (KH_2PO_4, 10 to 150 nM). Treatments were carried out in triplicate and the mean phosphate concentration (in nM) per treatment determined.

2.4. Statistics. Results are nonnormally distributed and as such are expressed as median with interquartile range (IQR). Two different treatments were compared using a Mann-Whitney t-test. Concentration response relationships were examined using a sigmoidal concentration response curve. All statistics were performed using Graphpad Prims (version 6) (San Diego, CA).

2.5. Materials. All cell culture reagents were purchased from Invitrogen (Mount Waverley, Australia). Bioluminescence ATP Assay kit and nucleotides were from Sigma-Aldrich (Sydney, Australia). All other reagents were of high analytical grade.

3. Results

3.1. Effect of Nucleotides on Urothelial Cell ATP Release. Hypotonic Krebs was used as a stretch stimulus and was seen to induce an approximate threefold increase in ATP release ($P = 0.0006$). Incubation of RT4 cells with ADP (100 μM for 10 minutes) induced ATP release to a level that was 40-fold higher compared to the hypotonic stimulus (Table 1, $P < 0.0011$). In the presence of AMP and adenosine, the level of

Table 1: ATP release in RT4 urothelial cells stimulated by 10-minute incubation with nucleotides (100 μM).

	ATP release in RT4 cells
Control	20.28 (13.24–40.22) nM (n = 33)
Hypotonic	64.63 (43.75–92.95) nM (n = 33)
ADP	2641 (2006–3598) nM (n = 13)
AMP	8.56 (4.74–189.2) nM (n = 17)
Adenosine	5.58 (4.52–65.85) nM (n = 4)
CTP	51.12 (37.4–99.96) nM (n = 11)
GTP	55.33 (34.42–173.4) nM (n = 15)
UTP	60.57 (36.51–126.1) nM (n = 11)
α,β-Methylene-ATP	26.6 (22.5–77.5) nM (n = 9)
ATP-γ-S	2294 (429–2538) nM (n = 16)

Table 2: ATP release in UROtsa urothelial cells stimulated by 10-minute incubation with nucleotides (100 μM).

	ATP release in UROtsa cells
Control	11.08 (4.3–30.2) nM (n = 6)
Hypotonic	68.3 (35.6–133.4) nM (n = 6)
ADP	3377 (752–6361) nM (n = 6)
UTP	61.2 (26.6–115.7) nM (n = 6)
2-Methylthio-ADP	17.7 (13.9–26.9) nM (n = 6)

ATP release was not significantly different to the control level (Table 1). The response to CTP and GTP was not significantly different to the ATP release induced by hypotonic media (Table 1).

When the concentration response effect of ADP induced ATP release was determined it was seen to only occur at high concentrations of ADP (Figure 1(a)) with an EC50 of 1.8 μM determined (n = 5). A concentration dependent inhibition of ATP release was seen with AMP (Figure 1(b)) with an EC50 of 0.33 μM (n = 7). A concentration dependent increase in ATP release was observed with UTP, up to a maximum of approximately 70% of the hypotonic response (Figure 1(c)). An EC50 of 0.62 μM was determined (n = 7).

In RT4 cells, concentration response relationships were also determined for P2 receptor agonists and antagonists. The P2X agonist, $\alpha\beta$-methylene-ATP, and P2Y agonist, ATP-γ-S, had no effect on ATP release (Figures 2(a) and 2(b), n = 9 and 15, resp.). The more selective $P2Y_1$ agonist, 2-methylthio-ADP, inhibited ATP release in a concentration dependent manner (Figure 2(c), EC50 0.16 μM, n = 8).

P2 receptor antagonists were investigated for their ability to inhibit ADP-stimulated ATP release. PPADS (100 μM) had no effect on ADP-stimulated ATP release (88.4 (65.2–113.4)% of ADP-stimulated release, n = 8, P = 0.4) while suramin (100 μM) significantly inhibited ADP-stimulated ATP release (43.3 (27.6–41.7)% of ADP-stimulated ATP release, P = 0.002, n = 4; IC50 8.3 μM, n = 8).

The results observed in RT4 urothelial cells were also confirmed in UROtsa cells (Table 2). Similar to the results observed in RT4 urothelial cells, treatment of UROtsa cells with hypotonic Krebs induced an increase in ATP release (P = 0.0087). Incubation of UROtsa cells with ADP (100 μM for 10 minutes) induced an almost 50-fold increase in ATP release compared to that seen with the hypotonic stimulus (P < 0.0022). UTP (100 μM) initiated ATP release similar to that seen with hypotonic stimulus while 2-methylthio-ADP (100 μM) elicited an ATP release response similar to the control level.

Table 3: Phosphate liberation from 30-minute incubation of RT4 urothelial cells with 100 μM nucleotides (n = 8).

	Phosphate liberated (nM)
ATP	1.3 (−0.9–3.8)
ADP	7.5 (1.9–12.1)
AMP	4.1 (3.3–7.7)
CTP	3.4 (1.2–4.8)
GTP	2.6 (0.3–6.2)
UTP	7.8 (6.5–9.9)

3.2. EctoATPase Activity of Urothelial Cells. RT4 urothelial cells were shown to have the capacity to liberate phosphate from nucleotides (100 μM), with the greatest amount of phosphate liberated from ADP and UTP (Table 3). In addition, a small amount of phosphate liberation was able to be demonstrated from endogenous nucleotides released during the experiment (Figure 3). When RT4 cells were incubated in either control or hypotonic phosphate free buffer for 10 or 30 minutes, significant ATP release was induced (Figure 3(a), n = 8). After a 10-minute incubation the level of release in the control was approximately 40 nM and in hypotonic phosphate free media was approximately 80 nM which was comparable to that seen using Krebs-Henseleit solution (Table 1). Over the 10- or 30-minute incubation some of this endogenous ATP was broken down by RT4 cells to liberate phosphate that could be detected (Figure 3(b), n = 8). Significantly more phosphate was detected in cells treated with hypotonic media for 10 and 30 minutes (P = 0.0002 and 0.0019, resp.). After a 10-minute incubation the level of phosphate liberated indicated that approximately 10% (7 to 15%) of the endogenously released ATP was broken down during the incubations in control and hypotonic media, respectively. In the presence of the ectoATPase inhibitor ARL67156 (100 μM) the amount of ATP detected in cells treated with hypotonic phosphate free buffer increased 5-fold after both 10- and 30-minute incubations (Figure 3(c), n = 4).

4. Discussion

The demonstration of ATP release from the rabbit bladder urothelium in response to stretch by Ferguson and colleagues in 1997 [1] has been pivotal to our understanding of signalling within the bladder. However, our understanding of the factors that modulate ATP release and the autocrine signalling that occurs at the urothelial cell layer is limited. The current study has demonstrated that P2 receptor agonists are able to stimulate ATP release from urothelial cells. In fact the P2Y agonist ADP was shown in two urothelial cell lines, to stimulate a level of ATP release that is far in excess of

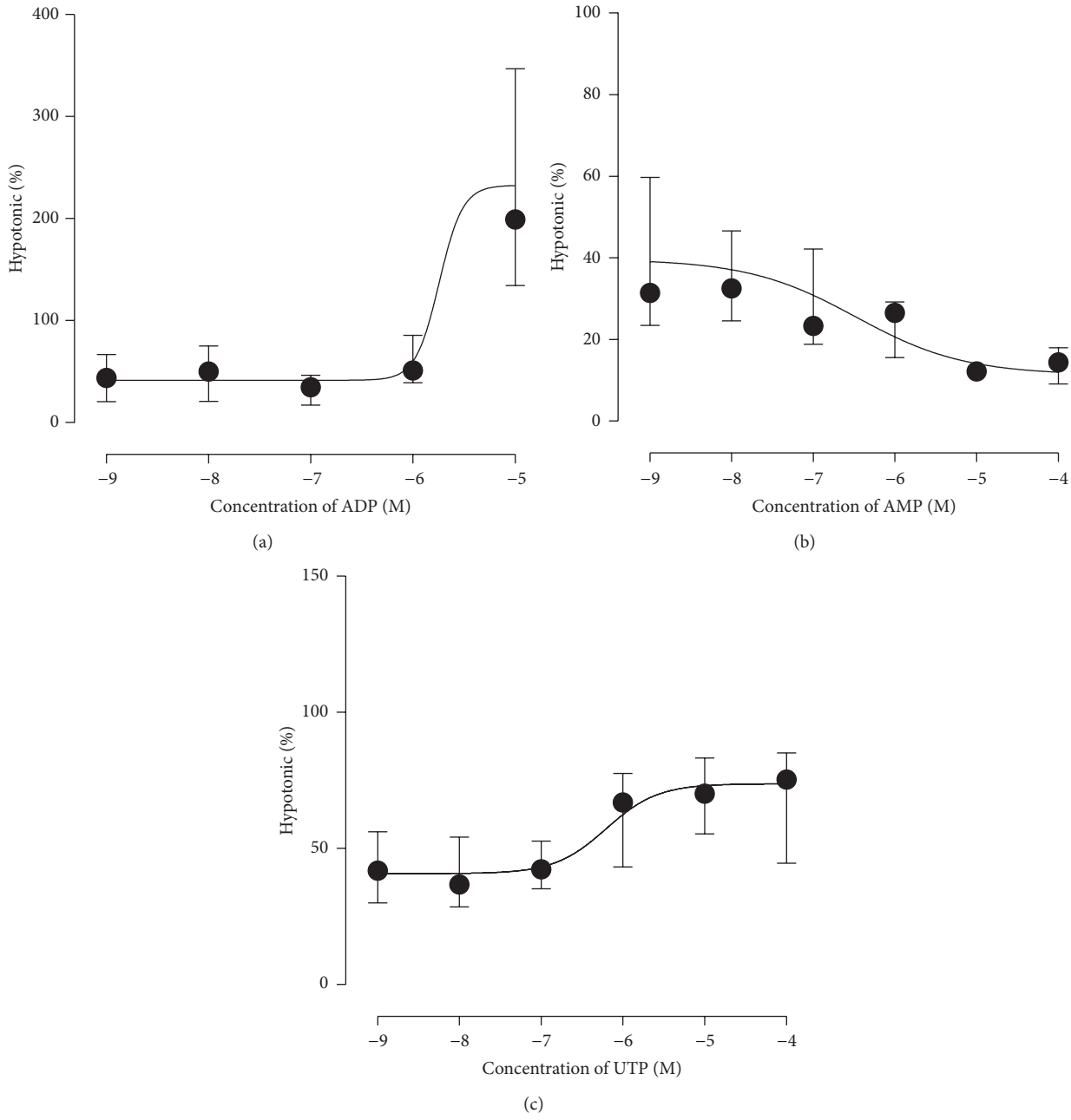

Figure 1: Concentration response relationships for nucleotides ADP (a), AMP (b), and UTP (c) on RT4 urothelial cell ATP release.

that stimulated by hypotonic media. Similarly, UTP, another P2Y agonist [22], was also shown to stimulate ATP release, although to a lesser extent. In contrast, P2X agonists such as α,β-methylene-ATP were not seen to stimulate ATP release from cultured urothelial cells. These results are similar to the recently published findings by Sui and colleagues who reported that the P2Y agonist UTP stimulated ATP release in guinea pig and human bladder mucosal strips, while the P2X agonist α,β-methylene-ATP had no effect [22]. UTP stimulated ATP release has also been reported from primary cultures of rat bladder urothelial cells [17].

It has been shown that $P2Y_1$ [5, 16], $P2Y_2$ [5, 16, 17], $P2Y_4$ [16, 17], and $P2Y_{11}$ [5] receptor subtypes are present in bladder urothelial cells (see introduction). While P2X receptors respond preferentially to ATP, P2Y receptors are divided on the basis of their sensitivity to adenine nucleotides ($P2Y_1$, $P2Y_{11}$, $P2Y_{12}$, and $P2Y_{13}$) or uracil nucleotides ($P2Y_2$, $P2Y_4$, $P2Y_6$, and $P2Y_{14}$) [23]. Of the P2Y receptors that respond to adenine nucleotides, $P2Y_1$, $P2Y_{12}$, and $P2Y_{13}$ respond to ADP while $P2Y_{11}$ receptors respond to ATP [23]. In the current study, ATP release was stimulated by both ADP and UTP, although the release elicited by ADP was far

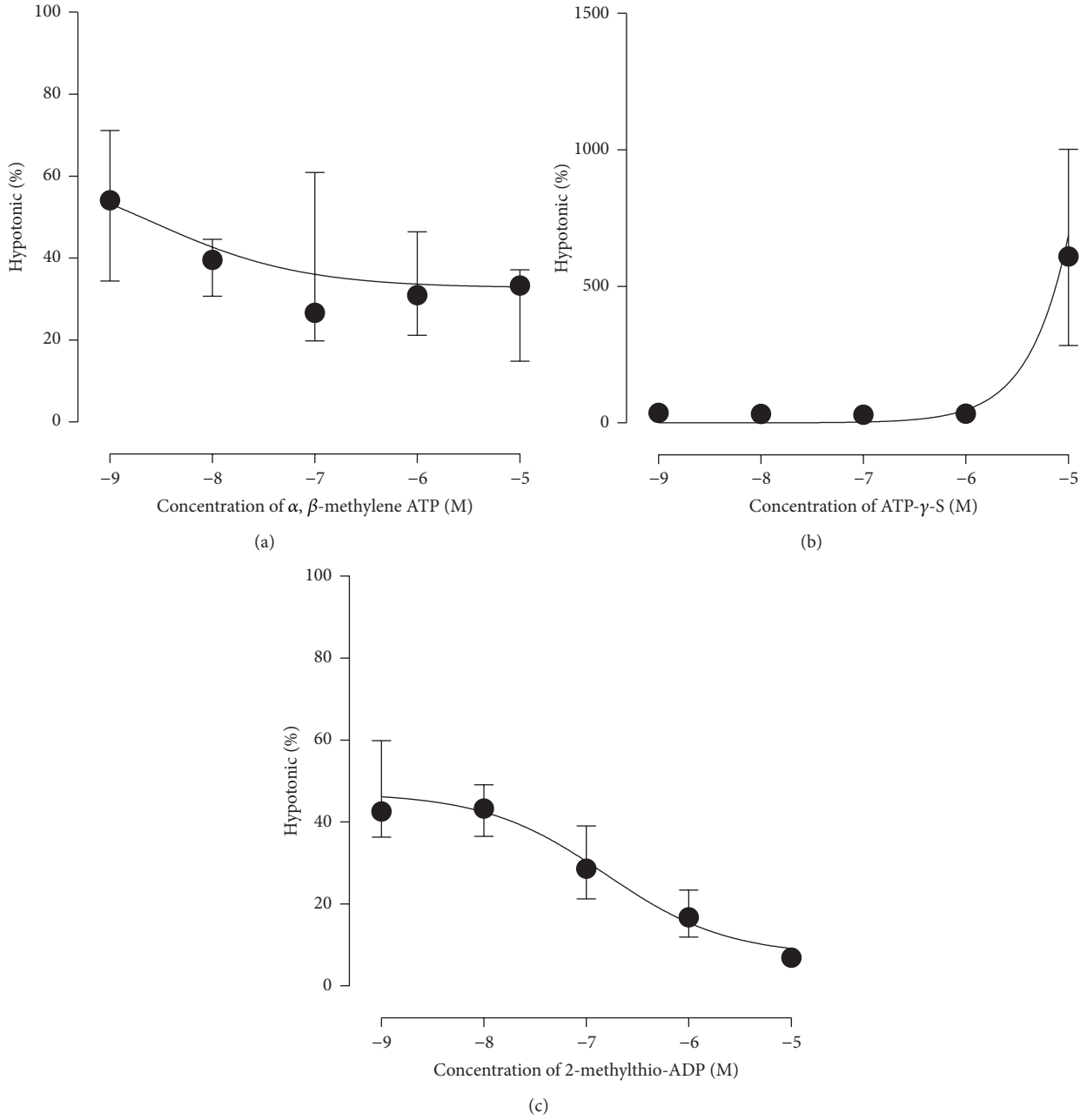

Figure 2: P2 receptor agonist stimulated ATP release. Incubation of RT4 urothelial cells with increasing concentration of α,β-methylene-ATP (a), ATP-γ-S (b), and 2-methylthio-ADP (c).

greater than that elicited by UTP, suggesting the involvement of more than one P2Y receptor subtype. The EC50 value of ADP-stimulated ATP release suggests activity at $P2Y_1$ or $P2Y_{11}$ receptors. This is in agreement with previously reported EC50s at $P2Y_1$ of 0.9–8 μM [24–26]. The effects of UTP were closely associated with EC50 values at $P2Y_2$ or $P2Y_4$ receptors, agreeing with previously reported EC50s (0.14–0.8 μM) at the $P2Y_2$ receptor [24, 25] and $P2Y_4$ receptor (2.5 μM) [24, 25]. In contrast, the selective $P2Y_1$ agonist, 2-methylthio-ADP [26], did not stimulate ATP release. The current study therefore suggests that more than one P2Y receptor subtype is likely to be involved in nucleotide stimulated ATP release. Unfortunately, the lack of subtype selective agonists for individual P2Y receptors makes it difficult to definitively identify the receptor subtypes involved in the observed responses.

It is possible that the apparent ATP release stimulated by high concentrations of ADP was due to the conversion of ADP to ATP by cell membrane-bound adenylate kinase. While this enzyme is predominately intracellular [27], it has been identified on the cell membrane of endothelial cells [28] and other cell types [27] although there have been no

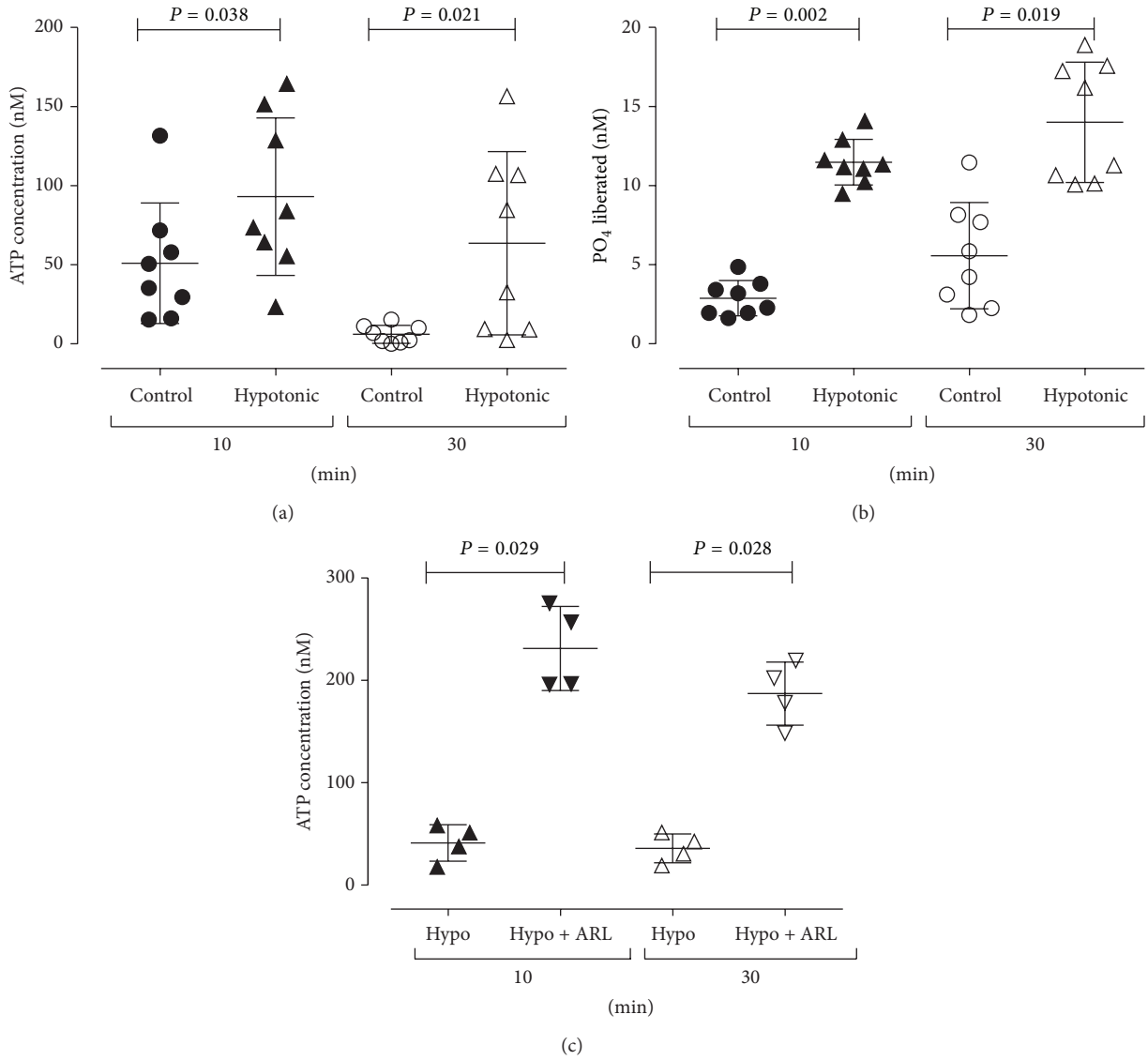

Figure 3: Phosphate liberation from endogenously released ATP. Incubation of RT4 urothelial cells in control or hypotonic phosphate free buffer for 10 or 30 minutes induced ATP release (a). Over the 10- or 30-minute incubation a small amount (approximately 10%) of this ATP was broken down by urothelial cells to liberate phosphate that could be detected (b). The presence of the ectoATPase inhibitor ARL67156 (100 μM) was seen to increase the amount of ATP detected after both 10 and 30 minutes (c).

reports of cell membrane-bound adenylate cyclase in the urothelium. However, coincubation of ADP with the P2 receptor antagonist suramin inhibited ADP-stimulated ATP release, suggesting that it is indeed P2Y receptors that are involved in this response (reported IC50s 3–50 μM) [15].

The source of ADP *in vivo* is unknown; however, nucleotides have recently been isolated from human urine samples [13]. In addition, urothelial cells express ectonucleotidase enzymes capable of degrading ATP and UTP to their respective nucleotides [29]. The basal and intermediate cells of the mouse bladder urothelium express NTPDase 3 [29]. Similarly, the human urothelial cell line RT4 used in the current study expresses NTPDase 3 and 5 [12] but not NTPDase 1 [12, 30]. NTPDase 1, 2, and 3 are known to face the extracellular environment and catalyse the breakdown of extracellular ATP [29]. It is thought that ectoATPase enzymes function to limit the exposure of P2 receptors to their ligands and to modulate the autocrine response to released ATP [29].

In the current study the activity of ectoATPase in urothelial RT4 cells was examined in three ways. Firstly, ectoATPase enzymes present on the RT4 urothelial cells were able to liberate phosphate from stretch-induced ATP release, indicating a capacity to generate mediators such as ADP from stretch-induced ATP release. Secondly, incubation of the ectoATPase inhibitor ARL67156 (100 μM) [31] resulted in a higher level of stretch-induced ATP release being detected. At this concentration, ARL67156 has been shown to inhibit the activity of NTPDase3 (Ki 18 μM) [32]. Finally, we demonstrated the capacity of ectoATPase enzymes associated with RT4 urothelial cells to liberate phosphate

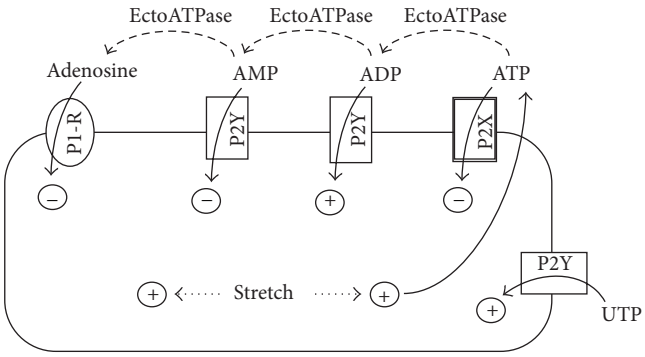

Figure 4: Hypothetical schematic representation of the complexity of the effect of nucleotides and stretch on urothelial cell ATP release.

from exogenous nucleotides including ATP, ADP, AMP, CTP, GTP, and UTP. Similarly, Stella and associates showed that RT4 urothelial cells were able to liberate phosphate from exogenous nucleotides [12].

The results obtained in the current study demonstrate that P2 receptor signalling in the urothelium is a complex interaction between ATP release and breakdown. To add to the complexity, it now seems likely that products formed from breakdown of nucleotides such as ATP also have affects at P2 receptors located on the urothelium. Our understanding of this complex interaction between ATP release and breakdown has been shown schematically in Figure 4. ADP has been previously postulated to stimulate urothelial ATP release [9] and to activate intracellular calcium transients [5, 33] that were greater than those activated by hypotonic solutions [33], a commonly used stimulus for ATP release. It was proposed that the ATP-dependent calcium transients were mediated by urothelial P2Y (rather than P2X) receptors [5, 33]; however, it is known that ATP has a low affinity at P2Y receptors [23, 25]. It is therefore possible that the calcium transients induced by endogenous ATP were in fact due to the generation of ADP by membrane-bound ectoATPases. This is supported by the findings of the current study which demonstrate that ADP is capable of stimulating ATP release. Adding to the complexity of P2 receptor signalling, further breakdown of ADP to AMP and adenosine was seen to inhibit ATP release. Inhibition of ATP release by adenosine has been previously reported in rabbit bladder mucosal strips [20]. These findings indicate that while the initial breakdown of ATP to ADP may exert positive feedback for ATP release which is short lived, further breakdown of ATP to AMP and adenosine may provide negative feedback for ATP release.

Activation of urothelial P2Y receptors has been proposed to be responsible for a number of physiological functions of the urothelium. Exogenous application of P2Y receptor agonists, including ADP and UTP, has been shown to increase spontaneous activity in rat bladder sheets [34]. Similar to the results seen in this study, it was unlikely that a single P2Y receptor subtype was responsible for the reported increase in spontaneous activity [34] with the involvement of $P2Y_1$, $P2Y_2$, $P2Y_4$, and $P2Y_6$ being hypothesised. In addition, activation of $P2Y_2$ receptors leads to release of the inflammatory mediators interleukins 8 and 6 from uroepithelial cells [35], indicating a role for activation of P2Y receptors in inflammatory responses in the bladder. Interestingly, expression of the $P2Y_2$ receptor decreased in a feline model of interstitial cystitis [16]. Intravesical instillation of a $P2Y_6$ selective agonist induced bladder overactivity characterised by increased voiding frequency in a rat cystometry model in addition to increased ATP release into the voided fluid [19].

Alterations in urothelial ATP release have been identified in bladder dysfunction including interstitial cystitis [9], painful bladder syndrome [10, 11], and overactive bladder [3]. The results of this study demonstrate the complexity of P2 receptor signalling in the urothelium by elucidating a role for a number of P2Y receptor subtypes in initiating ATP release. Recent literature has shown the important role of these receptors in normal bladder physiology, indicating that these receptors may represent a potential future target for the treatments of bladder dysfunction.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

The Role(s) of Cytokines/Chemokines in Urinary Bladder Inflammation and Dysfunction

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Bladder pain syndrome (BPS)/interstitial cystitis (IC) is a chronic pain syndrome characterized by pain, pressure, or discomfort perceived to be bladder related and with at least one urinary symptom. It was recently concluded that 3.3–7.9 million women (>18 years old) in the United States exhibit BPS/IC symptoms. The impact of BPS/IC on quality of life is enormous and the economic burden is significant. Although the etiology and pathogenesis of BPS/IC are unknown, numerous theories including infection, inflammation, autoimmune disorder, toxic urinary agents, urothelial dysfunction, and neurogenic causes have been proposed. Altered visceral sensations from the urinary bladder (i.e., pain at low or moderate bladder filling) that accompany BPS/IC may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). The goals for this review are to describe chemokine/receptor (CXCL12/CXCR4; CCL2/CCR2) signaling and cytokine/receptor (transforming growth factor (TGF-β)/TGF-β type 1 receptor) signaling that may be valuable LUT targets for pharmacologic therapy to improve urinary bladder function and reduce somatic sensitivity associated with urinary bladder inflammation.

1. Lower Urinary Tract (LUT)

1.1. Anatomy. The LUT (bladder and urethra) is a division of the renal system that functions to passively store kidney byproducts until it is appropriate to void. To accomplish this, the urinary bladder is a muscular and membranous organ whose structure embodies its reservoir function. Its external features can be organized into an apex, fundus, body, and neck. The apex, or vertex, is on the anterior surface of the urinary bladder and is associated with ligament remnants attached to the umbilicus [1]. The posterior surface is the fundus and its most inferior aspect is termed the base of the urinary bladder [1]. The body typically represents the area between the apex and the fundus and the bladder neck is the most caudal aspect of the inferior bladder surface that is perforated by the internal urethral orifice [1].

The urinary bladder wall is composed of three layers: tunica mucosa, tunica muscularis propria, and tunica serosa/adventitia. The tunica mucosa consists of transitional epithelium and a lamina propria. Transitional epithelial cells in the urinary bladder are termed the urothelium and are arranged in basal, intermediate, and apical cell layers. Basal cells are monolayers directly attached to the basement membrane [2]. Intermediate cells are generally larger in diameter than basal cells and range from one to multiple cell layers depending on the species [2]. The apical, or umbrella, cells are hexagonal in shape and range from 25 to 250 μm depending on urinary bladder distention [2, 3].

Several distinct features of the luminal surface of umbrella cells establish antiadherence and an impermeable barrier characteristic of the urinary bladder mucosa. First, tight junction complexes comprised of occludin and claudin proteins regulate paracellular transport between adjacent umbrella cells [3]. The apical membrane is also occupied by uroplakin, a crystalline plaque cell surface protein that forms an asymmetric unit membrane to maintain impermeability during bladder expansion [4]. Lastly, a layer of proteoglycans on the mucosal surface of umbrella cells serves as an antiadherence factor and provides yet another physical barrier between urinary constituents and the lamina propria [5].

The extracellular matrix of the lamina propria is deep to the basement membrane of the urothelium and contains a diverse array of interstitial cells, nerve terminals, and vasculature [3, 6]. It has been suggested that the lamina propria may have an important role in integrating epithelial and smooth muscle function due to its innervations and proximity to the urothelium and tunica muscularis propria [6]. The tunica muscularis propria consists of three smooth muscle layers termed the detrusor. The internal and external layers are arranged longitudinally, whereas those in the middle are circular [7, 8]. The smooth muscle cells in the muscularis propria retain their classic spindle shape and are bundled together by collagen-rich connective tissue [7]. External to the muscularis propria, the tunica serosa surrounds the superior and lateral surfaces of the urinary bladder wall, whereas the retroperitoneal aspects contain a vascular, loose connective tissue termed the tunica adventitia [8].

Caudal to the inferior surface of the urinary bladder is the urethra. Similar to the urinary bladder wall, the urethral wall is composed of a tunica mucosa, tunica muscularis propria, and tunica adventitia. The tunica mucosa consists of transitional epithelium proximal to the urinary bladder followed by nonkeratinized, stratified squamous epithelium distally [8, 9]. The tunica muscularis propria is composed of inner and outer smooth muscle arranged longitudinally and circularly, respectively [8]. In the male urethra, the circular smooth muscle fascicles join with urinary bladder smooth muscle at the urethrovesical junction to form the internal urethral sphincter [8, 9]. The smooth muscle fascicles along the proximal female urethra, however, do not appear to anatomically arrange into a sphincter [9]. Skeletal muscle of the urethral wall forms the external urethral sphincter and extends along the membranous urethra in males to generate voluntary pressure during bladder filling [10]. The skeletal muscle fibers in the female urethra join to form an "external" urethral sphincter comprised of a sphincter urethrae, compressor urethrae and sphincter urethrovaginalis to provide urinary continence through urethral and vaginal closure [11].

1.2. Neural Control. The LUT is regulated by supraspinal, spinal, and peripheral nervous system (PNS) input to maintain "switch-like" patterns of storage and elimination activity and has been previously reviewed in greater detail [10]. Briefly, bladder wall mechanoreceptors initiate visceral afferent (Aδ fibers) activity during the storage phase that synapse on spinal interneurons [10, 12]. Spinal reflex pathways then facilitate storage by directly enhancing thoracolumbar sympathetic outflow and somatomotor discharge or ascending, in some species, to keep metencephalic integration centers [10, 12].

Spinal interneurons activate preganglionic sympathetic fibers from the intermediolateral cell column of the lower thoracic (T10) through upper lumbar (L2) spinal cord that form thoracic and lumbar splanchnic nerves [13, 14]. The preganglionic fibers then synapse on the prevertebral inferior mesenteric ganglia or paravertebral ganglia and travel along the hypogastric and pelvic nerves, respectively [10]. Adrenergic neurotransmission on the urinary bladder smooth muscle β-adrenergic receptors promotes bladder wall relaxation and accommodation [13]. Bladder filling is also facilitated by the activation of α-adrenergic receptors on the internal urethral sphincter resulting in contraction of the urethral outlet [13]. Spinal reflex pathways not only enhance sympathetic outflow but also α-motoneuron discharge from Onuf's nucleus in the ventrolateral horn of the sacral (S2–S4) spinal cord [12]. Propagation of this signal along the pudendal nerve to the external urethral sphincter elicits skeletal muscle contraction by activating nicotinic acetylcholine receptors to provide voluntary control over urinary continence [13].

Upon reaching the tension threshold, bladder afferents (Aδ fibers) bypass local spinal reflexes and ascend to the mesencephalic periaqueductal gray (PAG). Unlike the reflexes underlying the storage phase, the elimination phase relies on supraspinal circuitry as evidenced by voiding dysfunction following lower thoracic spinal cord injury [14, 15]. After cortical processing, the PAG sends excitatory input to a region in the dorsolateral pontine tegmentum termed the pontine micturition center (PMC) [16]. The PMC then sends descending cortical projections that synapse on preganglionic parasympathetic neurons and inhibitory interneurons in the sacral spinal cord [14, 16].

The preganglionic parasympathetic fibers arise from the intermediolateral cell column of the sacral (S2–S4) spinal cord to form pelvic splanchnic nerves. Upon coursing through and exiting the hypogastric and pelvic plexus, the fibers join the pelvic and pudendal nerves to synapse on terminal ganglia and innervate the detrusor smooth muscle and urethra [12, 13]. Cholinergic and nonadrenergic/noncholinergic neurotransmission on the urinary bladder smooth muscle promotes bladder wall contraction by activating muscarinic acetylcholine receptors and purinergic receptors, respectively [14]. Elimination of urine is also facilitated by nitric oxide release onto the internal urethral sphincter resulting in a relaxation of the urethral outlet [14]. The PMC not only augments parasympathetic outflow but also attenuates preganglionic sympathetic and α-motoneuron discharge to the LUT [16]. The descending cortical projections terminating on inhibitory interneurons in the sacral spinal cord prevent excitatory input into the urethral sphincters resulting in dilation of the urethral orifice and continuous flow of urine. As distention of the urinary bladder decreases during the elimination phase, ascending excitation to the dorsolateral metencephalon is diminished and the storage phase is once again switched on.

1.3. Symptoms and Dysfunction. The terminology used in the following section is consistent with the standardization report of LUT symptoms and function by the International Continence Society and will refer to their definitions when appropriate [17]. Similar to other clinical indications, LUT symptoms are the patient's qualitative representation of a purported condition. These symptoms, in particular, refer to a spectrum of LUT functions that include storage, elimination, and postmicturition disturbances.

Symptoms associated with the storage phase include, but are not limited to, "increased frequency, urgency, and incontinence" [17]. The complaint of increased urinary frequency is prevalent among both men and women with LUT dysfunction and has been suggested to affect an individual's quality of life as demonstrated by a strong correlation between frequency and bothersome endorsements [18]. Increased urgency is a complaint of the "sudden compelling desire to pass urine" that may be accompanied by pain, pressure, or discomfort associated with the LUT [17]. Lastly, urinary incontinence includes a complaint of the "involuntary leakage of urine" and may manifest in various forms and severities [17]. It is important to note that incontinence is not representative of one particular LUT dysfunction but rather can arise from multiple sources including stress, comorbid disorders, and congenital abnormalities [19].

Symptoms associated with the elimination phase include "hesitancy, slow or intermittent stream, straining, and terminal dribble" [17]. These symptoms generally involve complaints of the initiation and continuation of voiding and alterations to their urine stream and appear to be more prevalent in men compared to women [17, 18]. Symptoms associated with the postmicturition phase occur after voiding and include "incomplete emptying and postmicturition dribble" [17]. Although equally bothersome, postmicturition dribble may be more prevalent in men, whereas, in women, incomplete emptying may be more prevalent [18]. As briefly mentioned above, LUT symptoms are not confined to urodynamic disturbances but may also include unpleasant sensations of pain or discomfort during storage or elimination. These sensations are generally perceived to emanate from the urogenital organs and may exacerbate storage and elimination symptoms [20].

2. Bladder Pain Syndrome (BPS)/Interstitial Cystitis (IC)

2.1. Background. LUT signs and symptoms resembling what is currently termed BPS/IC have been documented throughout history and its perspective has been previously reviewed in detail [21]. Briefly, Drs. Philip Syng Physick and Joseph Parish first recognized an inflammatory condition called tic douloureux of the bladder whose symptoms included chronic urinary frequency, urgency, and pelvic pain [22]. Skene [23] expanded the cystoscopic features of this concept in the late 19th century and introduced the term IC which included ulceration of the mucous membrane and inflammation within the bladder wall. Focal, ulcerative bleeding in the urinary bladder wall remained a hallmark of IC due, in part, to the work of Hunner [24] in the early 20th century [21]. Many patients, however, were misdiagnosed as current estimates suggest only 5–7% of those with BPS/IC present with bladder ulcerations [21, 25].

In the absence of a formal classification for IC, the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) attempted to standardize its research definition in 1987 by establishing a diagnostic criteria [26]. The criteria included the presence of "glomerulations on cystoscopic examination or a classic Hunner ulcer, pain associated with the bladder, urinary urgency" and eighteen exclusion conditions [21, 26]. After several iterations and international consultations, the term IC was expanded to include BPS [27, 28]. The patient selection for BPS is based on "chronic pelvic pain, pressure, or discomfort perceived to be related to the urinary bladder accompanied by at least one other urinary symptom such as persistent urge to void or frequency," whereas IC is "reserved for cystoscopic and histological features" [17, 28]. At this time, the terms BPS and BPS/IC are analogous and are defined by the American Urological Association Interstitial Cystitis Guidelines Panel as at least six weeks of LUT symptoms and unpleasant sensations perceived to be related to the urinary bladder and with no other clinically identifiable sources [27].

2.2. Epidemiology. The epidemiology of BPS/IC is limited due to the absence of standardized definitions, markers, and examinations [21, 25, 29, 30]. Taking into account this variability, it is estimated that there is a 5 : 1 female-to-male ratio among BPS/IC patients [21, 25]. It is estimated that 300 per 100,000 women worldwide suffer from BPS/IC [21]. In the United States alone, 3.3 to 7.9 million women are estimated to meet the criteria for BPS/IC [30]. As expected, BPS/IC puts an enormous financial burden on the individual and economy as a whole. Health care costs for an individual with BPS/IC range from 4 to 7 thousand dollars per year, while the economic burden approaches 500 million dollars per year in lost productivity and therapeutics [21, 31].

2.3. Pathophysiology. While the primary insult underlying BPS/IC is not known, it has been suggested that the pathophysiology is a "vicious circle" involving uroepithelial dysfunction, inflammation, afferent nerve hyperexcitability, and visceral hyperalgesia and allodynia (Figure 1) [32]. This section will explore the said mechanisms that have been proposed to feedforward to promote the chronicity of LUT symptoms observed in BPS/IC [32].

The urothelium is a specialized, stratified epithelium that when intact provides a nonadherent, passive barrier through tight junction proteins, plaque proteins, and surface proteoglycans [2]. Any perturbation to the components of this permeability barrier may lead to increased infiltration into the bladder wall and exposure of the interstitium to urinary constituents [33–36]. The diffusion of urinary constituents like potassium into the bladder interstitium may depolarize muscle and nerve cells, inflame tissues, degranulate mast cells, and cascade to the development of LUT symptoms (Figure 1) [35]. Uroepithelial dysfunction specific to BPS/IC, however, remains controversial. For example, Chelsky et al. [37] demonstrated that the permeability in IC was comparable to the variation seen in symptom-free controls, whereas Parsons et al. [36] demonstrated abnormal permeability and potassium absorption in those with IC [21]. The abundance of studies for or against uroepithelial dysfunction in BPS/IC suggests that it may not be a primary insult but rather may occur in a subset of patients to exacerbate LUT symptoms [21].

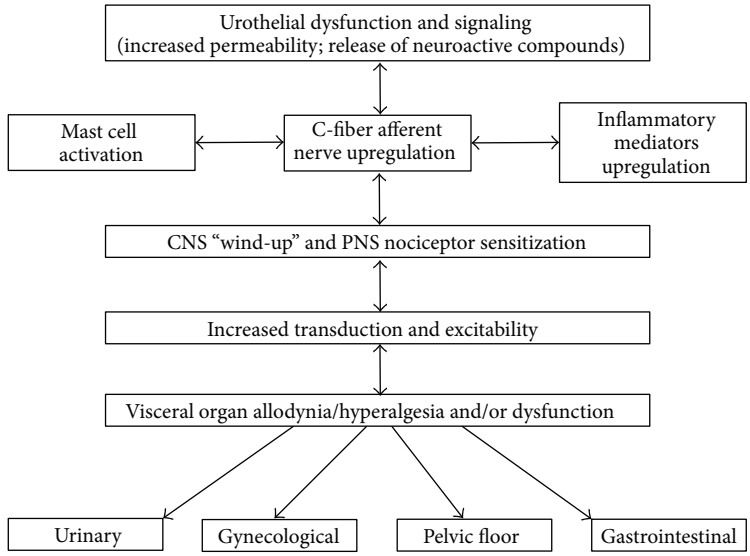

Figure 1: Potential etiologic cascade and pathogenesis underlying painful bladder syndrome (BPS)/interstitial cystitis (IC). It is likely that BPS/IC has a multifactorial etiology that may act predominantly through one or more pathways resulting in the typical symptom-complex. There is a lack of consensus regarding the etiology or pathogenesis of BPS/IC but a number of proposals include a "leaky epithelium," release of neuroactive compounds at the level of the urinary bladder with mast cell activation, "awakening" of C-fiber bladder afferents, and upregulation of inflammatory mediators including cytokines and chemotactic cytokines (chemokines). Inflammatory mediators can affect CNS and PNS neural circuitry including central "wind-up" and nociceptor sensitization resulting in chronic bladder pain and voiding dysfunction. BPS/IC is associated with diseases affecting other viscera and pelvic floors. See text for additional details. Figure adapted from [32].

In addition to the uroepithelial disruption, visceral inflammation also remains a central pathological process in BPS/IC and has been suggested to underlie the development of LUT symptoms (Figure 1). Inflammation within the urinary bladder viscera is characterized by increased vasculature, mucosal irritation that may result in barrier dysfunction, and infiltration of inflammatory mediators [38, 39]. The proliferation and activation of mast cells, in particular, have received considerable attention in the urinary bladder immune response [32]. Mast cells secrete vasoactive chemicals to promote innate and autoimmunity and their increased activity has been widely demonstrated in BPS/IC [40–43]. The subsequent exposure in the bladder interstitium to vasoactive chemicals, inflammatory mediators, and neuropeptides from visceral inflammation may lead to afferent nerve hyperexcitability and neurogenic inflammation (Figure 1) [44–46].

The loss of inhibition on peripheral afferents (Aδ and C fibers) increases input into the spinal cord and may eventually promote central sensitization [32]. An unregulated state of central and peripheral reactivity causes "wind-up" which is observed clinically as hyperalgesia and allodynia (Figure 1). In BPS/IC, hyperalgesia and allodynia are characterized by an elevated state of urinary bladder sensation that may cause pain, pressure, or discomfort and may result in increased urinary frequency and urgency [38]. The "vicious circle" continues as mast cell degranulation and infiltration of mediators from uroepithelial dysfunction and/or visceral inflammation sustain peripheral and central sensitization to establish visceral hyperalgesia/allodynia and chronic LUT symptoms (Figure 1) [32].

2.4. Animal Models. Numerous animal models have been implemented to determine the onset and chronicity of LUT dysfunctions like BPS/IC. While one model cannot currently account for the constellation of symptoms in BPS/IC, they each aid in identifying distinct mechanisms underlying part of its pathophysiology. This section will explore a naturally occurring cystitis model in felines and focus its review on experimental models of cystitis induced chemically. It is important to note that models of BPS/IC are not limited to what will be discussed in this section and exhaustive reviews have been previously published [47–49].

The natural development of spontaneous LUT symptoms has been documented in cats for several decades and is termed feline interstitial cystitis (FIC) [47, 50]. Though the primary insult for FIC is not known, the pathophysiology has marked similarities to BPS/IC including uroepithelial dysfunction and visceral inflammation. Cats with FIC have been shown to have a disruption to the epithelial cytoarchitecture that increased diffusion and infiltration of urinary constituents [51, 52]. Uroepithelial dysfunction in FIC further led to a peripheral upregulation of neuropeptides and inflammatory mediators that altered bladder afferent soma size and increased input to the central nervous system (CNS) [53]. As previously discussed, the alterations to central and peripheral reactivity following uroepithelial dysfunction and/or visceral inflammation may promote the development of LUT symptoms that is observed in FIC and, by extension, BPS/IC [32, 38, 53].

Despite these pathophysiological similarities, FIC as a model for BPS/IC is limited due to its spontaneity and epidemiology. Investigators are practically and financially

restricted to structural and functional alterations following its spontaneous induction and thus inadequately define insults preceding the development of FIC [49]. Furthermore, unlike BPS/IC, FIC occurs irrespective of biological sex [49]. While this may be due to a misdiagnosis of BPS/IC in males, one cannot discount hormonal differences that may affect LUT symptoms in humans [54, 55].

LUT symptoms have also been induced by an assortment of chemical irritants including, but not limited to, hydrochloric acid, acetic acid, protamine sulfate (PS), and cyclophosphamide (CYP). The inflammation induced by intravesical instillation of irritants like hydrochloric acid and acetic acid helps reveal the anatomical, organizational, and functional alterations attributable to the visceral immune response [47]. Specifically, the functional and histological features following acid instillation are similar to a BPS/IC subset and include urothelial hyperplasia, bladder ulceration, mucosal edema, inflammatory cell infiltration, and the development of LUT symptoms [56, 57]. Though acid instillation allows for a more controlled environment than FIC, the studies must be interpreted cautiously as the degree of inflammation resulting from exogenous irritants may not be representative of the naturally occurring BPS/IC [47].

Unlike acid instillation, PS lacks a pervasive inflammatory element but rather disrupts uroepithelial barrier function by targeting bladder surface proteoglycans [58]. Similar to the uroepithelial dysfunction observed in FIC, PS instillation is sufficient to induce LUT symptoms [59]. More recently, PS has been used in conjunction with bacterial induced cystitis. Instillation of both PS and *E. coli* lipopolysaccharide to, respectively, damage the urothelium and induce a visceral inflammatory cascade may help clarify the interaction(s) of multiple processes underlying LUT symptoms in BPS/IC [47, 60].

CYP is an antineoplastic prodrug that requires enzymatic activation to release phosphoramide mustard and the byproduct acrolein [61, 62]. A known adverse toxicity following systemic CYP administration is hemorrhagic cystitis [62]. Hemorrhagic cystitis is considered to arise from the bladder mucosal walls contact with acrolein, which has been shown to increase vascular permeability and result in bladder ulceration and hypertrophy [63]. In addition to hemorrhagic cystitis, systemic CYP treatment causes functional and histological changes similar to BPS/IC including mucosal edema, uroepithelial dysfunction, inflammatory cell infiltration, afferent nerve hyperexcitability, and the development of LUT symptoms [45, 64–67]. CYP administration also produces behavioral alterations consistent with the development of viscerosomatic pain including decreased breathing rate, closing of the eyes, and rounded back postures [66]. While the urinary bladder inflammatory response following systemic CYP administration is greater than what is observed in BPS/IC, this experimental model of cystitis is appealing because of its route of administration (intraperitoneal) and the chronicity and reproducibility of histopathological and functional alterations [47].

3. Inflammatory Mediators in Urinary Bladder Inflammation

We have hypothesized that pain associated with BPS/IC involves an alteration of visceral sensation/bladder sensory physiology. Altered visceral sensations from the urinary bladder (i.e., pain at low or moderate bladder filling) that accompany BPS/IC [68–72] may be mediated by many factors including changes in the properties of peripheral bladder afferent pathways such that bladder afferent neurons respond in an exaggerated manner to normally innocuous stimuli (allodynia). These changes may be mediated, in part, by inflammatory changes in the urinary bladder (Figure 1). Among potential mediators of inflammation, neurotrophins (e.g., nerve growth factor, NGF) have been implicated in the peripheral sensitization of nociceptors [73–75]. Proinflammatory cytokines also cause sensitization of polymodal C-fibers [74] and facilitate A-beta input to the spinal cord [76, 77]. Several studies from our laboratory have demonstrated increased expression of cytokines and chemokines (chemotactic cytokines) and the beneficial effects of receptor blockade in the urinary bladder after CYP-induced bladder inflammation [64]. In the next sections, we will present a summary of recent studies from our laboratory that addresses the role(s) of two chemokine/receptor pairs (CXCL12/CXCR4; CCL2/CCR2) and the cytokine/receptor pair (transforming growth factor (TGF-β)/TGF-β type 1 receptor) in urinary bladder inflammation and somatic sensitivity in a CYP rat model of urinary bladder inflammation.

Using the CYP-induced bladder inflammation model, we aimed to characterize further the role of inflammatory chemicals in the development and/or maintenance of neuronal sensitization and chronic pain states associated with BPS/IC. Inflammatory chemicals are released at sites of injury and inflammation by resident and infiltrating immune cells and endothelial and parenchymal cells. Proinflammatory molecules act to heal the injured/inflamed area and also to sensitize nociceptive neurons, thus increasing the pain response in order to prevent further insult [78]. While initial immune activation and sensitization of sensory neurons is protective, prolonged inflammatory processes and sensory sensitization occurring after tissue healing are associated with chronic pain syndromes, including BPS/IC. Various cytokines and chemokines have been detected in the urine and urinary bladder in models of cystitis and patients with BPS/IC and therefore may represent novel therapeutic targets or biomarkers for the syndrome.

4. Chemokines

4.1. Background. Chemokines are a large family of structurally and functionally related proteins that are important mediators of immune responses, inflammatory processes, and nociception. In the immune response, chemokines facilitate tissue recovery by causing the extravasation of leukocytes from blood plasma to the site of injury. Chemokine receptors

present on leukocytes sense increasing chemotactic concentration gradients and facilitate cellular motility towards them [79].

Chemokines are small, secreted proteins of approximately 100 amino acids in length that comprise 4 subfamilies: CC, CXC, CX3C and C (review see [80]). Each subfamily is named for the first cysteine residue motif from its amino terminus. Families of chemokines assert their actions by signaling via related G-coupled protein receptors. Within each subfamily, receptor and ligand pairing is not mutually exclusive; in other words, multiple ligands can bind the same receptor and vice versa (for review see [80]). The complexity of chemokine receptor binding presents challenges when examining the functional role of chemokine signaling. Despite difficulties, determining the role of chemokines and their receptors in both control and pathological states could provide insights to possible therapeutic interventions in a variety of chronic pain conditions including BPS/IC.

4.2. Chemokines and Peripheral Sensitization. Inflammatory mediators such as proinflammatory cytokines (e.g., tumor necrosis- (TNF-)α, interleukin- (IL-)6, IL-1β), COX-2, NGF, protons, prostaglandins, and bradykinin have been implicated in the direct sensitization of nociceptive afferents [81]. Traditionally, chemokines were not thought to assert direct effects on primary sensory neurons. Rather, chemokine/receptor interaction on the plasma membrane of leukocytes was thought to stimulate leukocyte release of nociceptive mediators via GCPR signaling mechanisms [78]. However, electrophysiological, expressional, and functional pain studies have demonstrated the possibility of direct chemokine-mediated neuronal hypersensitivity and pain. For example, *in vitro*, exogenous chemokine application can physiologically alter sensory neurons by changing membrane potentials [82], decreasing thresholds for action potential generation [82], increasing excitability, and evoking discharges [82, 83]. Various chemokines, including CXCL12, can modulate calcium ion currents in cultured DRG cells, potentially facilitating hyperexcitability [84–87]. In a neuronal injury model, chronic compression of the DRG elicits a depolarizing response to chemokines that was not detected in control (noncompressed) DRG [83]. Chemokine-mediated sensitization may involve members of the transient receptor potential family, including TRPV1. Various chemokines and receptors colocalize with neuronal TRPV1 as well as neuropeptides released in a TRPV1-dependent manner [87–90].

Following nerve injury or inflammation, expression of chemokines and associated receptors increases significantly in macrophages, infiltrating T cells, sensory neurons, and glia [79, 84, 85, 89, 91–93]. Additionally, increased neuronal activity, as would occur during injury or inflammation, has been shown to induce chemokine transcription in cultured DRG neurons [94]. Cytokines such as IL-1 can increase chemokine expression in neurons and astrocytes [95–97]. Chemokine expression in, and subsequent secretion from the various cell types (e.g., leukocytes, endothelial cells, neurons, or parenchymal cells) would enable chemokines, by diffusion, to interact with functional chemokine receptors on DRG neurons thus facilitating hyperexcitability changes such as those described above.

4.3. Chemokines and Central Sensitization. Increased primary afferent signaling can induce organizational and neurochemical changes in spinal cord synapses that underlie the phenomenon, central sensitization, which may contribute to chronic pain syndromes. During central sensitization, intensely heightened peripheral input decreases thresholds necessary to elicit action potentials in dorsal horn neurons. An increase in nociceptive neurotransmitter (e.g., SP and CGRP) release into the dorsal horn could increase the activity of spinal neurons that mediate both local reflexes and ascend to higher brain centers, thus facilitating the perception of pain [78, 87, 98].

Chemokine/receptor signaling may contribute to central sensitization via activation of either the peripheral or central afferent limbs of pain pathways [78, 98]. Chemokine activation of peripheral DRG neurons was described in the previous section. Additionally, chemokine application can evoke SP and CGRP release from DRG neurons that could cause chemokine-mediated central effects indirectly [87, 99]. Evidence suggests that chemokines may have direct central effects also. Jung et al. [89] detected large dense-core vesicles containing both CCL2 and CGRP in TRPV1-expressing DRG neurons. Dansereau et al. [88] demonstrated calcium-evoked release of CCL2 following incubation of DRG neurons with potassium or capsaicin. CCL2 may traffic anterogradely from the soma of peripheral sensory neurons and is increased in the supernatant following intense stimulation of mechanically injured DRG neurons [100]. Additionally, CCL2 application increases the frequency of spontaneous EPSCs in superficial dorsal horn neurons [101]. Chemokines released from primary afferent central terminals could exert either direct activation of superficial dorsal horn neurons via functional chemokine receptor expression or indirect sensitization via activation of microglia and astrocytes that subsequently release nociceptive mediators.

Chemokine receptors, including CCR2, and chemokines are detected in dorsal horn neurons and activated astrocytes and microglia in numerous models of neuropathic pain including peripheral or central nerve damage or tissue inflammation [102–107]. Chemokine cross signaling between bladder sensory afferents and microglia or astrocytes could modulate symptoms of BPS/IC especially considering that peripheral injury or inflammation (e.g., bladder) can induce central glial activation.

4.4. Chemokines and Nociception. Studies investigating nociceptive behavior illustrate a strong relationship between chemokines and pain. Exogenous administration of chemokines induces thermal hyperalgesia and mechanical allodynia [85, 87, 93] while certain chemokine knockout mice fail to develop somatic sensitivity [108, 109]. Oh and colleagues [87] published an early example of nociceptive chemokine function when they showed that intraplantar administration of various chemokines such as CXCL12, CCL5, and CCL22 induce mechanical hypersensitivity

lasting for at least 3 h. Since then numerous studies have reported that either exogenous peripheral (e.g., intradermal) or central (e.g., intrathecal) chemokine application induces mechanical hypersensitivity and/or thermal hyperalgesia [88, 90, 92, 99, 110, 111]. Intrathecal administration of CCL2 can produce mechanical hypersensitivity within 30 minutes and hyperalgesic effects can be detected up to 4 days after administration [88]. In contrast, CCL2-induced thermal hypersensitivity resolves 24 h after administration [88]. Interestingly, transgenic mice lacking the CCR2 receptor (principle receptor for CCL2) are resistant to the development of mechanical hypersensitivity following mechanical nerve injury; however, following complete Freund's adjuvant- (CFA-) induced neural inflammation, these mice display only a small, insignificant decrease in mechanical sensitivity compared to control animals and show no changes in thermal nociception [108]. These data suggest specificity for chemokine function with respect to type of injury and pain modality.

Studies utilizing antagonists against chemokine signaling provide evidence for a therapeutic role with respect to neuropathic pain. In two different models of HIV-1 associated neuropathy, Bhangoo et al. [85, 91] demonstrate that antiretroviral drug- or viral coat protein-, gp120-, induced mechanical hypersensitivity is attenuated by acute, systemic treatment with CCR2 or CXCR4 antagonists. Other pain eliciting models such as focal demyelination, CFA-induced inflammation and sciatic nerve constriction have demonstrated the therapeutic effects of chemokine receptor antagonists [84, 111, 112].

4.5. Chemokines and Cystitis. Clinical studies assessing patients with various pelvic inflammatory/pain syndromes and rodent models of visceral inflammation indicate a role for chemokines in the initiation or maintenance of visceral inflammation. CYP-induced inflammation increases the expression of CXCL12/CXCR4, CX3CL1/CX3CR1, CCL2/CCR2, and CXCL1 in the urinary bladder and CCL2 and CXCL1 in urine [113–117]. Blockade of CXCL10 signaling reduces severity of CYP-induced bladder inflammation by reducing hyperplasia, epithelial erosions, and infiltration of T cells, mast cells, and killer T cells in the bladder urothelium of rats [118]. Additionally, elevated chemokines levels have been detected in the seminal plasma and peripheral immune cells of patients with pelvic inflammatory/pain syndromes such as ulcerative colitis, chronic prostatitis, chronic pelvic pain syndrome, and BPS/IC [118–120]. Bladders from patients with ulcerative BPS/IC have increased mRNA expression of CXCL9, CXCL10, and CXCL11 in the interstitium and CXCR3 in the urothelial membrane [121]. Both Tyagi et al. [122] and Corcoran et al. [123] detected elevated chemokines, specifically CXCL1, CXCL10, CXCL12-α, and CCL7, in the urine of patients with ulcerative BPS/IC. Interestingly, CCL7 levels decreased following hydrostatic distention and were correlated with symptom relief [123]. Tyagi et al. [122] suggest the presence of urinary chemokines originates from bladder tissue because urinary CXCL10 levels are present at levels much higher than those detected in serum.

Considering the extensive data implicating a sensory and signaling role for the urothelium, it is possible that urothelial-derived chemokines, especially those detected in the urine of BPS/IC patients, contribute to symptoms of bladder dysfunction. Recently, the functional contribution of the urothelium has advanced beyond the view of a passive barrier and is now suggested to have "neuron-like" properties such as plasticity and sensory transduction capabilities, especially in the context of bladder inflammation [124, 125]. Functional receptor expression, in conjunction with secretion capabilities, allows the urothelium to respond to stimuli and reciprocally communicate with detrusor smooth muscle cells, suburothelial nerve plexus, or interstitial cells [126–130]. It is possible that chemokine signaling via receptor expression in urothelial cells may consequently activate downstream targets that promote either the transcription or the expression and release of other inflammatory mediators or excitatory amino acids. Urothelial derived mediators such as adenosine triphosphate or nitric oxide may then influence the suburothelial nerve plexus to affect micturition reflex function [129].

5. CXCL12 and CXCR4

Our lab examined the expression and therapeutic effect with receptor blockade of the chemokine CXCL12, and one of its two receptors, CXCR4, in a rodent model of cystitis. This chemokine/receptor pair was of interest because of its demonstrated role in visceral inflammation and pathology in other abdominopelvic organs. Mikami et al. [119] show that CXCR4 peripheral T-cell expression was increased in patients with ulcerative colitis and that expression levels correlated with disease activity. Additionally, chemically induced colitis in mice leads to an increase of CXCR4-positive leukocytes and CXCL12 expression in colonic tissue [119]. Administration of a CXCR4 antagonist reduced these inflammatory effects. To address the role of CXCL12/CXCR4 signaling in normal micturition and inflammation-induced bladder hyperreflexia, bladder inflammation in adult female Wistar rats was induced by injecting CYP intraperitoneally at acute (150 mg/kg; 4 h), intermediate (150 mg/kg; 48 h), and chronic (75 mg/kg; every third day for 10 days) time points. CXCL12 and its receptor, CXCR4, were examined in the whole urinary bladder of control and CYP-treated rats using complementary approaches including enzyme-linked immunosorbent assays (ELISAs), qRT-PCR, and immunostaining techniques. ELISAs, qRT-PCR, and immunostaining experiments revealed a significant increase in CXCL12 and CXCR4 expression in the whole urinary bladder and particularly in the urothelium, with CYP treatment [114]. CXCL12/CXCR4 interactions in micturition were evaluated using conscious cystometry with continuous instillation of saline and CXCR4 receptor antagonist (AMD3100; 5 μM) administration in control and CYP- (48 h) treated rats. Receptor blockade of CXCR4 using AMD3100 increased bladder capacity in control (no CYP) rats and reduced CYP-induced bladder hyperexcitability as demonstrated by significant increases in intercontraction interval, bladder capacity,

and void volume [114]. In these studies, AMD3100 is most likely acting at the level of the urothelium for several reasons: (1) both mRNA and histologic analyses showed that the greatest expressional increase for both CXCL12 and CXCR4 following CYP treatment was in the urothelium; (2) histologically, CXCR4 had a restricted presentation being expressed only in the urothelium in both control and CYP treated bladders; (3) repeated attempts did not demonstrate CXCL12- or CXCR4-IR in the suburothelial nerve plexus [114]. These results suggest a role for CXCL12/CXCR4 signaling in both normal micturition and with bladder hyperreflexia following bladder inflammation.

6. CCL2/CCR2

The chemokine, CCL2 (monocyte chemoattractant protein-1, MCP), and its high-affinity receptor, chemokine (C–C motif) receptor 2 (CCR2), have been implicated in hypersensitivity following neuronal inflammation or mechanical injury [88, 92, 99, 101, 108, 131, 132] in the central (i.e., spinal cord) and peripheral (i.e., DRG) nervous system. Blockade of CCR2 reduces established pain behaviors resulting from chronic nerve injury [88, 99, 101, 132] and exogenous application of CCL2, either centrally or peripherally, can elicit exaggerated sensory behavioral responses in rodents [88, 92, 99, 101, 132]. In addition, CCR2 null mice fail to develop somatic sensitivity following partial sciatic nerve ligation [108] whereas mice with CCL2 overexpression in astrocytes develop exaggerated thermal hyperalgesia following complete Freund's adjuvant-induced inflammation [131].

Our recent studies demonstrate novel findings with respect to the contribution of CCL2/CCR2 interactions with bladder inflammation-induced changes in bladder function and somatic sensitivity in female rats. We demonstrate that CYP-induced cystitis increases (1) CCL2 and CCR2 transcript and protein expression in the rat urinary bladder and (2) the number of bladder-associated CCR2-immunoreactive bladder afferent cells in the lumbosacral DRG [113]. Blockade of CCR2 receptor interactions with the highly selective receptor antagonist, RS504393 (5 μM), at the level of the urinary bladder, increased bladder capacity, decreased void frequency, and reduced somatic sensitivity of the hindpaw and pelvic region following CYP treatment [113]. These results extend previous findings [83, 88, 92, 133, 134] by demonstrating that CCL2/CCR2 interactions contribute to inflammation-induced bladder dysfunction and increased referred somatic sensitivity.

CCL2/CCR2 interactions at the level of the urothelium and suburothelial nerve plexus in the urinary bladder are likely to contribute to bladder dysfunction and increased somatic sensitivity following CYP-induced cystitis. Intravesical instillation of RS504393 likely makes direct contact with the urothelium that expresses CCR2 and the increased urothelial permeability due to CYP treatment makes it likely that intravesical RS504393 also contacts suburothelial nerves. Our studies did not differentiate between direct urothelial and nerve-mediated CCR2 effects versus indirect urothelial-mediated communication with the detrusor smooth muscle, suburothelial nerve plexus, and/or interstitial cells as previously suggested [126, 127]. It is possible that urothelial CCL2/CCR2 signaling facilitates the release of urothelial-derived mediators such as adenosine triphosphate or nitric oxide that may then influence underlying structures such as the suburothelial nerve plexus and/or detrusor smooth muscle [126, 127, 129].

Alternatively, or in addition to urothelial-mediated mechanisms, CCL2/CCR2 interactions in bladder associated DRG neurons may contribute to inflammatory-induced changes in bladder sensory physiology and function. CYP treatment triggered a robust increase in the percentage of bladder afferent cell bodies expressing CCR2-IR [113]. These results complement previous findings demonstrating an increase in the percentage of primary sensory afferent cells expressing CCL2 and/or CCR2 following focal nerve demyelination, sciatic nerve ligation, or chronic constriction injury [83, 84, 89, 92, 133, 135]. Jung and Miller [94] demonstrate that depolarization of cultured sensory neurons is sufficient to induce CCR2 mRNA expression suggesting that heightened sensory neuron activity during states of injury or inflammation may contribute to elevated levels of neuronal CCR2 expression. Increased receptor expression may explain why peripheral nerve damage or inflammation can also change the functional properties of sensory neuron populations such that an increasing percentage of DRG neurons responds to CCL2 application or neurons respond with increased intracellular calcium ion currents and/or frequency of EPSCs [82–84, 99, 101, 135]. Therefore, it is possible that CCL2 released, *in vivo*, by DRG neurons, glial cells, and urothelial cells could contribute to nociceptive sensations/behaviors by autocrine or paracrine signaling mechanisms.

7. Cytokines

In addition to the chemokine family, ample evidence suggests that other cytokines contribute to the development of hyperalgesia and allodynia following injury or inflammation [79, 136]. Cytokine receptors have been detected in neurons and glial cells, especially after peripheral neuropathy [136]. Cytokine/receptor interactions can activate signaling pathways that induce transcription and release of other proinflammatory/nociceptive mediators including NGF and other cytokines and chemokines from peripheral neurons or glial cells [95–97, 137]. Cultured human detrusor smooth muscle cells secrete low levels of cytokines (IL-6 and IL-8) and chemokines (CCL2 and CCL5) and exposure to the inflammatory cytokines, IL-1β and TNF-α, increases this release [138, 139]. The expression of cytokines, alone or in combination with other cytokines, growth factors, or other mediators, may form a bidirectional communication network between the nervous system and the immune system [140].

Studies examining cytokine expression using a CYP model of cystitis have detected elevated IL-6, IL-1α, and IL-4, among others, protein and mRNA levels in the urine and urinary bladder [64, 115]. Cytokine transcription and expression increase in the urinary bladder of patients with ulcerative BPS/IC [121, 123, 141]. Certain cytokine mRNAs, including IL-6 and TNF-α, have been detected in the interstitium and urothelium of these biopsies [121, 141]. Additionally, reports have repeatedly detected elevated IL-6 in the urine of patients with ulcerative BPS/IC. Increased levels have been suggested to indicate either severity of inflammation [39] or correlate with pain scores and nocturia [141, 142]. Lotz et al. [141] propose that the bladder is the primary source of urinary IL-6 because it was not detected in ureteral urine.

Recently we examined the expression and function of another cytokine, TGF-β, in the urinary bladder with inflammation. TGF-β has an extensive role in the immune system and has been implicated in nociception and detected in the urine and urothelium of rats treated with CYP-induced cystitis [143].

8. Transforming Growth Factor-Beta (TGF-β)

8.1. Background. The TGF-β superfamily is comprised of at least 35 pleiotropic proteins belonging to four subfamilies grouped by their sequence homology—decapentaplegic-Vg-related (DVR), activin/inhibin, TGF-β *sensu stricto,* and other divergent members [144]. Even though TGF-β superfamily members have distinct expression patterns and regulate a variety of functions, they are each translated as a preproprotein that contains a peptide sequence signaling to the endoplasmic reticulum, a N-terminal prodomain, and a C-terminal mature protein [144, 145]. After proteolytic processing and posttranslational modifications, the C-terminal fragment is either secreted as a mature protein dimer or forms a latent complex by maintaining a noncovalent bond to the prodomain [144, 145].

The canonical members of TGF-β *sensu stricto* are one such proprotein to form a latent complex. The interactions between the N-terminal prodomain, termed latency associated peptide (LAP), and the mature TGF-β dimer are sufficient to sequester its extracellular activity [146]. Additionally, LAP associates with a latent TGF-β binding protein (LTBP) that regulates TGF-β bioavailability by chaperoning the complex to the extracellular matrix [147]. The subsequent activation of latent TGF-β in the extracellular matrix via LAP cleavage occurs by protease-dependent or protease-independent (protons, integrins, reactive oxygen species, etc.) mechanisms [145, 148–151].

After its secretion, the mature or activated protein dimers process a signal through transmembrane Ser-Thr receptor kinases [144]. The TGF-β family of receptors is comprised of type I and type II receptors. Type II receptors selectively bind their respective ligands to define part of the specificity of signal transduction [144]. Ligand binding can either be "sequential" or "cooperative" and may involve an accessory receptor (type III) to enhance ligand presentation [152]. Following receptor-ligand interaction, the type II receptor forms a heterotetrameric complex with the type I receptor to transphosphorylate residues of the Gly-Ser (GS) box [152]. The activated type I receptors then phosphorylate Smad-dependent or Smad-independent substrates to regulate the transcription of target genes [153].

Smad proteins exist in three families: receptor-activated, common mediator, and inhibitory. Receptor-activated (R-) Smads dock onto type I receptors and are phosphorylated on distal serine residues following receptor activation [153]. Phosphorylated R-Smads dissociate from the receptor and interact with common mediator Smad4 [153]. The oligomeric R-Smad/Smad4 complex then translocates to the nucleus where it alters the transcription of target genes [153]. Type I receptors not only function through Smad signaling but may also directly activate Smad-independent pathways such as TGF-β-activated kinase 1 (TAK1), Ras, nuclear factor-κB (NF-κB), and the mitogen-activated protein kinase (MAPK) subfamily members [154–159]. The variety of direct and context-dependent downstream signaling pathways preserves the multifunctional role(s) of TGF-β superfamily ligands while providing the specificity required to control distinct target genes.

8.2. Immune Response. The canonical members of TGF-β *sensu stricto* maintain immunological function by regulating the initiation and resolution of the immune response and a comprehensive review has been previously published [160]. Briefly, activated TGF-β at the site of injury may initiate a proinflammatory milieu characterized by matrix remodeling and the recruitment and activation of leukocytes [160, 161]. TGF-β may then aid in resolving the primary immune response and support a milieu for tissue repair and immunological memory to progress by suppressing the proliferation, differentiation, and survival of a subset of lymphocytes [160].

To initiate an immune response, TGF-β may mobilize monocytes, mast cells, and granulocytes to the site of injury and influence their adhesion to the extracellular matrix [160, 162–164]. While TGF-β may also recruit monocyte-derived macrophages, their activation and function are typically inhibited to help resolve the immune response [161, 165, 166]. Since immune cells continue to infiltrate the site of injury, the extracellular matrix undergoes pathological remodeling characterized by protease secretion and matrix degradation [167]. TGF-β supports the remediation and repair of these tissues by increasing the deposition of matrix proteins and inhibiting protease activation [168].

To sustain the resolution of the immune response, TGF-β may regulate T-cell proliferation, differentiation, and survival [169]. TGF-β promotes T-cell growth arrest by suppressing interleukin-2 in areas of subthreshold antigen presentation [160, 170]. During the polarizing conditions of the immune response, TGF-β maintains peripheral immunological tolerance by inducing the transcription factor FoxP3 to promote CD4+ CD25+ T-cell differentiation to regulatory T cells [160, 171]. CD4+ T-cell differentiation to the T helper (Th) 1 and Th2 cell lineages, however, is inhibited by TGF-β mediated repression of the transcription factors T-bet and GATA-3, respectively [169, 172]. In addition to its effects

on CD4+ T cells, TGF-β may also attenuate the cytotoxicity of CD8+ T cells by inhibiting its cytolytic genes [173].

TGF-β not only stabilizes T-cell expression and function to resolve the immune response but also regulates B-cell proliferation, survival, and development [174]. TGF-β inhibits both the proliferation and cell cycle progression of B cells through Smad-dependent or Smad-independent pathways [160, 175–177]. TGF-β utilizes comparable B-cell growth arrest pathways, as well as a distinct Smad-independent pathway, to induce the apoptosis of B cells [160, 178]. Lastly, TGF-β may regulate the maturation and activation of B cells through its induction of isotype switching, suppression of B-cell antigen receptor signaling, and inhibition of immunoglobulin secretion [160, 179, 180].

8.3. Nociception. The members of TGF-β *sensu stricto* contribute to both the peripheral and central processing of noxious stimuli. TGF-β1 and TGF-β2 have been demonstrated to increase de novo neuropeptide synthesis in the DRG that may directly sensitize primary afferent nociceptors [181, 182]. TGF-β may also influence DRG excitability by regulating several ion channels including the voltage-gated potassium (Kv) channel and TRPV-1. Application of recombinant TGF-β1 *in vitro* has been demonstrated to downregulate KCNA4 gene expression and decrease A-type Kv currents in primary DRG cultures [183]. Additionally, TGF-β1 Smad-independent signaling may phosphorylate TRPV-1 on Thr residues and potentiate capsaicin-evoked calcium influx in the DRG [184, 185]. The subsequent prolonged depolarization and an impaired repolarization may lead to an amplification of nociceptive transmission and CNS input.

Unlike its role in the periphery, TGF-β in the CNS appears to be neuroprotective by regulating neuronal and nonneuronal response to inflammatory injury [186]. Non-neuronal glial cells have recently been recognized to enhance the proinflammatory milieu and facilitate the central processing of nociception [187]. Activated TGF-β in the CNS may inhibit the proliferation and activation of these spinal glial cells to attenuate the induction of neuropathic pain [188–190]. TGF-β may further reduce excitatory synaptic transmission of second-order neurons by directly suppressing the proinflammatory milieu in the spinal cord [189]. As a result of its biphasic and modulatory role in the peripheral and central transmission of nociception, TGF-β appears to have a profound impact on the perception of pain and may initiate, in part, pathological pain syndromes.

8.4. Role(s) in Cystitis. TGF-β ligands and its cognate receptors are expressed at low, basal levels in rat urinary bladder tissues [191]. Following chemically (CYP) induced cystitis of varying durations, TGF-β ligand, and receptor expression appears to display a time- and tissue-dependent regulation. TGF-β exhibits a delayed, but sustained, increase in urinary bladder gene and protein expression 8–48 h after CYP treatment [143, 191, 192]. Furthermore, urinary excretion of active and latent TGF-β1 is increased up to 100-fold 24 h after acute CYP treatment [143]. The aforementioned regulation of TGF-β gene and protein expression has been suggested to be more pronounced in the afferent limb of the micturition reflex suggesting a possible role in the development of LUT symptoms [191]. Its role in micturition reflex dysfunction was confirmed following the pharmacological inhibition of aberrant TGF-β signaling with cystitis. Inhibition of TGF-β type I receptors 48 h after CYP-induced cystitis decreased urinary frequency and increased bladder capacity, void volume, and intercontraction intervals [191]. These studies raise the possibility of targeting TGF-β at the level of the urinary bladder to alleviate voiding dysfunction with cystitis.

9. Perspectives and Future Directions

Blockade of cytokine/receptor and chemokine/receptor signaling may represent a potential therapeutic target for inflammation-associated bladder dysfunction. In addition, the presence of certain inflammatory molecules in patient urine may be useful biomarkers for BPS/IC or other bladder disorders such as overactive bladder (OAB). Similar to BPS/IC, the etiology of OAB remains elusive; however, based on patient biopsies an inflammatory contribution has been suggested [193–195]. Tyagi et al. [196] detected a 10-fold increase of CCL2 and the soluble fraction of the CD40 ligand (CD40L) in the urine of OAB patients versus controls. Various cytokines, epidermal growth factor (EGF), and the oncogene GRO-a were also elevated (3-5-fold) in the urine of OAB patients [196]. Whether certain inflammatory mediator/receptor interactions and downstream signaling pathways are redundant or unique across diverse bladder dysfunction or pelvic pain syndromes remains to be determined. Identification of urinary biomarkers in BPS/IC, OAB, or other bladder dysfunctions would improve diagnostic strategies and reduce invasiveness to the patient, improving exclusionary criteria, reducing time to diagnosis and aid in patient selection for pharmacological trials.

Abbreviations

BPS: Bladder pain syndrome
CCR2: Chemokine (C-C motif) receptor 2
CGRP: Calcitonin gene-related peptide
CNS: Central nervous system
CYP: Cyclophosphamide
DRG: Dorsal root ganglia
DVR: Decapentaplegic-Vg-related
EGF: Epidermal growth factor
ELISA: Enzyme-linked immunosorbent assay
EPSC: Excitatory postsynaptic current
FIC: Feline interstitial cystitis
GS: Glycine-serine
h: Hour(s)
HIV: Human immunodeficiency virus
IC: Interstitial cystitis
Kv: Voltage-gated potassium
L: Lumbar
LAP: Latency associated peptide

LTBP: Latent transforming growth factor-beta binding protein
LUT: Lower urinary tract
MAPK: Mitogen-activated protein kinase
MCP: Monocyte chemoattractant protein
NF-κB: Nuclear factor-kappa B
NGF: Nerve growth factor
NIDDK: National Institute of Diabetes and Digestive and Kidney Diseases
OAB: Overactive bladder
PAG: Periaqueductal gray
PMC: Pontine micturition center
PNS: Peripheral nervous system
PS: Protamine sulfate
qRT-PCR: Quantitative reverse transcriptase polymerase chain reaction
R-: Receptor-activated
S: Sacral
SP: Substance P
T: Thoracic
TAK1: Transforming growth factor-beta-activated kinase 1
TGF-β: Transforming growth factor-beta
Th: T helper
TRP: Transient receptor potential
V: Vanilloid.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

An Update of the Interstitial Cell Compartment in the Normal Human Bladder

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Aims. Interstitial cells, also called myofibroblasts, most probably play a major role in the pathogenesis of the overactive bladder. However, no specific phenotypic marker has been identified. We investigated whether N-cadherin could play a role as a discriminatory marker for interstitial cells in the human bladder. *Methods*. Bladder biopsies (n = 16) were collected from macroscopically nonpathological locations during cystectomy which was performed because of bladder cancer. Tissue was analyzed for expression of N-cadherin. N-cadherin+ cells were phenotyped using antibodies against PGP9.5, smoothelin, vimentin, and C-kit. Findings were related to bladder tissue histology and ultrastructure of myofibroblastic cells. *Results*. N-cadherin+/vimentin+ cells with branched cell bodies were found in the lamina propria and detrusor layer. They were closely associated with neurons and showed no colocalization of PGP9.5 or smoothelin. A second type of N-cadherin+ cells was found at the boundary of detrusor bundles and in the lamina propria. These cells colocalization C-kit. We assumed that N-cadherin+/vimentin+ cells are similar to the ultrastructurally defined myofibroblasts. *Conclusions*. N-cadherin can play a role as a discriminatory marker for interstitial cells in the human bladder, as the interstitial compartment of the human bladder houses a population of cells from mesenchymal origin, immunopositive for N-cadherin, vimentin, and C-kit.

1. Introduction

The human bladder shows spontaneous contractile activity during the filling phase of the micturition cycle [1]. As this activity is also found in bladders that are isolated from the central nervous system, it seems that it is generated within the bladder wall. The overactive bladder (OAB) syndrome is associated with complaints of frequency and urgency that typically occur during the filling phase [2, 3]. Spontaneous contractile activity of the bladder may share characteristics with peristaltic activity in the gastrointestinal tract [4]. Specialized pacemaker cells of the gut, also known as interstitial cells of Cajal (ICCs), are thought to behave as pacemaker cells that transmit their electrical activity to the smooth muscle [5, 6]. Interstitial cells (ICs) are also found in the human bladder [7, 8]. These cells are located throughout the lamina propria and detrusor layer. It has been suggested that they form a network integrating signals and responses in the bladder wall between various types of cells. However, ICs have several subtypes based on morphological appearance and differential expression of markers, like myofibroblasts, making the search for their exact functional role in the human bladder a contentious subject.

Recent studies have identified various surrogate histochemical markers for ICs, such as the stem cell receptor C-kit and cyclic guanosine monophosphate (cGMP) [8]. However, the specific immunophenotype of ICs is still controversial. C-kit is not expressed by all types of ICs and it can also be detected in other cell types [9]. Additionally, many C-kit antibodies fail to detect ICs in positive control tissues. cGMP is a marker for ICs in the bladder but it is also expressed by urothelial cells [8]. Thus, so far, no marker has been identified that can be considered as a specific phenotypic marker for ICs in the human bladder. Therefore, irrefutable confirmation

of the interstitial phenotype still depends on application of transmission electron microscopy (TEM), which is highly time consuming.

Cadherins constitute a superfamily of glycoproteins that participate in cell-cell recognition by functioning as signaling centers [10, 11]. We have previously shown that cadherin-11 is expressed by ICs in the lamina propria [12]. Cadherins may therefore play a role in regulating an intramural network of these cells. As subpopulations of bladder ICs exist, another subclass of cadherins might account for a specific discriminatory marker for interstitial cells in the interfascicular planes of the detrusor layer. N-cadherin is known to regulate mesenchymal cell development [13] and is the most commonly expressed cadherin in stromal cells [14, 15]. We therefore investigated the expression of N-cadherin in the normal human bladder. We used additional immunohistochemical cell markers as well as transmission electron microscopy (TEM).

2. Material and Methods

2.1. Patients. Bladder biopsies ($n = 16$) were collected from sixteen individuals in whom radical cystectomy was performed because of muscle invasive bladder cancer. Mean patient age was 62 years (52–75), nine males and seven females. Samples were dissected from tumor-free bladder areas at least 3 cm distant from tumor zones. Biopsies were all taken from the vesical dome from functionally stable bladders. All of the patients underwent primary resection in terms of early cystectomy and none of the patients underwent intravesical installation. The local ethics committee approved the study and informed consent was obtained from all patients. Full thickness specimens were collected and placed in a mould containing Tissue-Tek (Sakura) for cryosectioning. Specimens were snap frozen in isopentane at −80°C. Tissue was checked for intact urothelium using a hematoxylin-eosin stain.

2.2. Immunohistochemistry. Sections of 4 μm specimens were prepared using a cryostat and mounted on Super Frost Plus slides (Menzel-Gläser). The unfixed sections were immersed in 3% paraformaldehyde for ten minutes and stained for N-cadherin (M142 Takara; C2542 Clone GC-4 Sigma). Cell membranes were permeabilized in 0.2% Triton X-100 for 5 minutes. For cytoskeletal protein staining, samples were fixed in acetone for ten minutes and air dried at room temperature for 2 hours. Each step was separated by wash in magnesium and calcium containing PBS (PBS-Extra: 40 mL 25x PBS, 960 mL demi-water, 100 μL 1 M $MgCl_2$, 100 μL 1 M $CaCl_2$). Sections were incubated for 1 hour using primary antibodies diluted in PBS 1% bovine serum albumin for blocking. Sections again were washed three times in PBS-Extra. Next, the sections were incubated with Alexa Fluor 488 (A-11017, A-11070 Molecular Probes) or Alexa Fluor 594 (A-20185, A-11032 Molecular Probes). Finally, treatment with DAPI (24653 Merck) was performed for staining the nuclei. All sections were mounted in Fluorescent Mounting Medium (S3023 Dako Cytomation). Negative controls included omission of primary antibodies. The following antibodies were used to further phenotype N-cadherin+ cells: PGP9.5 (a pan-neuronal marker) (7863-0504 AbD Serotec), smoothelin (specific marker for smooth muscle cells [16]) (R4A ab8969 Abcam), vimentin (marker for fibroblasts) (RV203 Eurogentec), and C-kit (CD117 DAKO). For the latter antibody, specimens of human jejunum were used as positive controls.

2.3. Transmission Electron Microscopy. Sixteen human normal bladder biopsies were also processed for standard transmission electron microscopy (TEM). Processing for TEM was done according to the standard protocol using Somogyi fixative [17]. Ultrathin sections were photographed using a TEM 1010 electron microscope (JEOL, Peabody, Massachusetts).

2.4. Analysis. Immunostained sections were examined by binocular epifluorescent microscopy (Leica DFC FX). Four times ten slides were analyzed per full-thickness specimen. Each set of ten slides was separated by approximately 5 mm of tissue. Cryosections were also stained with hematoxylin-eosin to interpret the fluorescent images. Morphology, phenotypic expression of above mentioned markers, and the ultrastructure of myofibroblastic cells were evaluated.

3. Results

3.1. N-Cadherin Expression in Normal Human Bladder. Throughout the entire bladder wall, N-cadherin positive structures were found. These structures were located immediately below the urothelium, throughout the lamina propria and in the detrusor layer (Figure 1). Counterstaining with DAPI showed that the N-cadherin+ structures embodied branched cells provided with multiple processes (Figure 2). N-cadherin expression showed a punctate pattern distributed throughout the entire cell body.

Suburothelial N-cadherin+ cells had branched morphology with multiple processes that seemed to form a network. In the detrusor, N-cadherin+ cells were found at different levels. N-cadherin+ cells with stellate morphology were also located at the boundaries of smooth muscle bundles. They seemed to interact with elongated N-cadherin+ cells running in the interfascicular planes, continuing as slender N-cadherin+ processes between smooth muscle cells.

3.2. Phenotyping of N-Cadherin Positive Cells. Staining for smoothelin confirmed that N-cadherin+ but smoothelin- cells were housed at the border of smooth muscle fascicles (Figure 2). Inside the fascicles, they continued as elongated processes running in parallel with smooth muscle orientation spanning numerous smooth muscle cells. Irregularly arranged bundles of cells expressing smoothelin were found midway between the urothelium and the detrusor smooth muscle bundles. Those so-called muscularis mucosae varied considerably in diameter and formed a discontinuous layer of cells, densely surrounded and traversed by N-cadherin+ structures.

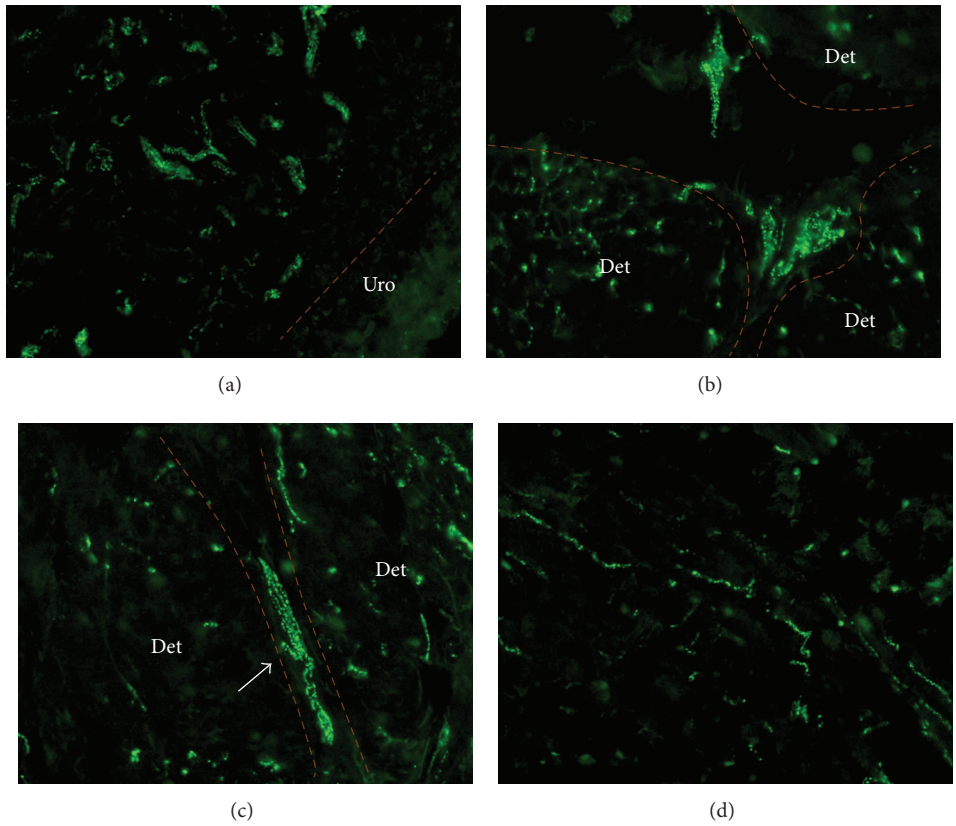


Figure 1: N-cadherin+ structures in the normal bladder wall. (a–d) A punctate signal for N-cadherin (green) reveals numerous positive N-cadherin+ cells within the bladder wall. (a) N-cadherin+ cells with multiple processes in the lamina propria. (b) Closely associated N-cadherin+ cells at the boundary of smooth muscle bundles. Note large size and stellate morphology. (c) N-cadherin+ cells running between smooth muscle fascicles show elongated instead of stellate morphology and reveal lateral branches (arrow) running into the muscle fascicles. (d) Intrafascicular, slender elongated N-cadherin+ branches run in parallel with the smooth muscle bundles. Magnification ×400. Uro: urothelium; Det: detrusor muscles.

PGP9.5 immunoreactivity was found in both the lamina propria and detrusor layer. In the detrusor layer, primary nerve trunks run close to the detrusor bundles. Secondary neuronal structures were found in the connective tissue between smooth muscle fascicles, whereas smaller fibers run between small groups of smooth muscle cells. Double staining of N-cadherin and PGP9.5 showed no colocalization. However, close association between N-cadherin+ cells and PGP9.5+ neuronal structures was found (Figure 2).

N-cadherin and vimentin were coexpressed by cells of ramified morphology in the suburothelial layer and deeper lamina propria (Figure 3). These cells seemed to form a suburothelial network. However, many vimentin+ cells did not express N-cadherin. These N-cadherin−/vimentin+ cells showed different morphology. They were smaller and less elongated, had little perinuclear cytoplasm, and presumably embodied fibroblasts.

N-cadherin+/vimentin+ cells were also found at the border of detrusor smooth muscle bundles. Similar to the suburothelial region, these cells seemed to interconnect with each other, expanding into and throughout the smooth muscle fascicles like a network of N-cadherin+/vimentin+ processes.

The punctuate pattern of N-cadherin expression was expressed throughout the entire cell body and at the cell membrane. In general, the small vimentin+ cells with little perinuclear cytoplasm did not express N-cadherin.

Most N-cadherin+ cells coexpressed vimentin. However, a second type of N-cadherin+ cells was found that did not coexpress vimentin. These cells were not housed between smooth muscle cells but were restricted to the edge of smooth muscle fascicles and were also found in the lamina propria (Figure 3(f)). In contrast to the N-cadherin+/vimentin+ cells, these N-cadherin+/vimentin− cells showed different morphology: they had small appearance with little perinuclear cytoplasm sprouting into multiple cytoplasmatic processes.

3.3. N-Cadherin Positive Cells and Interstitial Cell Marker C-Kit. C-kit+ cells were found throughout the entire bladder wall (Figure 4). A large number of these cells coexpressed N-cadherin and showed similar morphology to the previously described N-cadherin+/vimentin− cells (Figure 4(b)). They were located on the boundary of smooth muscle fascicles and in the lamina propria.

Specimens of the human jejunum were used as a positive control for C-kit. The gut showed a large population of

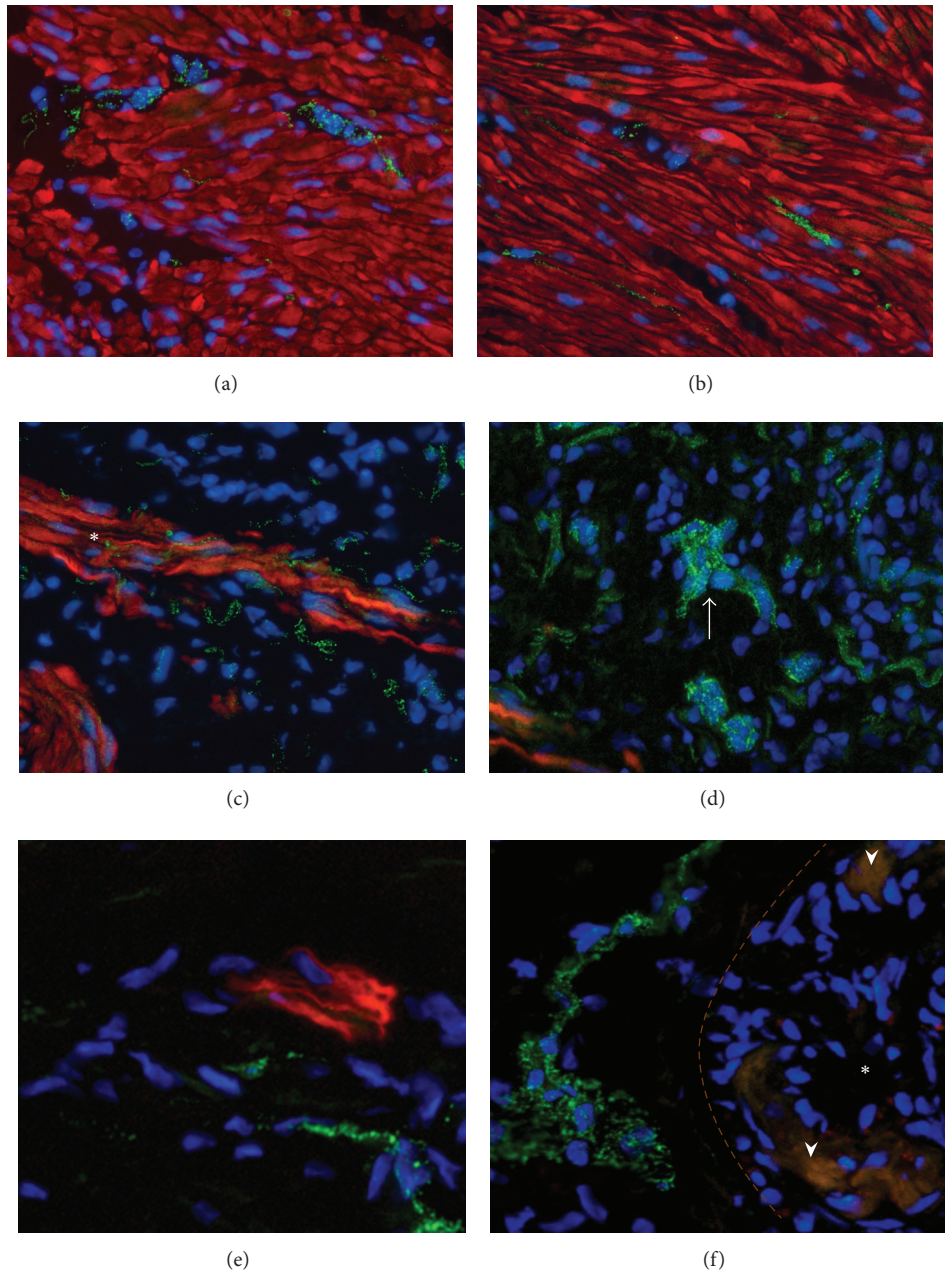


Figure 2: Double staining of N-cadherin with smoothelin and PGP9.5. (a–d) Costaining of N-cadherin (green) and smoothelin (red) in the bladder wall shows no colocalization. (a) Transversal and (b) longitudinal sections. (c) N-cadherin+ structures intermingle with smooth muscle cells in the region of the muscularis mucosae (asterix) (d) Note the aggregation of multiple N-cadherin+ cells in the lamina propria (arrow). (e) PGP9.5 is expressed by a nerve trunk (red). (f) Aggregated N-cadherin+/PGP9.5− cells are closely associated with a ganglion region (asterisk). Ganglion coexpressesbackground signal of N-cadherin, resulting in orange bodies (arrowheads). Note high contrast to the punctate expression of N-cadherin in the N-cadherin+/PGP9.5− cells. DAPI (blue) for nuclei. Magnification ×400.

cells coexpressing N-cadherin and C-kit. Although most cells showed coexpression of N-cadherin and C-kit, N-cadherin+/C-kit− and N-cadherin−/C-kit+ cells were also found.

3.4. Ultrastructure of ICs in Lamina Propria and Detrusor Muscle. In electron microscopy, interstitial cells with stellate morphology were found in the lamina propria and the musculus detrusor layer. They had characteristic features of myofibroblasts, such as cytoplasmatic filaments, focal densities and membranous attachment plaques, interrupted basal lamina of extracellular matrix, numerous mitochondria, and prominent rough endoplasmic reticulum (Figure 5). From a morphological point of view, they appear to be similar to the N-cadherin+ cells as described in Figure 1.

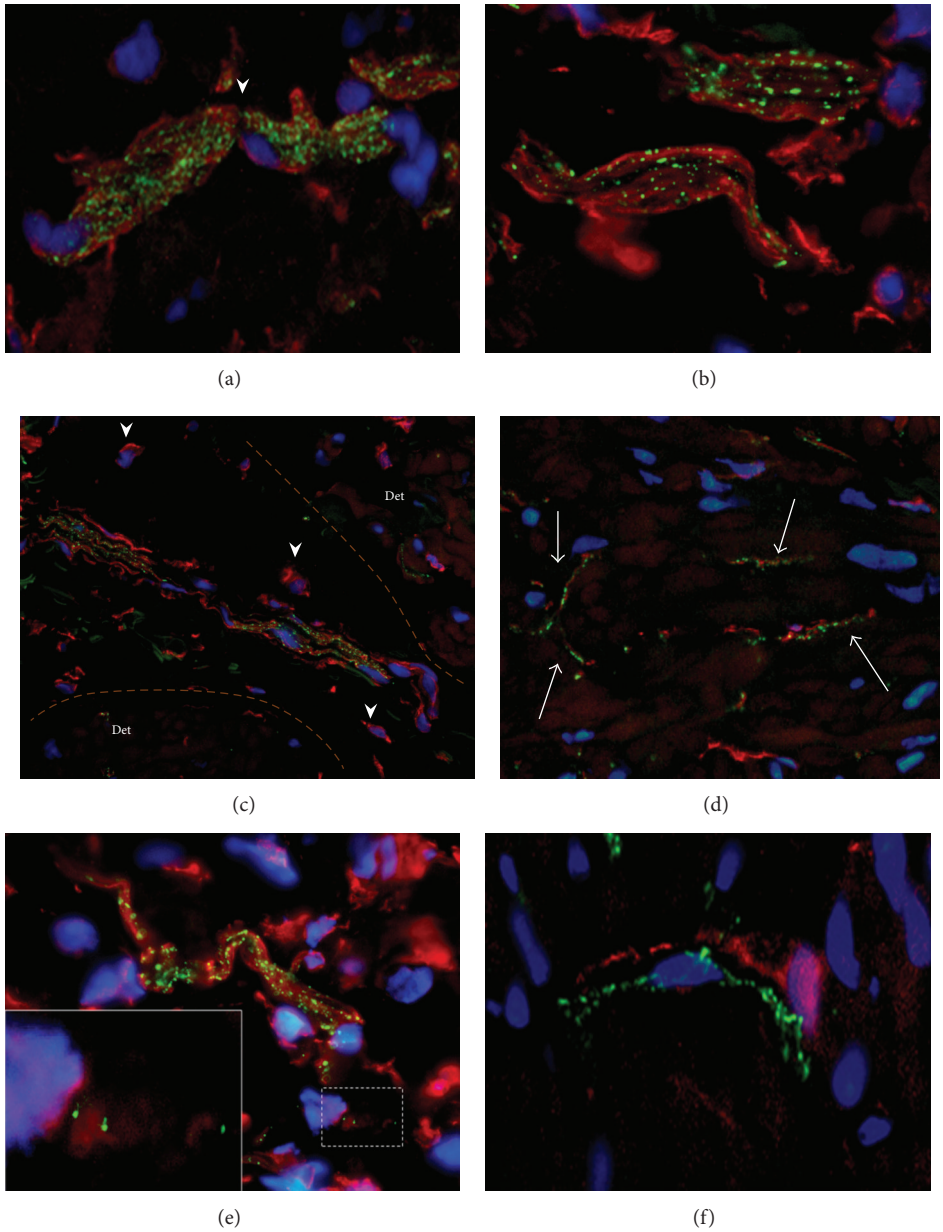
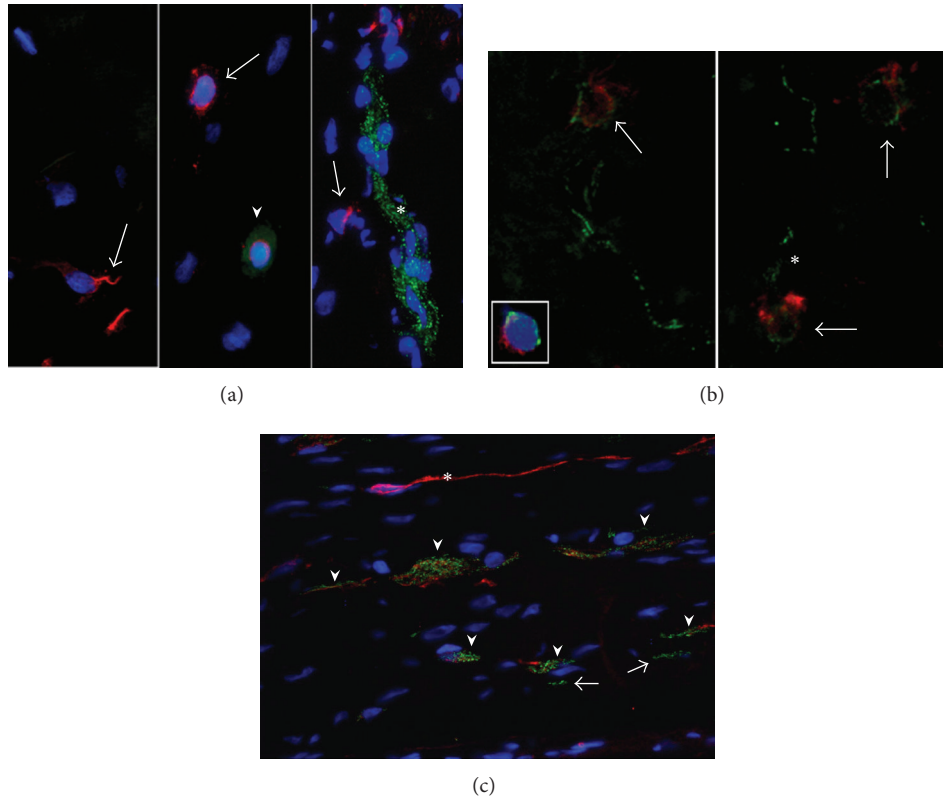


Figure 3: Double staining of N-cadherin and vimentin. (a) Two suburothelial cells coexpressing N-cadherin (green) and vimentin (red) are closely associated with each other (arrowhead). (b) Note punctate pattern of N-cadherin in contrast to the vimentin+ filaments, which both are expressed within the same cell body. Magnification ×1000. (c) Multiple elongated N-cadherin+/vimentin+ containing cells are closely associated at the border of detrusor smooth muscle bundles. (d) Detrusor fascicles show numerous N-cadherin+/vimentin+ processes (arrows) running between muscle cells. Magnification ×630. (e) Higher magnification shows expression of N-cadherin that is located at the cell membrane of some vimentin+ cells. Magnification ×1000. (f) Two cells localized at the edge of a smooth muscle fascicle. N-cadherin+/vimentin− cell is neighboured by a N-cadherin−/vimentin+ cell. Magnification ×1000.

4. Discussion

During the filling phase, the human bladder shows contractile activity which is generated within the bladder wall and does not result in intravesical pressure rise. It is also called autonomous activity [1–3]. It has been proposed that this activity shares characteristics with electrical rhythmicity in gastrointestinal muscles. Throughout the gastrointestinal tract, a network of interstitial cells of Cajal (ICCs) acts as pacemakers and conductors of electrical activity along the gut wall [5]. Initially, interstitial cells (ICs) were thought to represent a specialized type of neurons, but it is now concluded that they are a unique class of cells [6]. As ICs are also found in the human bladder, it has been proposed that they mediate autonomous bladder activity [2].

Currently, ICs are identified by various surrogate phenotypic markers, but no immunophenotype has been identified that is fully characteristic for ICs in the human



(a) (b) (c)

Figure 4: Double staining of N-cadherin and C-kit. (a) Arrows show cells in the bladder expressing C-kit (arrows, red). They lack expression of N-cadherin (green). Arrowhead shows a round cell with perinuclear expression of C-kit and diffuse cytoplasmic background signal for N-cadherin, highly resembling a mast cell. Elongated clusters of punctate N-cadherin+ cells lack expression of C-kit (asterisk). Magnification ×630. (b) Staining lacking DAPI for better orientation. Arrows show cells in the bladder expressing C-kit and punctate N-cadherin. These cells seem to give rise to N-cadherin+ branches (asterisk). Magnification ×1000. (c) N-cadherin+/C-kit+ cells running parallely (arrowheads) are neighboured by a slender elongated N-cadherin−/C-kit+ cell body (asterisk) and several smaller N-cadherin+/C-kit− cells (arrows) in the jejunum. Magnification ×630.

bladder [9]. Irrefutable confirmation of the interstitial phenotype depends on application of electron microscopy, which is highly time consuming. This study was performed in search of a specific marker for interstitial cells in the human bladder and electron microscopic investigations of the same biopsies had to confirm the immunohistochemical findings.

It has been suggested that ICs of the human bladder form a network of interacting cells [7, 8]. Cadherin complexes participate in cell-cell recognition by functioning as signaling centers [10, 11]. The subtype N-cadherin is known to regulate mesenchymal cell development [13] and is the most commonly expressed cadherin in the interstitial compartment [14, 15]. We therefore investigated the expression of N-cadherin in the human normal bladder.

N-cadherin+ cells were found in the lamina propria and the detrusor layer. They showed abundant punctuate expression of N-cadherin at their cell membrane and throughout their cell body. We cannot fully explain why N-cadherin was not exclusively expressed at the plasma membrane. However, other investigators also found that cadherins can be localized intracellular, rather than being characteristically concentrated at regions of cell-cell contact [18].

Additional cell markers were used to further analyze our findings. Smoothelin is a smooth muscle cell specific marker [16]. As no colocalization of N-cadherin and smoothelin was found, we believe that the N-cadherin+ cells do not represent smooth muscle cells.

Vimentin is expressed by fibroblastic cells [9]. A large population of N-cadherin+ cells coexpressed vimentin. They showed elongated or stellate morphology with multiple processes that seemed to form a network. However, many vimentin+ cells did not express N-cadherin. These cells were smaller and less elongated, had little perinuclear cytoplasm compared to the N-cadherin+/vimentin+ cell, and appeared to be regular fibroblasts.

PGP9.5 was chosen as a pan-neuronal marker as it is generally accepted that this protein is expressed by all neuronal structures of the bladder wall [19]. No colocalization of N-cadherin+/vimentin+ cells and PGP9.5 was found. However, N-cadherin+ cells and PGP9.5+ neurons were closely associated. Furthermore, as mature neurons lack expression of vimentin [20], we believe that the N-cadherin+/vimentin+ cells do not represent neuronal structures.

C-kit is a widely used marker for ICCs of the gut [21]. We used specimens of the human gut as a positive control. ICCs

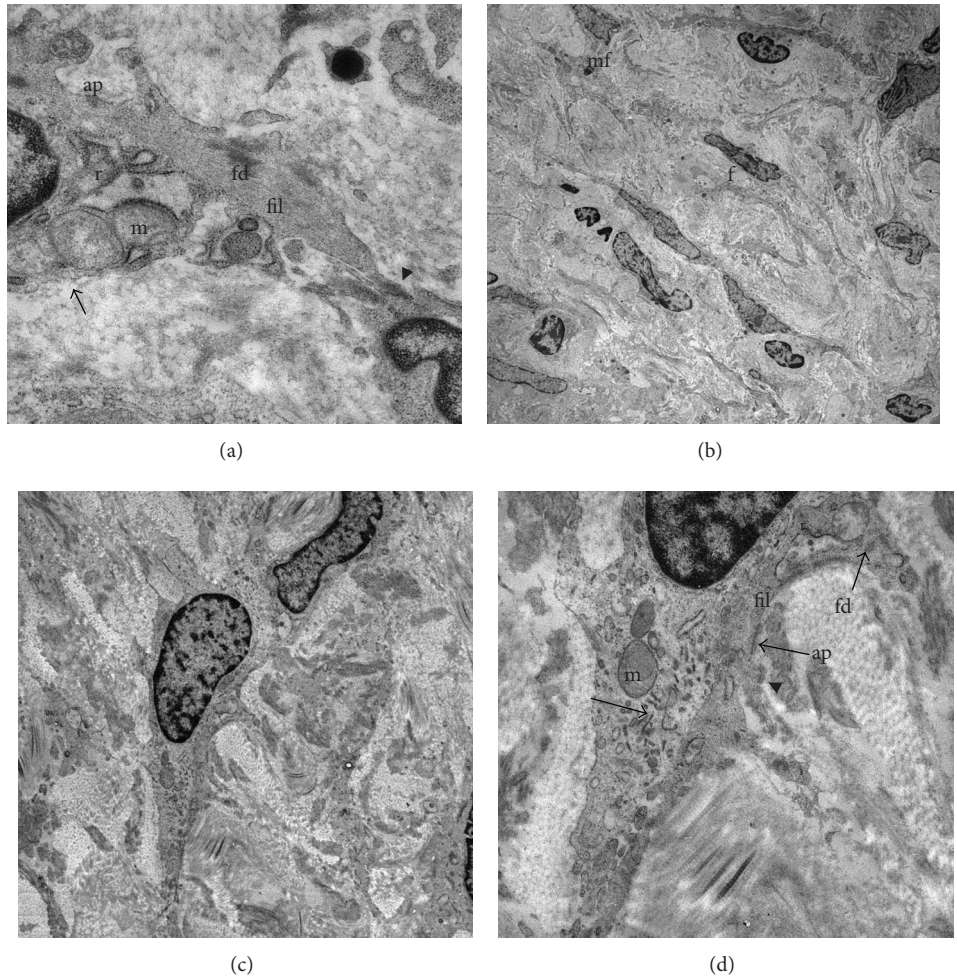


Figure 5: Ultrastructure of interstitial cells. (a) A myofibroblast located in the lamina propria. The cell is identified by filaments (fil), focal densities (fd), membranous attachment plaques (ap), subsurface vacuoles (arrow), mitochondria (m), and prominent rough endoplasmic reticulum (r). Cytoplasmic filaments cohere to a membranous attachment plaque, showing intimate association with its neighboring cell (arrowhead). Magnification ×420,000. (b) Overview of cells in the lamina propria. The left upper corner shows a myofibroblast (mf). Note its stellate morphology with multiple branches. The cell is accompanied by fibroblasts (f). Magnification ×10,500. (c) Two closely associated interstitial cells in the detrusor layer. (d) Higher magnification of (c). Branched interstitial cell shows mitochondria (m), interrupted basal lamina (arrowhead), peripheral filaments (fil), membranous attachment plaques (ap), focal densities (fd), and prominent rough endoplasmic reticulum (r). Numerous tubulovesicular structures (open arrow) were found exclusively in this cell type.

of the gut coexpressed N-cadherin and C-kit. In the bladder, N-cadherin and C-kit were coexpressed by cells with little perinuclear cytoplasm seeming to sprout into N-cadherin+ processes. As these cells seemed highly similar to C-kit+ cells in human detrusor as found by others [22], we believe that they embody interstitial cells.

Interstitial cells are found in both the lamina propria and the detrusor layer of the human bladder. Myofibroblasts are a recently documented interstitial cell type housed in the interstitial compartment. They share characteristics with smooth muscle cells and fibroblasts. In a previous study, no ultrastructural evidence for myofibroblast differentiation in the detrusor layer was discerned [23]. Interstitial cells within this layer were identified as fibroblasts. It is therefore generally believed that myofibroblasts in the human bladder solely refer to a specific group of interstitial cells within the suburothelial layer [24]. However, during our study, TEM revealed interstitial cells with stellate morphology in the lamina propria and the musculus detrusor layer. They had characteristic features of myofibroblasts. We previously showed that a suburothelial layer of cells expresses alpha smooth muscle actin myofilaments [12], most probably embodying the cytoplasmic filaments as shown during TEM in this study. Furthermore, fully differentiated myofibroblasts express alpha smooth muscle actin [25]. We therefore conclude that both the lamina propria and the detrusor layer house myofibroblasts, a unique class of interstitial cells.

From a morphological point of view, ultrastructurally defined myofibroblasts in the lamina propria and detrusor layer appear to be similar to the N-cadherin+/vimentin+ cells. Both techniques identified specified cells of mesenchymal origin with highly branched morphology and multiple

processes that were closely associated with neighbouring homotypic cells.

This study shows a population of cells from mesenchymal origin with multiple phenotypes, immunopositive for N-cadherin, vimentin, and C-kit. These cells are housed in the interstitial compartment throughout the entire human bladder wall. The findings are in accordance with a recent study of Monaghan et al. in which multiple subgroups of vimentin+ cells with distinctive morphology were found in all layers of the bladder wall [26]. It is therefore likely that not all IC's may be labeled with the same markers.

Heterogeneity of the interstitial compartment could be explained by a model in which C-kit and N-cadherin regulate mesenchymal cell differentiation. C-kit is an important stem cell marker used to identify certain types of progenitor cells [27]. Signaling through C-kit plays a role in cell differentiation, proliferation, and survival. The cadherins constitute a superfamily of glycoproteins that participate in cell-cell recognition [10]. Like C-kit, they play a crucial role in cellular differentiation and embryogenesis.

From a functional point of view, one should consider the following characteristics associated with the markers used. Similar to the gut, C-kit+ cells in the human bladder possibly act as pacemaker cells from which spontaneous calcium transients originate [28]. Cadherin complexes play a major role in cell-cell recognition and function as signaling centers [11]. Therefore, the population of N-cadherin+/vimentin+ cells may participate in specialized events such as spread of pacemaking activity. Although our results are promising, this study relies on morphological evidence. In order to illuminate functional properties of these cells, future research is needed using functional cell analyses, such as Ca-imaging and patch clamp techniques.

Although dissection of our specimens was performed distant from tumor sites, an influence of cancer cannot be ruled out. However, no thickening of the urothelial layer or abnormal urothelial morphology was found (Figure 6). Also, urothelium did not express N-cadherin, which is often seen in urothelial bladder cancer [29]. We therefore believe that our findings are unaffected by tumor-related factors.

5. Conclusions

This study shows that the interstitial compartment of the human bladder houses a heterogenous population of cells from mesenchymal origin, immunopositive for N-cadherin, vimentin, and C-kit. Due to characteristics associated with these proteins, this population of cells may participate in specialized events of the human bladder. We assume that N-cadherin/vimentin is a specific marker for a subpopulation of interstitial cells in the human bladder, that is, the ultrastructurally defined myofibroblasts. These cells may participate in spread of pacemaking activity. Although further insight is needed in the correlation between morphology and function of these cells, these findings could be promising in understanding normal and overactive bladder behaviour. Furthermore, we question the possible existence of one specific marker which defines the entire group of ICs in the human bladder.

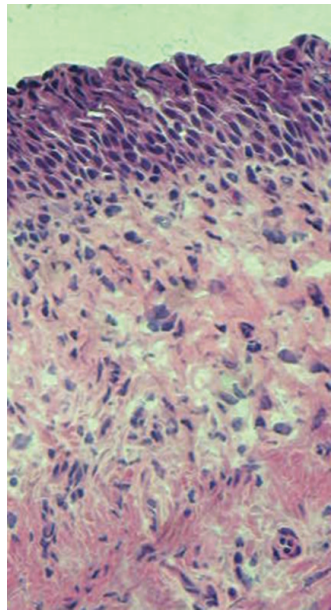


Figure 6: Representative hematoxylin and eosin stain of urothelial area of the specimens used. Normal urothelium consists of approximately 3–5 cell layers. Note that no abnormal thickening of the urothelial layer or abnormal suburothelial morphology is found.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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