Review Article
Int Neurourol J 2011;15:184-191
http://dx.doi.org/10.5213/inj.2011.15.4.184
pISSN 2093-4777 · eISSN 2093-6931

International Neurourology Journal
INJ

Antiproliferative Factor Signaling and Interstitial Cystitis/Painful Bladder Syndrome

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A unique glycopeptide, antiproliferative factor (APF), has been suggested as a urinary biomarker and potential mediator of long-term bladder disorder Interstitial Cystitis/Painful Bladder Syndrome. There is no known cause for this disease. Several mechanistic approaches have been employed to address the underlying mechanism whereby APF regulates cellular responses in the bladder epithelium. A summary of recent literature is provided, and is focused on signal transduction pathways and networks that are responsive to APF.

Keywords: Human antiproliferative factor APF; Interstitial Cystitis; Signal transduction

INTRODUCTION

Interstitial Cystitis/Painful Bladder Syndrome (IC/PBS)

A chronic bladder disorder, IC/PBS, affects over 1 out of 77 Americans (3 to 8 million women and 1 to 4 million men). Clinopathological processes underlying this disease have not yet been elucidated, clinical biological markers of the condition are not available, and the type and severity of symptoms can vary. Consequently, an unambiguous clinical definition of the condition is not yet possible [1-8]. It is not understood whether IC/PBS is a systemic disease or whether it originates from the bladder and/or other pelvic organs [5,9]. Thus, IC/PBS is a controversial subject and there is still much to be leaned about this disease [10].

Diagnosis

IC/PBS patients are generally diagnosed according to certain clinical criteria, such as National Institute of Diabetes and Digestive and Kidney Diseases guidelines, which contain inclusion clinical diagnostic criteria using symptoms combined with cystoscopic observations, in the absence of other bladder diseases (e.g., overactive bladder, bacterial cystitis, neurogenic bladder, stress incontinence, urological cancer [bladder or prostate cancers], or benign prostatic hypertrophy, and chronic pelvic prostatic symptoms) [1,2,5,6,10-14]. Hydrodistension has been used as part of a diagnostic algorithm. Accumulating scientific data on diagnostics show that cystoscopy and hydrodistension may not be sufficiently sensitive or specific [13,14]. Other diagnostic tests can involve results from urine analysis, urine culture, biopsy of the bladder wall and urethra, specific questionnaires to assess the condition (e.g., pain score), the potassium sensitivity test, and/or the anesthetic bladder challenge [1,15,16]. However, there is no single diagnostic gold standard. In the absence of agreed-upon diagnostic tests, IC/PBS criteria have not been consistently applied for diagnosis in clinical medicine [4].

Recently, new guidelines by the American Urological Association (AUA) IC/PBS guidelines committee suggest a management strategy for the IC/PBS patient [17]. This recommendation was based on an extensive literature review of 86 articles containing information on application of inclusion and exclusion criteria toward IC/PBS patients. The AUA guidelines suggest conservative, noninvasive approaches involving diet change,

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Submitted: November 30, 2011 / Accepted after revision: December 20, 2011

physical therapy for symptom relief and stress management [8,16,17]. Overall, this recommendation outlines five levels of treatment, including pain management.

Treatment

Treatment of IC/PBS remains challenging. Although medical treatments and procedures, such as bladder instillation, hydrodistension, and oral pharmaceutical drugs are prescribed for those afflicted with the disease [1,13,17,18], these therapies are effective in only a small percentage of patients and most cause undesirable side effects [19]. Oral medications include pentosan polysulfate (Elmiron), antihistamines, tricyclic antidepressants, and immune modulators. Intravesical medications include dimethyl sulfoxide (DMSO), pentosan polysulfate, and heparin. Elmiron is the only oral therapy, and DMSO-based therapy is the only intravesical therapy, with Food and Drug Administration-approval for the treatment of IC/PBS [18]. The most commonly prescribed IC/PBS medication is Elmiron; however, symptom relief by this drug is only for 30 to 60% of patients. Side effects such as hair loss and gastrointestinal disturbance are commonly observed after treatment is initiated [18].

POTENTIAL BIOMARKERS OF IC/PBS

Since current IC/PBS diagnosis is generally based on symptoms and exclusion of other bladder disorders, identification of objective biomarkers, phenotyping and characterization of pathology would represent a major advance [11,20-22]. Although there are substantial limitations and roadblocks to implementing biomarker discovery, there is a tremendous need for specific and sensitive biomarkers in this field [9,15,23]. Biomarkers can be used for diagnosis and/or for tracking the treatment response of patients, which can reduce clinical burden. Biomarkers in urine or blood are favorable, since their application requires less invasive methods [23].

Searches for an objective urinary IC/PBS biomarker have led to the identification of several candidates such as substance P, uroplakin III-δ4 mRNA, interleukin-6, cyclic guanosine monophosphate, uromodulin, kininogens, inter-α-trypsin inhibitor heavy chain H4, nitric oxide, nerve growth factor, heparin-binding epidermal growth factor-like growth factor (HB-EGF) and antiproliferative factor (APF) [15,24-33]. There are still a lot of discrepancies on strong correlation to severity (e.g., pain and symptom score), clinical course and responsiveness to treatments (e.g., steroid or oral prednisolone) in IC/PBS patients, compared to their age-, race- and gender-matched controls.

HB-EGF, EGF and APF

Three urinary biomarkers identified from IC/PBS patient material were found by Keay et al. [25,34]: epidermal growth factor (EGF) and APF concentrations in urine were positively correlated with presence of disease, while HB-EGF exhibited an inverse correlation in urine of IC/PBS patients, compared to controls (urine from patients without IC/PBS).

EGF and HB-EGF are potent urothelial and smooth muscle cell mitogens, and enhance proliferation [35,36]. EGF is a product of many epithelial cells and was the first activating ligand for the epidermal growth factor receptor (EGFR) identified [37]. HB-EGF is initially expressed as a transmembrane precursor (proHB-EGF), with the soluble form generated by the regulated metalloproteinase-dependent ectodomain shedding. Secreted soluble HB-EGF activates mitogenic and cell survival functions, while proHB-EGF acts as a juxtacrine factor and also mediates translocation of diphtheria toxin from the cell surface to the cytosol. In addition to activation of the EGFR, HB-EGF is also a direct activating ligand for the related tyrosine kinase, ErbB4/HER4 [36,38]. EGF and HB-EGF are present under normal conditions in human urine, and the levels are altered in patients with IC/PBS [25,34]. Concentrations of HB-EGF and EGF change in an inverse manner (EGF levels are higher and HB-EGF levels are lower) in IC/PBS patients [34].

APF is a small, 8 amino acid sialoglycosylated peptide identified in IC/PBS patients. Bioactivities attributed to APF include: suppression of cell growth; increases of transcellular permeability; lowering of the expression of proteins that form intercellular junctional complexes; and reduction of HB-EGF production from urothelial cells [39]. APF is originally purified from the urine specimens of IC/PBS patients using high performance liquid chromatography [24,40,41], and recently a bioactive, synthetic form of the glycopeptide was reported [42]. APF activity is detected in the urine of approximately 95% of IC/PBS patients (as compared to approximately 9% of controls).

APF AND IC/PBS

The accumulation in urine of the bioactive factor APF, which is capable of altering the physiology and behavior of urothelial cells, is consistent with clinical observations of epithelial thinning and denudation observed in IC/PBS bladder tissue. *In vitro*, APF is synthesized and secreted from bladder epithelial cells

derived from patients diagnosed as IC/PBS, but not from asymptomatic controls [41]. APF is 100% homologous to the 6th transmembrane domain of membrane receptor frizzled-8, in the Wnt signaling network. APF is acidic, heat-stable and glycosylation is required for biological activity. The structure of APF (Neu5Acα2-3Galβ1-3GalNAcα-O-TVPAAVVVA) was deduced by a series of processing steps, involving ion trap mass spectrometry, enzymatic digestion, lectin affinity chromatography, and microcapillary reversed-phase liquid chromatography/mass spectrometry (MS) [24].

Both chemically synthesized and native APF (purified from urine or explants from biopsies from patients with IC/PBS) had identical biological activity in normal bladder epithelial cells and several types of cancer cells including T24 bladder cancer cells [24,42,43]. These findings suggest that APF is not only a urinary biomarker but also a potential mediator of the pathogenic changes seen in IC/PBS.

APF SIGNALING

In order to determine the potential etiologies of IC/PBS, and to identify molecular and cellular differences between IC/PBS patients and controls, understanding the molecular mechanisms of the various effects induced by APF has been attempted. Denudation or thinning of the epithelium is commonly observed in bladder biopsies from patients with IC/PBS, suggesting cell proliferation is hindered in IC/PBS cells [40,44]. Although the exact etiology is unknown, the impermeability of the bladder barrier is increased, resulting in a leaky bladder epithelium and potassium leakage, potentially a source of origin for IC/PBS symptoms (such as frequency, urgency, pelvic pain or incontinence) [45]. Additional bladder dysfunction includes increased nerve fiber density and inflammatory infiltrates (e.g., mast cell and/or lymphocytic), which are correlated to the increased pain and cytokine production in IC/PBS bladder [46-48]. To understand the underlying mechanisms, several laboratories have focused on abnormal cell signaling induced in presence of APF in bladder epithelial cells. As we will discuss below, these led to the following observations that 1) cytoskeleton-associated protein 4 (CKAP4 or CLIMP63) plays a role as an APF receptor [42,49, 50]; 2) the palmitoylation status of CKAP4 is important for APF signal transduction [39,49]; 3) mitogen-activated protein kinase (MAPK) signal pathways were altered by APF [39,51]; 4) phosphorylation of Akt and its target, glycogen synthase kinase (GSK), is induced by APF treatment [42,52]; 5) the p52-p21 signal pathway is enhanced in the presence of APF, leading to inhibition of cell proliferation [52]; and 6) a quantitative proteomics analysis and bioinformatics identified β-catenin as a prominent hub molecule in APF-induced signaling [53].

APF Receptor, CKAP4/CLIMP63

Zhang et al. [54] found that APF binds to a high affinity cell surface receptor, CKAP4/CLIMP63, which mediates APF signaling in bladder epithelial cells, using the palmitoyl-cysteine identification capture and analysis method. In general, palmitoylation (S-acylation) of transmembrane proteins regulates subcellular trafficking and/or protein degradation [55]. S-acylation of CKAP4/p63 is required for its proper localization into the plasma membrane by Asp-His-His-Cys protein (DHHC), a specific palmitoyl acyltransferase and a putative tumor suppressor [54]. APF binds to membrane resident palmitoylated CKAP4, resulting in an inhibition of cell proliferation and alteration of the expression of genes relevant to cell-to-cell permeability (e.g., E-cadherin, vimentin, and tight junction protein, ZO-1) [54,56]. Interestingly, DHHC2-mediated CKAP4/p63 palmitoylation mediates CKAP4/p63 trafficking from the ER to the surface membrane and/or to nucleus, suggesting that DHHC2 activity is critical to the APF-mediated signaling requiring CKAP4/p63 [49,55]. The potential correlation of expression of CKAP4/p63, DHHC2 and sensitivity to APF remains to be tested.

MAPK Signal Pathways

Various biological responses in response to APF accompany the perturbed signal transduction pathways [39]. The possible role of the MAPK pathways (including Erk/MAPK, p38MAPK, and c-Jun N-terminal kinases/stress-activated protein kinase [JNK/SAPK] signaling) in IC/PBS are not well understood, in spite of importance of MAPK signaling in epithelial, endothelial, and fibroblast cell proliferation [57,58]. Although there are important differences between tissues and cell types, Erk, p38 and JNK/SAPK MAPK pathways often exert opposing functions and undergo extensive cross-talk in regulating proliferation and cell death.

APF treatment has been reported to result in the increased p38MAPK phosphorylation and suppressed cell growth, which were both reversed by a p38MAPK-selective inhibitor [51]. Very recently, Li et al. [59] reported that a transcription factor in the JNK/SAPK pathway, c-Jun, involves in growth suppression by APF. APF also induced a significant decrease in c-Jun expression in concert with growth inhibition. Enforced c-Jun expres-

 http://dx.doi.org/10.5213/inj.2011.15.4.184

sion reversed APF inhibitory effects on cell growth. These results show that JNK/SAPK expression may also be important for control of uroepithelial cell proliferation, with JNK1 levels decreased in both IC/PBS cells (compared to controls) and APF-treated normal bladder epithelial cells (compared to control peptide-treated) [45,59].

Akt Pathway

Activation of the a serine-threonine kinase, Akt/protein kinase B, is generally important to cell proliferation, migration and survival [60-62]. There are three isoforms, Akt1, Akt2, and Akt3, which show differential functions in cancer cells [62,63]. Phosphorylation of Akt/PKB occurs at Ser473 and Thr308 [61-63]. Recent studies have shown that the rapamycin-insensitive rictor-mTOR complex (mTORC2) and DNA-dependent protein kinase both directly phosphorylates Akt at Ser473 [64,65]. However, it has not been shown whether Akt or Akt-related molecules (e.g., PI3-Kinase and GSK) are related to IC/PBS, except a very recent paper showing that APF treatment downregulates Akt signaling in bladder epithelial cells [42]. Phosphorylation of Akt (at Ser473 and Thr308), GSK3β (at Tyr216) and β-catenin (at Ser45/Thr41) were decreased by APF treatment, suggesting that APF downregulates Akt and its downstream signaling network, which is consistent with the growth arrest observed in IC/PBS cells. It has not been tested whether this downregulation of Akt signaling in response to APF is dependent on PI3-kinase or the PTEN pathway, which is an important upstream inhibitor of Akt signaling [66,67].

p53-p21 Signaling

Activation of the p53 pathway is stimulated by stress signals (such as DNA damage and oxidative stress) and activated oncogenes [68-70]. As a transcription factor, p53 controls the expression of p53-regulated genes [71,72], resulting in cell cycle arrest, cellular senescence, apoptotic cell death, or repair of damaged DNA [73,74]. Loss of p53 function is one of the common genetic alterations observed in human cancer cells [73,75,76].

The signaling mechanisms by which APF induces its antiproliferative activity are of considerable interest. Treatment of normal human urothelial cells, and T24 human bladder carcinoma cells with APF increased p53 levels [52]. Changed p53 level altered the APF effect on cell growth, indicating that p53 is involved in the mechanism of APF-induced growth suppression observed in the context of IC/PBS. p53 down-regulation enhanced the suppressive effect of APF on cell growth, while ectopic p53 expression induced cell cycle arrest in the absence of APF. One of the p53 targets, p21 was induced by APF treatment in presence of p53, implying that p53-p21 signaling is an important mediator of APF–induced effects on bladder epithelial cells [52]. Some p53-interacting proteins, including herpesvirus-associated ubiquitin-specific protease, homeodomain-interacting protein kinase 2, ataxia telangiectasia mutated (ATM), ATM and RAD3-related, and heterogeneous nuclear ribonucleoprotein K, are known to stabilize p53 and increase expression level [68,77-80]. Phosphorylation of p53 at specific sites protects p53 from degradation by murine double minute [81,82]. However, the specific mechanism by which APF regulates p53 and p21 levels in the IC/PBS context is not clearly understood. Additional studies on the p53 modification status in response to APF remain to be undertaken.

β-catenin in IC/PBS

In order to identify IC/PBS-specific pathogenetic mechanisms and biomarkers, efforts have been focused on genomic, proteomic and metabolomic patterns to distinguish IC/PBS from other bladder diseases. These studies were often combined with a systems-biology approach with microarray, MS and proton nuclear magnetic resonance (1H-NMR)-based spectral patterns [45,83].

Over about 100 significantly changed proteins following APF treatment were identified by stable isotope labeling by amino acids in cell culture (SILAC) quantitative proteomics [53]. β-catenin was found to be the most prominent and functionally relevant protein in the APF signaling network by various functional validation assays. Bioinformatics analysis with data integration of the *in vitro* SILAC data with *in vivo* RNA expression data obtained from IC/PBS patients identified cyclooxygenase-2 (COX-2) as an effector of β-catenin in IC/PBS. In bladder epithelial cells from IC/PBS patients, the expression level of β-catenin was correlated to level of COX-2 [53]. These findings suggest that inflammation associated with IC/PBS may be caused, at least in part, by APF. These findings imply that targeting the β-catenin-COX-2 signaling pathway in addition to APF may be a rational approach toward treating IC/PBS.

THERAPEUTIC IMPLICATION OF APF RESEARCH

HB-EGF as an APF Antagonist

One interesting feature of the existing urinary biomarkers of

IC/PBS is the functional relationship between APF and HB-EGF. First, levels of these two biomarkers are inversely correlated in IC/PBS patient urine [25,41]. Second, this inversed pattern has been observed after cystoscopic hydrodistention, a common clinical procedure to relieve IC/PBS-related symptoms. Application of hydrodistention significantly increased HB-EGF levels and decreased APF activity in urine [84]. Third, APF was shown to decrease HB-EGF production by normal human bladder epithelial cells *in vitro*, and concentrations of recombinant human HB-EGF, similar to those found in normal human urine specimens, were shown to abrogate the effects of APF on human bladder epithelial cells [40]. Lastly, removal of APF from the culture medium induced HB-EGF production and recovery of cell proliferation [51]. Together, these results suggest that HB-EGF and APF are functionally antagonistic biopeptides.

Further investigation on the underlying mechanisms of the bioactivity relationship between HB-EGF and APF has revealed the involvement of signal transduction pathways perturbed by them. HB-EGF and APF are functionally antagonistic due to signals through parallel MAPK signaling pathways: activated Erk/MAPK by HB-EGF is inhibited by APF, and APF cannot stimulate p38MAPK in the presence of soluble HB-EGF (or when cells overexpressed constitutively secreted HB-EGF) [51].

Although the mechanism of the hydrodistention effect has not been clearly known hydrodistension therapy has been introduced to relief IC/PBS symptoms [14]. IC/PBS symptom scores were decreased 56% in a cohort of patients after hydrodistension [14]. One suggested hypothesis is that bladder distention may increase the capacity to reduce pain signals transmitted by the nervous system. Hydrodistention also results in an increase of HB-EGF levels in urine and a reduction of urine APF activity [84]. The functional correlation of APF and HB-EGF through crosstalk between signal pathways provides a mechanistic basis for the reported therapeutic effects of hydrodistension to relief IC/PBS symptoms, one consequence of which is increased intraluminal concentrations of HB-EGF, an APF antagonist.

Inactive APF Derivatives

Chemically synthesized asialylated APF (as-APF) exhibited similar antiproliferative effects on normal bladder epithelial cells. Removal of the sialic acid unit from as-APF did not affect its biological activity. Over 40 synthetic APF analogues were recently screened to identify inactive APF derivatives for their ability to block antiproliferative activity of as-APF in normal bladder cells [43]. Two inactive as-APF derivatives, D-proline as-APF, Galβ1-3GalNAcα-O-TV-(d-pipecolic acid)-AAVVVA, and D-pipecolic acid as-APF, Galβ1-3GalNAcα-O-TV-(d-proline)-AAVVVA were found to block as-APF antiproliferative activity in normal bladder epithelial cells. Furthermore, these as-APF derivatives were able to recover the altered tight junction protein expression, paracellular permeability, and/or proliferation of IC/PBS cells [43].

CONCLUDING REMARKS

The purpose of this review was to revisit the published literature showing evidence for a biological role of APF in IC/PBS, with a focus on APF-induced gene expression and signaling network alterations. We also discuss some potential therapeutic strategies for this bladder disorder based on the functions of APF antagonists.

Clinical diagnosis of IC/PBS largely relies on the exclusion of other bladder diseases. Pathologic and biologic tools for diagnosis are sorely needed. However, it is encouraging that IC/PBS biomarkers have been identified in patient urine, suggesting that non-invasive methods using quantitative and accurate diagnostic criteria may be possible. Our understanding of the origins and molecular mechanisms of this debilitating disease are limited and contradictory. Much work needs to be undertaken to refine the currently existing candidate biomarkers. Ongoing efforts in the IC/PBS field include mechanistic studies to enhance our understanding of etiology and disease progression, and to develop IC/PBS drugs using the inactive analogues of APF. Accumulating evidence also suggests the potential for APF signaling network-targeting therapy and deserves greater experimental attention.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

ACKNOWLEDGEMENTS

This manuscript is supported by grants from NIH grants R01 DK087806, R01 CA143777 and P50 DK65298 (to M.R.F.); the Fishbein Family IC Research Foundation/Interstitial Cystitis Association (ICA), Pilot Research Program/ICA, New York

 http://dx.doi.org/10.5213/inj.2011.15.4.184

Academy of Medicine, and Children's Hospital Boston Faculty Development (to J.K.). J.K. is an American Urological Association Foundation Research Scholar and a Harvard Medical School Eleanor and Miles Shore Scholar.

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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