Published in final edited form as:

BJU Int. 2009 February ; 103(4): 541–546. doi:10.1111/j.1464-410X.2008.08097.x.

HB-EGF Functionally Antagonizes Interstitial Cystitis Antiproliferative Factor (APF) via MAPK Pathway Activation

Jayoung Kim^{1,2,*}, Susan K. Keay³, and Michael R. Freeman^{1,2}

¹The Urological Diseases Research Center, Children's Hospital Boston, Boston, MA 02115, USA

²Departments of Surgery and Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, MA 02115, USA

³Division of Infectious Diseases, Department of Medicine, the University of Maryland School of Medicine and VA Medical Center, Baltimore, MD 21201, USA

Abstract

OBJECTIVE—Interstitial cystitis (IC) is a chronic syndrome of unknown etiology with variable symptoms including pelvic and or perineal pain, urinary frequency, and urgency. Antiproliferative factor (APF) is a sialoglycopeptide biomarker found at increased levels in the urine of IC patients, coincident with a decline in urine concentration of the urothelial and smooth muscle cell mitogen, HB-EGF. APF has previously been shown to decrease the proliferation rate of normal bladder epithelial cells and the amount of HB-EGF produced by these cells. The present study was directed toward exploring the mechanisms underlying the bioactivity relationship between HB-EGF and APF.

MATERIALS AND METHODS—APF-responsive T24 transitional carcinoma bladder cells were treated with HPLC-purified native APF with or without HB-EGF to determine the involvement of signaling pathways and proliferation by Western blot analysis, p38MAPK and Erk/MAPK assays, and MTT assay.

RESULTS—Cyclic stretch induced the secretion of HB-EGF from T24 cells overexpressing the HB-EGF precursor, resulting in enhanced proliferation. Treatment of T24 cells with APF resulted in increased p38MAPK activity and suppressed cell growth, events which were both reversed by treatment with a p38MAPK-selective inhibitor. Activation of Erk/MAPK by HB-EGF was inhibited by APF, and APF did not stimulate p38MAPK in the presence of soluble HB-EGF or when cells overexpressed constitutively secreted HB-EGF. Lastly, APF inhibitory effects on cell growth were attenuated by HB-EGF.

CONCLUSIONS—These results indicate that HB-EGF and APF are functionally antagonistic and signal through parallel MAPK signaling pathways in bladder cells.

Keywords

HB-EGF; APF; interstitial cystitis; mitogen activated protein kinase

*Correspondence: Jayoung Kim, PhD, Enders Research Laboratories, 1148, 300 Longwood Avenue, Boston MA 02115, Tel: 617 919-2639, Fax: 617 730-0238, ja.kim@childrens.harvard.edu.

Introduction

Interstitial cystitis (IC) is an idiopathic condition characterized by long-term pelvic symptoms of pain, with increased urinary frequency and urgency [1]. Thinning or ulceration of the bladder epithelial lining in the absence of urinary tract infection is frequently present in IC [2]. Urothelial defects associated with symptoms can be caused by disruptions in urothelial tissue integrity, proliferation, metabolism or function [1]. Although multiple hypotheses have been proposed for the primary cause of IC, the underlying molecular mechanism remains largely undefined.

Three biomarkers have been identified in urine from IC patients: the EGFR/ErbB1/HER1 peptide ligands heparin-binding epidermal growth factor-like growth factor (HB-EGF) and epidermal growth factor (EGF), and an antiproliferative factor, APF [3-5]. APF is present and levels of EGF are significantly increased in IC patient urine specimens in comparison to specimens from unaffected control subjects, while levels of HB-EGF are decreased significantly [5]. APF, an unusual soluble glycosylated peptide related to the membrane receptor frizzled-8, is also synthesized by bladder epithelial cells from IC patients and has been shown to elicit several responses from this cell type [6-10]. Recently, APF was found to bind to a high affinity cell surface receptor, CKAP4/p63 [11], suggesting that an interaction between APF and this cognate protein may play a role in IC. APF suppresses primary bladder epithelial cell proliferation, increases transcellular permeability by lowering expression of proteins involved in intercellular junctional complexes, and reduces HB-EGF production from primary bladder epithelial cells [8, 9, 12].

In addition to activation of EGFR, HB-EGF is also a direct activating ligand for the related tyrosine kinase, ErbB4/HER4. HB-EGF is initially expressed as a transmembrane precursor (proHB-EGF), with the soluble form generated by 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 [13-15].

One interesting feature of the existing biomarker data on IC is the potential functional relationship between APF and HB-EGF, IC biomarkers whose levels vary inversely in the urine of IC patients. 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 [16]. Upon removal of APF from the culture medium, HB-EGF production was shown to increase just prior to recovery of cell proliferation [12]. These results, combined with the lower levels of urine HB-EGF seen clinically in IC patients, suggest that HB-EGF and APF are functionally antagonistic biopeptides.

The goal of the present study was to delineate the mechanism underlying the potential functional relationship between APF and HB-EGF. Our observations indicate that mechanical stretch of a human bladder epithelial cancer cell line expressing proHB-EGF resulted in suppression of APF activity via increased secretion of soluble HB-EGF (sHB-EGF), as well as HB-EGF-mediated inhibition of the p38MAPK pathway, which we found

to be activated by APF. These findings indicate that MAPK pathways relay growth-promoting and -inhibiting signals in urothelial cells in response to the presence of APF and HB-EGF in the external medium.

Materials and Methods

Reagents

APF was purified from the supernatant of bladder epithelial cells explanted from one IC patient [16]. Mock APF was prepared using cells from an age-, race- and gender-matched normal control subjected to the same purification procedure. Antibodies against p38MAPK, phospho-p38MAPK, Erk/MAPK and phospho-Erk/MAPK were obtained from Cell Signaling Technology Inc. (Danvers, MA). Anti-HB-EGF antibody was purchased from R&D Systems, Inc. (Minneapolis, MN). SB203580 and DMSO were purchased from Calbiochem (San Diego, CA).

Cell culture and transfection

T24 cells (ATCC, HTB-4) were cultured in McCoy's 5A containing 10% FBS, 1% L-glutamine, and 1% antibiotic/antimycotic solution (all from Sigma) routinely at 37°C / 5% CO₂ condition in humidified incubator. T24 cells were transiently transfected with 2 µg of DNA plasmid vectors (vector only, proHB-EGF (full length HB-EGF construct) or HB-EGF_{cs} (constitutively secretable HB-EGF mutant lacking the transmembrane region) using Nucleofector (Amaxa Inc., Gaithersburg, MD) using the manufacturer's suggested protocols.

In vitro cyclic stretch

T24 cells transiently transfected with a proHB-EGF expression plasmid, and verified to express high levels of the protein, were seeded on type I collagen-coated BioFlex culture plates (Flexcell International, Hillsborough, NC) at a density of 1×10⁵ cells. Cells were subjected to cyclic-relaxation stretch the following day. One cycle consisted of 5 s of stretch and 5 s of relaxation (0.1 Hz) and elongation to 20% maximum radial stretch at the periphery of the membrane as previously described [15]. Medium was collected 1 h after cyclic stretch and subjected to pull-down assay using heparin-sepharose beads, followed by Western blot analysis to evaluate HB-EGF secretion levels as described previously [17]. T24 cells transiently transfected with vector plasmid alone were used as a control.

Western blot analysis

Cells were solubilized with whole cell lysis buffer [1% Nonidet P-40; 50 mM Tris pH 7.4; 10 mM NaCl; 1 mM NaF; 5 mM MgCl₂; 0.1 mM EDTA; 1 mM PMSF; and protease inhibitor cocktail tablet (Roche Diagnostocs Corp., Switzerland)] and centrifuged at 12,000× g for 15 min. The supernatant was assayed for protein concentration and equal amounts of protein were used for Western blotting.

p38MAP kinase assay

T24 cells were preincubated at 37°C with a specific p38MAPK inhibitor (SB203580) or DMSO for 30 min, followed by treatment of APF for 5 min. Cell lysates were prepared using 1x lysis buffer provided by the company (p38 MAP Kinase assay kit, Cell Signaling Technology Inc.). After immunoprecipitation with phospho-specific p38MAPK antibody-conjugated beads overnight at 4°C, samples were incubated in kinase buffer [25 mM Tris pH 7.5; 5 mM β -Glycerolphosphate; 2 mM DTT; 0.1 mM Na_3VO_4 ; 10 mM MgCl_2] with 200 μM ATP to measure kinase activity using 1 μg recombinant ATF-2 protein as a substrate for p38MAPK. After incubation at 30 °C for 30 min for kinase reaction, samples were boiled in SDS-containing buffer and applied to SDS-PAGE gels to detect the phosphorylation levels of ATF-2 by western blotting using anti-phospho ATF-2 and anti-ATF-2 antibodies.

Cell proliferation assay

Cells were plated onto 24-well tissue culture plates at a density of 1×10^4 cells/well in standard growth medium. Cells were then serum-starved for 12 h prior to proliferation analysis. To observe the effect of APF as an antagonist of HB-EGF, 10 ng/ml APF or Mock APF was added to the medium 5 min before treatment with 100 ng/ml HB-EGF. To determine the involvement of p38MAPK activity in APF function, cells were pretreated with 10 μM SB203580 for 1h, and were incubated with APF-containing medium for the indicated times. Cell viability was determined by uptake of 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) as described [17].

Statistical Analysis

Data were compared using a paired Student's t-test. P values < 0.05 were considered significant.

Results

Cyclic stretch induces HB-EGF secretion and cell proliferation

Bladder epithelial cells have been shown to respond to cyclic mechanical deformation (stretch) with release of cytokines, growth factors and other soluble molecules, such as ATP [18, 19]. We have reported that cyclic stretch induced secretion of soluble HB-EGF in various cell types, including bladder smooth muscle cells *in vitro* [20, 21]. It has been shown in humans that bladder stretch via hydrodistension changes urinary levels of HB-EGF and APF activity [18]. To begin to understand the possible network relationship between APF and HB-EGF, T24 human bladder cells stably expressing the precursor form of HB-EGF (T24_{proHB-EGF} cells) and vector-only (T24_{vector} cells) were constructed. T24_{proHB-EGF} cells were employed in the following experiments because they secrete bioactive, soluble HB-EGF (sHB-EGF) into the medium in a regulated (inducible) manner, and downstream effects of the growth factor can be monitored [15]. Cyclic stretch was applied for 1 h, followed by collection of conditioned medium. Pull down assay using heparin-sepharose beads demonstrated that substantially greater sHB-EGF was secreted by the T24_{proHB-EGF} cells under the stretch conditions than under non-stretch conditions (Fig 1).

HB-EGF activates the Erk/MAPK pathway in T24 cells, but not in the presence of APF

HB-EGF has been shown to be an autocrine regulator of growth of human bladder epithelial cells [15] and to induce a time- and concentration-dependent phosphorylation of Erk/MAPK, Akt1 and p70S6K, but not p38 MAPK in these cells. Western blot analysis demonstrated that HB-EGF treatment of T24 cells transiently stimulated Erk/MAPK phosphorylation, reaching a maximum level after 15 min (Fig 2A). HB-EGF activated the Erk/MAPK pathway without a discernable effect on Erk/MAPK protein expression (Fig 2A). Phosphorylation levels of Erk/MAPK were measured in response to HB-EGF in a dose-response format. Western blotting demonstrated marked Erk/MAPK phosphorylation when cells were stimulated with 100ng/ml HB-EGF for 15 min (Fig 2B).

In subsequent experiments, we tested the hypothesis that APF exposure inhibits the largely pro-proliferative signaling cascades induced by HB-EGF. Cells were pre-incubated in APF-containing medium for 5 min, after which the medium was replaced and cells were stimulated with 100 ng/ml recombinant HB-EGF for 15 min. Remarkably, this brief exposure of cells to APF completely inhibited subsequent stimulation of Erk/MAPK phosphorylation by HB-EGF (Fig 2C). These findings provide the first direct evidence for inhibitory crosstalk between HB-EGF and APF.

APF suppresses bladder cell proliferation through p38MAPK activation

APF has been shown to inhibit cell proliferation of T24 cells via inhibition of cell cycle transit at the G₂/M phase and an increase in p53 protein expression [10]. Time- dependence experiments showed that APF treatment activated p38MAPK (Fig 3A and 3B). This APF-induced effect is specific within this experimental system because APF did not alter other, alternative signaling pathways (Erk/MAPK, JNK/SAPK or Akt (data not shown)). The level of p38MAPK phosphorylation was elevated within 5 min after APF treatment (Fig 3A). Kinase activity of p38MAPK was also increased in response to APF as shown in figure 3B, and kinase activity was suppressed when cells were preincubated with a specific p38MAPK inhibitor, SB203580, before APF treatment. Cell proliferation rate was reduced by about 40% following addition of APF in comparison to the Mock peptide control (Fig 3B). This growth suppressive effect was completely abrogated by pretreatment of cells with SB203580 demonstrating that p38MAPK is an important mediator of APF-induced cell growth arrest (Fig 3B).

HB-EGF suppresses APF-stimulated p38MAPK activation

To expand our observations and test directly whether the bladder epithelial cell growth factor HB-EGF antagonizes APF-induced growth arrest, we performed two experiments to assess the effect of exogenous treatment of HB-EGF on p38MAPK activation induced by APF treatment. T24 human bladder cells were stimulated with HB-EGF for 15 min, followed by treatment with APF for the indicated time points. Consistent with the above observations (Fig 2A), HB-EGF stimulated the Erk/MAPK pathway 15 min after treatment. The purified native APF activated p38MAPK (Fig 4A) and this activation was completely inhibited by the presence of exogenous HB-EGF (Fig 4A). Furthermore, as demonstrated in Figure 4B, APF stimulated p38MAPK activation in vector-only (T24_{vector}) cells; in contrast, APF did not activate p38MAPK in T24 cells constitutively expressing sHB-EGF

(T24_{HB-EGFcs}). These data strongly suggest that HB-EGF suppresses APF bioactivity by regulating the p38MAPK signal transduction pathway.

To further test our hypothesis that the HB-EGF and APF-induced pathways are antagonistic, T24 cells were pretreated with recombinant HB-EGF for 15 min, followed by incubation in APF-containing medium. When cell proliferation was assessed by MTT cell growth assay, proliferation of control cells treated with APF alone for 7 d was decreased by about 40% compared to control. Cells pretreated with HB-EGF and then incubated in APF-containing medium in the absence of HB-EGF showed partial recovery of proliferation in comparison to cells cultured in APF-containing medium alone (Fig 5).

Discussion

The data presented herein demonstrate that HB-EGF is secreted following application of a repetitive mechanical force on bladder-derived epithelial cells, and they confirm that the growth inhibition caused by APF is abrogated by HB-EGF. Cyclic stretch-relaxation resulted in the secretion of HB-EGF into the extracellular space from T24 cells engineered to overexpress the cell surface-localized, precursor form of HB-EGF (proHB-EGF), and pretreatment of cells with recombinant HB-EGF prior to treatment with APF decreased the antiproliferative effect of APF in T24 bladder cells. Our data also indicate that (a) *in vitro* treatment of T24 cells with HB-EGF activates the Erk/MAPK pathway; (b) APF activates the p38MAPK pathway in these cells, and (c) both growth inhibition and activation of p38MAPK pathway by APF treatment are suppressed by pre-incubation of cells with HB-EGF. These data highlight an important biological role for the p38MAPK pathway for mediating APF activity in bladder epithelial cells, and are also consistent with previous papers showing inhibition of Erk/MAPK pathway signaling by p38MAPK pathway activation (or the inverse) [22, 23]. In addition, these data suggest the testable hypothesis that the balance between urinary APF and HB-EGF effects on bladder epithelial cells might influence the rate of turnover of cells in the bladder mucosa in IC patients.

Notably, some IC patients have seen an improvement in symptoms after a bladder distention is performed [24]. Distention, which stretches both bladder smooth muscle and epithelium *in vivo*, has also been shown to result in increased HB-EGF levels in urine and a reduction of urine APF activity [18]. In previous studies, exogenous treatment of cells with recombinant HB-EGF effectively reversed the effects of APF activity on bladder epithelial cell proliferation [16], consistent with our present observations. Differences in the experimental design between the former study (in which cells were simultaneously exposed to APF and HB-EGF for the duration of the experiment) and the current study (in which cells were exposed sequentially to APF or HB-EGF) may explain the slight difference between the two studies in the degree to which HB-EGF could reverse APF-induced inhibition of proliferation.

The role of the MAPK pathway (generally considered to consist of Erk/MAPK, p38MAPK, and JNK/SAPK signaling transduction pathways) in IC is currently undefined, although all three of these signaling systems appear to be important for epithelial, endothelial, and fibroblast cell proliferation. While Erk/MAPK and JNK/SAPK pathways are generally

considered to mediate pro-proliferation signals, the p38MAPK pathway is usually associated with inhibition of cell proliferation, and the two pathways often function in opposition to one another to control proliferation [22, 23]. For example, during the initial phases of wound healing, corneal epithelial cells migrate but do not proliferate, coincident with p38MAPK activation and Erk/MAPK inhibition [25]. Similarly, suppression of hepatocellular carcinoma cell growth by certain agents is associated with p38MAPK activation and Erk/MAPK inhibition [26]. The Erk/MAPK pathway is activated by HB-EGF through binding to the EGFR tyrosine kinase, and triggers diverse biological responses in bladder cancer cells [17]. Studies using a MAPK-dependent promoter demonstrated that Erk/MAPK activation is also associated with proliferation of normal uroepithelial cells [27]. Enhanced p38MAPK expression levels in the urothelium were also evident in Chernobyl cystitis, a urological disease caused by chronic exposure to low-dose Cs radiation [28], suggesting that alterations in the p38MAPK cascade are early molecular events in the pathogenesis of bladder epithelial cells. In addition, JNK/SAPK expression may also be important for control of uroepithelial cell proliferation, with JNK1 levels decreased in both IC (compared with control) and APF-treated (compared with mock APF-treated) normal bladder epithelial cells [8].

In conclusion, we used an *in vitro* model to show that mechanical distention induces secretion of soluble HB-EGF in bladder epithelial cells expressing the precursor protein, proHB-EGF. We also identified a role for the p38MAPK pathway as an important mediator of the antiproliferative effects by APF in bladder epithelial cells, and further showed that HB-EGF can decrease the effects of APF on cell proliferation via stimulation of Erk/MAPK signaling. Our findings are consistent with the hypothesis that bladder distention (or stretching) *in vivo* is able to increase the urinary levels of endogenous HB-EGF, which may in turn suppress inhibition of bladder epithelial cell proliferation by APF. This hypothesis is also supported by a previous report showing that APF activity may be involved in the regulation of HB-EGF production by bladder epithelial cells [16]. These findings suggest a new mechanism for altering APF-stimulated pathologic effects on the bladder wall and provide new insight into possible therapeutic strategies for symptom relief in patients with IC.

Acknowledgments

We thank Dr. Rosalyn Adam for providing advice and for critical reading of the manuscript. This research was supported by NIH grants: R37 DK47556, R01 DK 57691, P50 DK65298 (to M.R.F.) and R01 DK52596 (to S.K.K.), and a grant from the Fishbein Family IC Research Foundation / Interstitial Cystitis Association (ICA) (to J.K.). J.K. is an American Urological Association Foundation Research Scholar.

References

1. Bogart LM, Berry SH, Clemens JQ. Symptoms of interstitial cystitis, painful bladder syndrome and similar diseases in women: a systematic review. *J Urol.* 2007 Feb.177:450–6. [PubMed: 17222607]
2. Tomaszewski JE, Landis JR, Russack V, et al. Biopsy features are associated with primary symptoms in interstitial cystitis: results from the interstitial cystitis database study. *Urology.* 2001 Jun.57:67–81. [PubMed: 11378053]
3. Keay S, Zhang CO, Kagen DI, et al. Concentrations of specific epithelial growth factors in the urine of interstitial cystitis patients and controls. *J Urol.* 1997 Nov.158:1983–8. [PubMed: 9334654]

4. Keay S, Zhang CO, Hise MK, et al. A diagnostic in vitro urine assay for interstitial cystitis. *Urology*. 1998 Dec.52:974–8. [PubMed: 9836539]
5. Keay S, Zhang CO, Marvel R, Chai T. Antiproliferative factor, heparin-binding epidermal growth factor-like growth factor, and epidermal growth factor: sensitive and specific urine markers for interstitial cystitis. *Urology*. 2001 Jun.57:104. [PubMed: 11378066]
6. Keay SK, Szekely Z, Conrads TP, et al. An antiproliferative factor from interstitial cystitis patients is a frizzled 8 protein-related sialoglycopeptide. *Proc Natl Acad Sci U S A*. 2004 Aug 10.101:11803–8. [PubMed: 15282374]
7. Rashid HH, Reeder JE, O'Connell MJ, Zhang CO, Messing EM, Keay SK. Interstitial cystitis antiproliferative factor (APF) as a cell-cycle modulator. *BMC Urol*. 2004 Apr 6.4:3. [PubMed: 15068487]
8. Keay S, Seillier-Moiseiwitsch F, Zhang CO, Chai TC, Zhang J. Changes in human bladder epithelial cell gene expression associated with interstitial cystitis or antiproliferative factor treatment. *Physiol Genomics*. 2003 Jul 7.14:107–15. [PubMed: 12847144]
9. Zhang CO, Wang JY, Koch KR, Keay S. Regulation of tight junction proteins and bladder epithelial paracellular permeability by an antiproliferative factor from patients with interstitial cystitis. *J Urol*. 2005 Dec.174:2382–7. [PubMed: 16280852]
10. Kim J, Keay SK, Dimitrakov JD, Freeman MR. p53 mediates interstitial cystitis antiproliferative factor (APF)-induced growth inhibition of human urothelial cells. *FEBS Lett*. 2007 Aug 7.581:3795–9. [PubMed: 17628545]
11. Conrads TP, Tocci GM, Hood BL, et al. CKAP4/p63 is a receptor for the frizzled-8 protein-related antiproliferative factor from interstitial cystitis patients. *J Biol Chem*. 2006 Dec 8.281:37836–43. [PubMed: 17030514]
12. Keay S, Zhang CO, Shoenfelt JL, Chai TC. Decreased in vitro proliferation of bladder epithelial cells from patients with interstitial cystitis. *Urology*. 2003 Jun.61:1278–84. [PubMed: 12809929]
13. Miyamoto S, Yagi H, Yotsumoto F, Kawarabayashi T, Mekada E. Heparin-binding epidermal growth factor-like growth factor as a novel targeting molecule for cancer therapy. *Cancer Sci*. 2006 May.97:341–7. [PubMed: 16630129]
14. Raab G, Klagsbrun M. Heparin-binding EGF-like growth factor. *Biochim Biophys Acta*. 1997 Dec 9.1333:F179–99. [PubMed: 9426203]
15. Freeman MR, Yoo JJ, Raab G, et al. Heparin-binding EGF-like growth factor is an autocrine growth factor for human urothelial cells and is synthesized by epithelial and smooth muscle cells in the human bladder. *J Clin Invest*. 1997 Mar 1.99:1028–36. [PubMed: 9062361]
16. Keay S, Kleinberg M, Zhang CO, Hise MK, Warren JW. Bladder epithelial cells from patients with interstitial cystitis produce an inhibitor of heparin-binding epidermal growth factor-like growth factor production. *J Urol*. 2000 Dec.164:2112–8. [PubMed: 11061938]
17. Kim J, Adam RM, Freeman MR. Trafficking of nuclear heparin-binding epidermal growth factor-like growth factor into an epidermal growth factor receptor-dependent autocrine loop in response to oxidative stress. *Cancer Res*. 2005 Sep 15.65:8242–9. [PubMed: 16166300]
18. Chai TC, Zhang CO, Shoenfelt JL, Johnson HW Jr, Warren JW, Keay S. Bladder stretch alters urinary heparin-binding epidermal growth factor and antiproliferative factor in patients with interstitial cystitis. *J Urol*. 2000 May.163:1440–4. [PubMed: 10751853]
19. Sun Y, Chai TC. Augmented extracellular ATP signaling in bladder urothelial cells from patients with interstitial cystitis. *Am J Physiol Cell Physiol*. 2006 Jan.290:C27–34. [PubMed: 16107499]
20. Park JM, Borer JG, Freeman MR, Peters CA. Stretch activates heparin-binding EGF-like growth factor expression in bladder smooth muscle cells. *Am J Physiol*. 1998 Nov.275:C1247–54. [PubMed: 9814973]
21. Park JM, Adam RM, Peters CA, et al. AP-1 mediates stretch-induced expression of HB-EGF in bladder smooth muscle cells. *Am J Physiol*. 1999 Aug.277:C294–301. [PubMed: 10444406]
22. Ranganathan AC, Adam AP, Aguirre-Ghiso JA. Opposing roles of mitogenic and stress signaling pathways in the induction of cancer dormancy. *Cell Cycle*. 2006 Aug.5:1799–807. [PubMed: 16929185]

23. Kim J, Adam RM, Freeman MR. Activation of the Erk mitogen-activated protein kinase pathway stimulates neuroendocrine differentiation in LNCaP cells independently of cell cycle withdrawal and STAT3 phosphorylation. *Cancer Res.* 2002 Mar 1.62:1549–54. [PubMed: 11888934]
24. Ottem DP, Teichman JM. What is the value of cystoscopy with hydrodistension for interstitial cystitis? *Urology.* 2005 Sep.66:494–9. [PubMed: 16140064]
25. Saika S. TGF-beta signal transduction in corneal wound healing as a therapeutic target. *Cornea.* 2004 Nov.23:S25–30. [PubMed: 15448476]
26. Sutter AP, Maaser K, Hopfner M, Huether A, Schuppan D, Scherubl H. Cell cycle arrest and apoptosis induction in hepatocellular carcinoma cells by HMG-CoA reductase inhibitors. Synergistic antiproliferative action with ligands of the peripheral benzodiazepine receptor. *J Hepatol.* 2005 Nov.43:808–16. [PubMed: 16083991]
27. Swiatkowski S, Seifert HH, Steinhoff C, et al. Activities of MAP-kinase pathways in normal uroepithelial cells and urothelial carcinoma cell lines. *Exp Cell Res.* 2003 Jan 1.282:48–57. [PubMed: 12490193]
28. Romanenko A, Morimura K, Wanibuchi H, et al. Urinary bladder lesions induced by persistent chronic low-dose ionizing radiation. *Cancer Sci.* 2003 Apr.94:328–33. [PubMed: 12824899]

Figure 1. Bladder cell stretch results in secretion of soluble HB-EGF (sHB-EGF) into the medium

(A) T24 bladder cells were transiently transfected with an expression plasmid containing proHB-EGF (T24_{proHB-EGF}) or vector only (T24_{vector}), and were subsequently seeded onto stretch plates. 48 h after transfection cells were cyclically stretched (20% elongation, 0.5Hz) for 1 h. Medium from cells under each set of conditions was collected, and a pull-down assay using heparin-sepharose column was performed to measure HB-EGF levels under -/+ stretch conditions. Secretion of sHB-EGF from T24_{proHB-EGF} or T24_{vector} cells was confirmed by immunoblot using anti-HB-EGF Ab (CM, conditioned medium).

Figure 2. Erk/MAPK activation by recombinant human HB-EGF is inhibited by APF

(A) T24 bladder cancer cells were serum starved for 16 h, followed by exposure to 100 ng/ml HB-EGF for the times indicated. Western blot analysis was performed using anti-Erk/MAPK and anti-phospho-Erk/MAPK Abs. (B) After serum starvation, T24 cells were stimulated with various doses of HB-EGF (0, 10, 10^2 , and 10^3 ng/ml) for 15 min. Cell lysates were prepared and applied to western blotting. (C) T24 cells were treated with 10 ng/ml APF for 5 min, and then incubated with medium containing 100 ng/ml recombinant HB-EGF for another 15 min. Western blot analysis was performed to determine Erk/MAPK activation status.

Figure 3. Abrogation of APF-induced growth arrest by p38MAPK inhibition

(A) T24 cells were stimulated by treatment with HPLC-purified APF. At various time points, whole cell lysates were prepared, and Western blot analysis was performed. Activation of p38MAPK was determined using anti-phospho p38MAPK and anti-p38MAPK Abs. (B) To determine the involvement of p38MAPK, a specific pharmacological inhibitor of p38MAPK, SB203580, was added 1 h before APF treatment. Medium was changed every 2 d and APF was added to the new medium. Kinase activity of p38MAPK was analyzed by measuring ATF-2 phosphorylation levels after IP with phospho-p38MAPK antibody. Cell proliferation was measured at 7 d by MTT analysis (* and **, $p < 0.05$).

Figure 4. HB-EGF antagonizes APF-induced p38MAPK activation

(A) T24 cells were serum-starved and stimulated with HB-EGF for 15min. After removing of medium, cells were treated with 10 ng/ml APF time-dependently. Western blot analysis was done to determine phosphorylation status of p38MAPK at the indicated time points. The protein expression level of p38MAPK was determined by anti-p38MAPK Ab. (B) To observe the ability of HB-EGF to suppress APF-induced signaling, T24 cells were transiently transfected with expression constructs containing a constitutively secreted form of HB-EGF (HB-EGF_{CS}) or vector only. After serum starvation for 16 h, cells were treated with APF in the absence of serum. APF increased p38MAPK activation rapidly and transiently. However, T24_{HB-EGF_{CS}} cells did not show this activation following APF treatment.

Figure 5. Recombinant HB-EGF antagonizes APF-induced growth arrest

(A) T24 cells were serum starved, pretreated with 100 ng/ml HB-EGF for 15 min, after which they were incubated in medium containing 10 ng/ml APF (without HB-EGF) for 7 d. Medium was changed every 2 d and APF replenished. Cell viability was analyzed by MTT assay as described in Materials and Methods (* and **, $p < 0.05$).