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

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Abstract

The epidermal growth factor receptor (HER1) has been implicated in regenerative growth and proliferative diseases of the human bladder epithelium (urothelium), however a cognate HER1 ligand that can act as a growth factor for normal human urothelial cells (HUC) has not been identified. Here we show that heparin-binding EGF-like growth factor (HB-EGF), an activating HER1 ligand, is an autocrine regulator of HUC growth. This conclusion is based on demonstration of HB-EGF synthesis and secretion by primary culture HUC, identification of HER1 as an activatable HB-EGF receptor on HUC surfaces, stimulation of HUC clonal growth by HB-EGF, inhibition of HB-EGF–stimulated growth by heparin and of log-phase growth by CRM 197, a specific inhibitor of HB-EGF/HER1 interaction, and identification of human urothelium as a site of HB-EGF precursor (proHB-EGF) synthesis in vivo. ProHB-EGF expression was also detected in the vascular and detrusor smooth muscle of the human bladder. These data suggest a physiologic role for HB-EGF in the regulation of urothelial proliferation and regeneration subsequent to mucosal injury. Expression of proHB-EGF is also a feature of differentiated vascular and detrusor smooth muscle in the bladder. Because proHB-EGF is known to be the high affinity diphtheria toxin (DT) receptor in human cells, synthesis of the HB-EGF precursor by human urothelium also suggests the possibility of using the DT-binding sites of proHB-EGF as an in vivo target for the intraluminal treatment of urothelial diseases. (*J. Clin. Invest.* 1997. 99:1028–1036.) Key words: cell proliferation • urothelium • urinary tract • EGF receptor • ErbB • HER1

Introduction

The mammalian urinary collecting system, including the renal pelvis, ureters, bladder, and urethra is lined by a multilayered transitional epithelium, commonly referred to as urothelium. Urothelial cell turnover rates in situ under normal conditions are extremely slow (1), but elevated rates of cell proliferation in urinary tract mucosa are associated with premalignant and malignant conditions in humans and in experimental models. Mechanisms of urothelial cell cycling and tumor growth are poorly understood, although a variety of studies have shown that urine contains bioactive substances that may play important roles in proliferative urothelial abnormalities (2–6). Urine promotes hyperplasia of normal urothelium (7) and tumor growth in animals treated with carcinogens (8, 9). This growth- and tumor-enhancing activity has been attributed, at least in part, to several polypeptide mitogens, including transferrin and EGF-like molecules (4, 10, 11). Urinary EGF, produced by the distal segments of the kidney nephron, stimulates clonal growth (12) and ornithine decarboxylase activity (4) of transitional cell carcinoma (TCC) cell lines in vitro. The urothelium has also been reported to synthesize EGF (13, 14) and basal cells of the bladder mucosa express high levels of the EGF receptor (HER1)[1] (15). A high molecular weight, glycosylated form of proEGF with a moderate affinity for heparin was recently isolated from human urine (16), consistent with earlier studies predicting the presence of EGF-like growth factors other than the mature form of EGF in the bladder reservoir (4).

Immunohistochemical evidence suggests that HER1 is functionally involved in proliferative diseases of the urothelium in humans. HER1 expression has been reported by a number of groups to be an informative prognostic indicator for TCC (17–20). Increased HER1 expression has also been linked spatially to local increases in cell proliferation (21), suggesting a role for one or more HER1 ligands in aberrant urothelial cell growth. However, several reports have shown that EGF does not promote the growth of normal human urothelial cells (HUC) in serum-free medium, despite the presence of high-affinity EGF binding sites in these cells (22–24). In contrast, human TCC cell lines have been shown to proliferate in response to EGF (22, 23). These observations have led to the suggestion that, possibly as a result of an adaptive mechanism present to compensate for high urinary EGF concentrations, the normal urothelium might be refractory to EGF. If EGF is not a physiologic growth factor for normal urothelium, the presence of HER1 in nonpathologic urothe-

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1. *Abbreviations used in this paper:* bFGF, basic fibroblast growth factor (FGF-2); BPE, bovine pituitary extract; DT, diphtheria toxin; HB-EGF, heparin-binding EGF-like growth factor; HER1 and EGF-R, EGF receptor; HER2, HER3, HER4, EGF receptor-related tyrosine kinase receptors; HUC, human urothelial cells; proHB-EGF, HB-EGF precursor; TCC, transitional cell carcinoma; TPA, 12-*o*-tetradecanoyl-phorbol 13-acetate.

lium in vitro and in vivo suggests that activating HER1 ligands other than EGF might be involved in normal urothelial cell function or growth regulation.

Heparin-binding EGF-like growth factor (HB-EGF) is a 76–86 amino acid glycosylated protein that was originally cloned from macrophage-like U937 cells (25–27). HB-EGF is an activating ligand for HER1 and a potent mitogen for smooth muscle cells and fibroblasts. Soluble HB-EGF appears to be an inflammatory mediator because expression of the HB-EGF gene is rapidly activated by inflammatory cytokines such as TNFα, IL-1β, PDGF, and basic fibroblast growth factor (bFGF) (28, 29) and because HB-EGF is a secreted product of monocyte-derived macrophages and $CD4^+$ T lymphocytes (25, 30). HB-EGF is also expressed by macrophages and smooth muscle cells residing in human atherosclerotic plaques (31) and by eosinophils infiltrating lung tissue subsequent to hypoxia (32). The mature form of HB-EGF is proteolytically processed from a membrane-anchored HB-EGF precursor (proHB-EGF) that was identified independently as the high-affinity receptor for diphtheria toxin (DT) in human cells (33, 34). Studies with cultured cell lines have shown that HB-EGF has the potential to function as an autocrine or paracrine factor in its processed form and as a juxtacrine factor and cell-surface DT receptor in its cell-associated proform (35–38).

HB-EGF was originally identified as a secreted factor with a high affinity for immobilized heparin (25). A physiologic role for heparin sulfate proteoglycans (HSPGs) in the functional activity of soluble and cell-surface forms of HB-EGF has now been demonstrated in several studies. Cell-associated HSPGs facilitate HB-EGF–stimulated migration of vascular smooth muscle cells (26) and appear to be required for HB-EGF–mediated cell growth and for DT-binding to membrane-anchored proHB-EGF and subsequent cell intoxication (39, 40). The superficial urothelium is decorated with a thin layer of complex polysaccharides that has been proposed to act as a permeability barrier important in protecting the mucosal and submucosal tissues from cytotoxic effects of substances in urine (41, 42). The disruption of this glycosaminoglycan layer and the subsequent activation of a cytokine network have been suggested to be involved in the pathological consequences of several inflammatory bladder diseases, including interstitial cystitis and mycobacterial infection (41, 43, 44). Because HB-EGF has been identified as an inflammatory cytokine and also because of its affinity for heparin-like glycosaminoglycans, we decided to examine the possibility that HB-EGF might play a role in normal bladder function. This hypothesis is supported by the recent identification of HB-EGF as an autocrine growth factor for human keratinocytes (36), as normal HUC proliferate avidly in media optimized for keratinocyte growth (24).

In this study we demonstrate that HB-EGF is an autocrine growth factor for HUC in primary culture, and we have identified in vivo sites of HB-EGF synthesis in the human bladder by immunohistochemical localization of proHB-EGF.

Methods

Urothelial cell cultures. Normal human urothelial cells were obtained from discarded human surgical material obtained from donors between the ages of 2 and 10 yr as described previously (24). Cells were cultured and passaged in K-SFM (GIBCO BRL, Grand Island, NY) serum-free medium supplemented with 50 mg/ml bovine pituitary extract (BPE). HUC were typically passaged at a 1/5 dilution of cells at 90% confluency in the absence of serum and were used for experiments between the second and sixth passages.

Human tissues and immunohistochemistry. Histologic specimens fixed in 10% formalin and embedded in paraffin were from cystectomy tissue obtained from adult cancer patients. Sections of normal bladder biopsies were chosen for immunostaining and previewed by a genitourinary pathologist (A.A. Renshaw). Immunostaining for expression of proHB-EGF was carried out using the avidin-biotin detection system as previously described (44). The 3100 antibody (46), which specifically recognizes the carboxyl terminus of proHB-EGF, was used at a 1/500 dilution. Preimmune serum was used as a negative control.

Northern blot hybridization. Total RNAs from cultured cells were isolated using a rapid guanidinium thiocyanate procedure (47). RNAs were electrophoresed in 2 M formaldehyde/1.2% agarose gels and blotted onto nylon membranes by capillary transfer. Human HB-EGF cDNA was labeled to high specific activity by the random oligonucleotide method using the Multiprime kit (Amersham Corp., Arlington Heights, IL). Hybridization conditions were as described (48). Blots were washed for extended times at 65°C in 0.5× SSC and exposed to Kodak XAR film for autoradiography (Eastman Kodak Co., Rochester, NY).

Diphtheria toxin sensitivity. Subconfluent HUC plated in 24-well tissue culture plates were incubated with DT at the indicated concentrations for 90 min at 37°C. DT-containing medium was removed and replaced with 1 μCi [^{3}H]leucine and incubated in 200 μl leucine-free DMEM (GIBCO BRL) for 3 h. Cells were fixed in methanol and protein precipitated with 5% trichloroacetic acid. After rinsing with distilled water, acid-precipitable material was solubilized in 0.3 M NaOH/0.1% (vol/vol) Triton X-100. Radioactivity incorporated into the precipitate was measured by scintillation counting. TPA-treated cells were handled identically to control cells except for treatment with 324 nM TPA for 30 min before the assay (all references to TPA in the text refer to a concentration of 324 nM).

Western blot analysis. Urothelial cell lysates enriched in heparin-binding proteins were prepared from whole cell lysates by heparin-Sepharose chromatography as described (27). Heparin-binding proteins which eluted from the column with 2 M NaCl were dialyzed against water, lyophilized, and subjected to SDS-polyacrylamide gel electrophoresis. Protein was then electrophoretically blotted onto Immobilon P membranes (Millipore Corp., Bedford, MA). Blots were probed with an anti–HB-EGF polyclonal antibody (197) raised against recombinant 77-amino acid human HB-EGF (a gift of Judy Abraham; Scios Nova, Mountain View, CA). Detection of antibody–antigen complexes was carried out by chemiluminescence using the ECL system (Amersham Corp.).

Heparin affinity fast protein liquid chromatography (FPLC). Medium was conditioned for the indicated times in the presence or absence of TPA. ~ 200 ml of conditioned medium was applied to a 5-ml heparin-Sepharose column (Pharmacia LKB Biotechnology, Uppsala, Sweden) and heparin-binding proteins were batch-eluted with 2 M NaCl. The column eluate was diluted 1:10 and applied to a TSK-heparin 5PW FPLC column (8 × 75 mm, Tosohaas). Bound protein was eluted with a 40-ml linear gradient of 0.2–2 M NaCl in 0.01 M Tris-HCl, pH 7.4, at a flow rate of 1 ml/min. 1-ml fractions were collected and 5 μl of each fraction was tested for mitogenic activity using HER1-transfected (EGF-R+), parental (EGF-R−), Swiss 3T3 NR6 cells (49), or Balb/c3T3 cells. NR6 cells were kindly provided by Dr. Gordon Gill (University of California at San Diego, CA).

Affinity cross-linking and growth factor binding. ^{125}I-EGF was purchased from New England Nuclear (Du Pont-NEN, Boston, MA) and was used at a specific activity of ~ 400,000 cpm/ng. The iodination of HB-EGF was carried out using the IODO-GEN kit (Pierce Chemical Co., Rockland, IL) according to the manufacturer's instructions and as previously described (50). Briefly, 2 μg of HB-EGF were iodinated with 0.2 μCi of ^{125}I-sodium. ^{125}I–HB-EGF was purified by heparin affinity chromatography and gelatin was added to a final concentration of 2 mg/ml. The specific activity of ^{125}I–HB-EGF was 40,000

cpm/ng. Binding and cross-linking experiments using ^{125}I–HB-EGF and ^{125}I-EGF were performed as previously described for ^{125}I-VEGF (50). Briefly, for binding experiments cells were grown in 48-well dishes and the binding reactions were done at 1.5 ml/dish. ^{125}I–HB-EGF and ^{125}I-EGF were added to the binding reactions at 5–10 ng/ml and the nonlabeled competitors were added along with the labeled proteins. ^{125}I–HB-EGF and ^{125}I-EGF binding were quantitated by measuring cell-associated radioactivity in a gamma counter. The counts represent the average of three wells. After cross-linking, cells were lysed and samples were boiled for 3 min. Proteins were resolved by 6% SDS/PAGE and the gels were dried and exposed to x-ray film.

Tyrosine phosphorylation of ErbB receptors. HUC were grown to confluence in 10-cm dishes, in KSF-M medium supplemented with EGF and BPE. 24 h before the experiment, cells were starved by removal of growth medium and addition of fresh medium lacking the supplements. One plate of cells was used per growth factor tested, and a control was also included. Medium was aspirated from plates of cells and 5 ml fresh medium without supplements was added; to this was added EGF, HB-EGF at a concentration of 100 ng/ml or no growth factor (control). Cells were incubated on ice for 10 min, followed by incubation at 37°C for 5 min. At the end of incubation, medium was aspirated and 5–10 ml PBS was added to wash cells and to remove all traces of growth factor. 1 ml lysis buffer was added, cell layers were scraped, pipetted into microfuge tubes, and centrifuged at 10,000 rpm, for 10 min at 4°C to pellet debris. Cell lysates were precleared by the addition of 100 μl per lysate of G50 sephadex and incubation for 1 h at 4°C. Lysates were incubated in the presence of the appropriate anti-ErbB antibody (HER1, HER2, HER3, or HER4; Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C. Immune complexes were precipitated by addition of 40 μl/sample protein G and incubation for 1 h at 4°C on a rotary mixer. Tertiary complexes were pelleted by centrifugation at 13,000 rpm, for 5 min, at 4°C. Pellets were washed in lysis buffer and the final pellet resuspended in 20 μl SDS-PAGE sample buffer. Samples were electrophoresed on a 6% SDS-PAGE gel, transferred to Immobilon P, and probed successively with antiphosphotyrosine antibody (4G10) and anti–mouse HRP-conjugated antibody. Protein bands were visualized using the ECL system.

Log-phase cell growth assays. HUC were plated at 300 cells/well in 96-well dishes. Cultures were maintained for 7 d, with changes of medium including the indicated additives (CRM 197, a diphtheria toxin mutant containing a point mutation that abolishes the enzymatic function of the toxin, and heparin), at the concentrations shown, every other day. At the end of the growth period, quantitation of relative cell number was performed using the MTT method essentially as described (51). Statistical analysis for the MTT assay and the clonal growth assay (below) was performed using the Student's unpaired *t* test. CRM 197 (Bactoxin) was purchased from USA Burma Products (Coral Gables, FL).

Clonal growth assays. Cells were plated in 24-well tissue culture plates at a density of 50 cells/cm^2 in insulin-free, K-SFM medium supplemented with 50 μg/ml BPE. On the day after plating, the medium was replaced with K-SFM without BPE containing the indicated concentrations of recombinant HB-EGF (R & D Systems, Minneapolis, MN). BPE-supplemented K-SFM served as a positive control and K-SFM medium without BPE was the negative control. Medium was subsequently replaced with identical medium at 2-d intervals. At 10 d after plating, cells were fixed with 3.7% formaldehyde and stained with 0.1% crystal violet. Absorbance (550 nm) was measured with a Multiscan microplate reader (Titertek, Elfab Oy, Finland).

Results

We have previously reported that normal HUC obtained from human bladder tissue can be expanded extensively under serum-free conditions in medium optimized for keratinocyte growth (24). EGF, which is used as a supplement for keratinocyte cultures, can be omitted from this medium with no detectable change in growth kinetics (24). To determine if HUC cultured under these conditions synthesize HB-EGF, Northern blot analysis was performed. HB-EGF mRNA was detected in log-phase HUC using total RNA blots (Fig. 1). Stimulation with the phorbol ester 12-*O*-tetradecanoyl-phorbol 13-acetate (TPA; 324 nM) resulted in a dramatic increase in HB-EGF mRNA levels (greater than 10-fold), with a peak occurring 4 h after treatment. Elevated HB-EGF mRNA was still apparent 24 h after challenge with TPA. These results are consistent with previous reports of rapid elevation of HB-EGF mRNA by phorbol ester in vascular smooth muscle cells which synthesize HB-EGF (52).

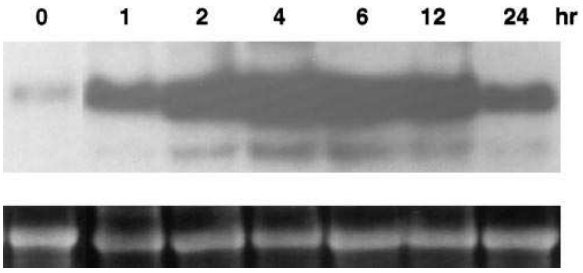

Figure 1. HB-EGF mRNA expression in HUC in response to treatment with the phorbol ester TPA. (*Bottom*) The 28S rRNA shows equivalence of loading.

To determine if HUC synthesize and export HB-EGF, HUC-conditioned medium was fractionated by heparin affinity chromatography and fractions were tested for the presence of active mitogen. Medium was conditioned under two experimental conditions: (*a*) over 4 d during log-phase growth, and (*b*) over 4 h after treatment with TPA. Heparin-binding proteins eluted from a heparin-Sepharose column with 2.0 M NaCl buffer were subsequently eluted from a heparin affinity FPLC column with a continuous gradient of NaCl (0.2–2.0 M NaCl). Fractions were tested for growth factor activity using two types of Swiss 3T3 NR6 sublines as target cells: cells that express transfected HER1 and which respond to EGF-like mitogens (EGF-R+ cells) and parental NR6 cells that do not express HER and which do not respond to EGF-like factors (EGF-R− cells). A peak of growth factor activity eluting from the heparin column near 0.8–1.1 M NaCl (peak I) was detected when EGF-R+ cells were used for mitogenic assays (Fig. 2 *A*). Medium conditioned briefly (4 h) by TPA-treated cells demonstrated an enhanced mitogenic peak across approximately the same range. The heparin-binding profile of the peak I mitogen(s) is consistent with the presence of HB-EGF, which elutes from heparin columns within this range of NaCl concentration (26). EGF-R cells were not responsive to this activity (Fig. 2 *B*), indicating that peak I contained a factor(s) that induced DNA synthesis by HER1 activation. A second peak of mitogenic activity (peak II), which was present in medium conditioned over 4 d and over 4 h after TPA treatment, was detectable using both target cells, indicating that this activity is not an EGF-R ligand. The high heparin affinity of peak II activity is consistent with the presence of bFGF (FGF-2) in HUC conditioned medium (bFGF is the only known heparin-binding 3T3 mitogen with such a high affinity for immobilized heparin that concentrations of NaCl greater than 1.5 M are re-

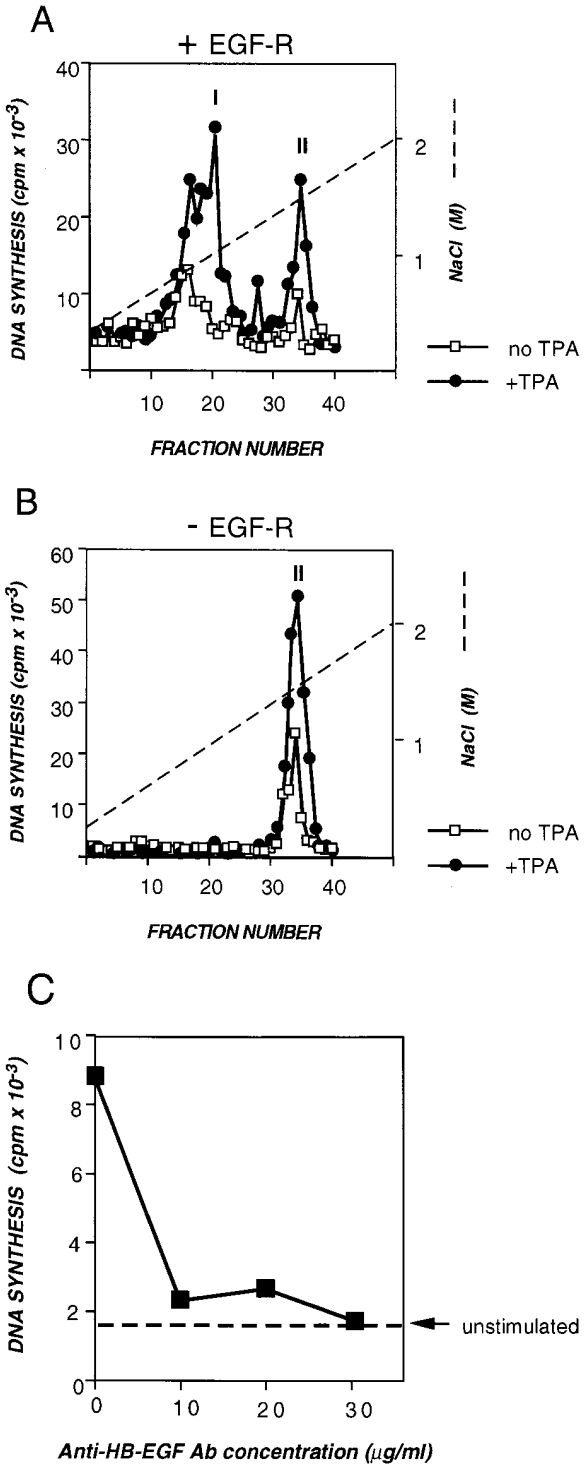

Figure 2. (*A* and *B*) Elution profiles from heparin-affinity columns showing Swiss 3T3 (NR6) cell mitogens secreted by TPA-stimulated and TPA-nonstimulated HUC. NR6 cells expressing HER1 (EGF-R+ cells, *A*) and parent NR6 cells (EGFR−, *B*) were used for this experiment. HUC conditioned medium was eluted from TSK-heparin FPLC affinity columna with a continuous gradient of NaCl and fractions were subsequently tested for mitogenic activity. A heparin-binding mitogen capable of stimulating the EGF-R was identified (*peak I*) that was not evident in the same fractions when no EGF-R cells were used as target cells. Peak II activity was evident using both sublines, and is therefore not an EGF-R ligand. The high heparin affinity of the peak II mitogen suggests that it is bFGF/FGF-2.

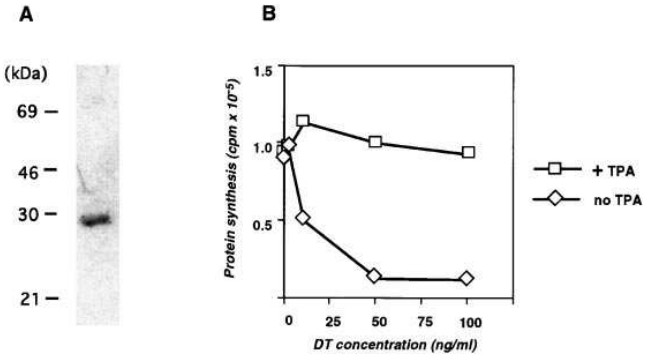

Figure 3. (*A*) Western blot of an HUC lysate probed with an antibody to human HB-EGF. The immunoreactive band migrates as a 29-kD protein, consistent with the major HB-EGF species in cell-associated material being present in the precursor form. Glycosylated forms of proHB-EGF have been shown previously to migrate in SDS-PAGE gels as 21–30 kD proteins (38). (*B*) Loss of DT sensitivity in HUC treated with TPA, as measured by protein synthesis assay. DT-treated cells were challenged with 324 nM TPA for 30 min before the assay. This result is consistent with rapid loss of cell-surface proHB-EGF in response to challenge with phorbol ester.

quired to displace it from heparin-affinity columns). In a parallel experiment, essentially all of the mitogenic activity in the 1 M NaCl fraction was inhibited in a dose-dependent manner by preincubation with a neutralizing anti–HB-EGF antibody (Fig. 2 *C*), indicating that HB-EGF is the predominant heparin-binding HER1 ligand secreted by phorbol ester-stimulated HUC. The relatively low level of HER1-stimulating activity seen in medium conditioned for a prolonged period by growing HUC, in comparison to medium conditioned for a short time by TPA-treated cells, suggests that HUC synthesize HB-EGF and retain it in a cell-associated form.

To confirm the observation of HB-EGF synthesis by HUC, heparin-binding proteins were prepared from HUC lysates by heparin-Sepharose chromatography and tested for expression of the cell-associated, precursor form of HB-EGF (proHB-EGF) by Western blot analysis. A ~ 29-kD protein was identified in Western blots with antisera raised against recombinant human HB-EGF (Fig. 3 *A*). Identification of proHB-EGF in cellular fractions suggests that HUC should be highly sensitive to DT because cell-surface proHB-EGF is the DT receptor in primate cells and mediates cellular entry of the toxin by endocytosis (33, 34). DT sensitivity was examined in log-phase HUC before and after treatment with TPA. Log-phase cultures were sensitive to DT in a dose-dependent manner, indicating the presence of functional proHB-EGF/DT receptors (Fig. 3 *B*). Treatment with TPA resulted in total loss of DT sensitivity, indicating the clearance of DT receptors from the cell surface. This provides evidence that TPA rapidly induces proteolytic processing of membrane-anchored proHB-EGF in HUC, consistent with published data on the effects of TPA on

(*C*) Mitogenic potency of 10 μl aliquots of the 1M NaCl fraction after heparin affinity FPLC fractionation of TPA-treated HUC conditioned medium. Column eluate was tested after incubation with the indicated concentrations of Ab 197, which specifically recognizes HB-EGF (30, 56). Target cells were serum-starved Balb/c3T3 fibroblasts.

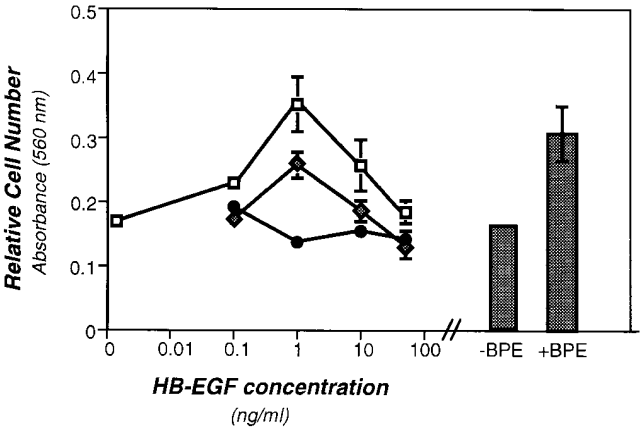

Figure 4. Effect of HB-EGF and heparin on clonal growth of HUC. Supplementation with 50 μg/ml BPE, which stimulates HUC growth, served as a positive control (*bars*, *right*). Open squares represent K-SFM medium plus the indicated concentrations of HB-EGF. Diamonds represent identical conditions to above with 10 μg/ml heparin. Closed circles represent identical conditions with 100 μg/ml heparin. Error = SEM. This result is representative of three independent experiments using HUC strains derived from different individuals.

proHB-EGF processing in MDA MB 231 breast cancer cells and kidney Vero cells (53, 38). Overall, these data indicate that under log phase conditions, secretion of mature HB-EGF appears not to occur at high rates in HUC and, instead, is retained in the pro- form at the cell surface (Figs. 2 and 3).

HUC cultured under serum-free conditions express high affinity binding sites for EGF (22, 23). Because HB-EGF stimulates cells by HER1 activation, our results suggest that HB-EGF may be an autocrine growth factor for HUC. To examine this possibility, recombinant HB-EGF was tested in clonal growth assays using HUC as target cells. Fig. 4 shows representative results from these experiments. At clonal density, HB-EGF induced a maximal growth response at 1 ng/ml, which was comparable to that seen in the presence of 50 μg/ml bovine pituitary extract (BPE), shown previously to stimulate HUC growth (23). In most cases (three independently derived strains), at higher concentrations HB-EGF either did not stimulate growth or did so to a lesser extent, suggesting that a biphasic response to HB-EGF may be typical for this cell type in serum-free conditions. However, one strain exhibited a reproducible, dose-dependent response to HB-EGF between the concentrations of 1 and 50 ng/ml (not shown); this was not seen with any other strain. The presence of heparin in the clonal growth assay inhibited the HB-EGF–induced growth response in a dose-dependent manner, suggesting a loss of growth factor activity by nonproductive binding interactions of HB-EGF with the large excess of soluble heparin.

To examine the role of HB-EGF in HUC growth further, log phase cultures were incubated with CRM 197, a noncytotoxic DT mutant that acts as an HB-EGF inhibitor by binding the soluble molecule in a similar fashion to native DT (34). The presence of 10 μg/ml CRM 197 for 7 d in log-phase cultures inhibited growth by 30% (Fig. 5 *A*) with no cytotoxic effect (not shown). Growth was also inhibited in a dose-dependent manner by heparin under identical conditions (Fig. 5 *B*). As exogenous HB-EGF was not added to the culture medium under these conditions, these results indicate that endogenous HB-EGF plays a role in growth stimulation.

To confirm the presence of cell-surface receptors for HB-EGF, chemical cross-linking of ^{125}I–HB-EGF and ^{125}I-EGF to cell surface proteins on HUC monolayers was carried out. A single cross-linked product of ~ 170 kD relative molecular mass was detected using both ligands (Fig. 6 *A*), suggesting that HER1 is the major high affinity HB-EGF receptor expressed by HUC. To confirm the identity of the receptor, HUC were challenged with EGF and HB-EGF, and the HER1, HER2, HER3, and HER4 tyrosine kinases were immunoprecipitated using monospecific antibodies. Immunoprecipitates were subjected to anti-phosphotyrosine Western blot analysis. Phosphorylation of HER1 in response to EGF and HB-EGF was detected in this experiment, while phosphorylation of HER3 and HER4 was not observed (Fig. 6 *B*). Weak stimulation of HER2 phosphorylation was detected (data not shown), most likely a result of transactivation of HER2 (which is not an HB-EGF receptor) by HER1 (54). HB-EGF has recently been identified as an activating ligand for at least one form of HER4 (55), however our results indicate that the major receptor activated by both HB-EGF and EGF in HUC is HER1.

To determine if HB-EGF and EGF binding properties in HUC are similar, competitive binding analysis with HB-EGF and EGF affinity probes was performed. Soluble HB-EGF and EGF were found to be essentially identical in their ability to

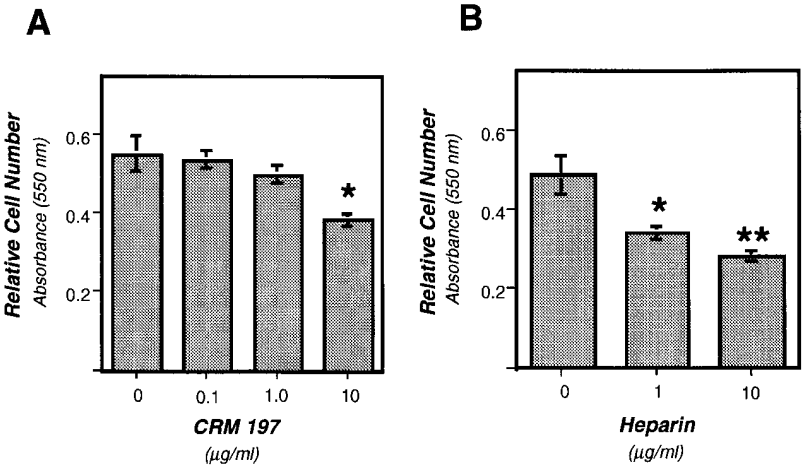

Figure 5. (*A*) Effect of CRM 197, a noncytotoxic mutant DT, which binds to HB-EGF and therefore inhibits HB-EGF–mediated mitogenesis. Cells were grown in log-phase in this experiment and cell density after 7 d was evaluated by MTT assay. Cells grown in the presence of 10 μg/ml CRM 197 showed an ~ 30% inhibition of cell growth ($^*P < 0.033$). (*B*) Inhibitory effect of heparin on log-phase growth of HUC. ($^*P < 0.022$; $^{**}P < 0.003$). Error for *A* and *B* is SEM.

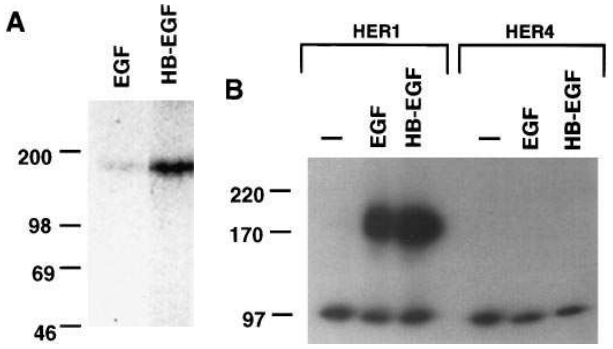

Figure 6. (*A*) Affinity cross-linking of ^{125}I-EGF and ^{125}I–HB-EGF to HUC monolayers. Both ligands recognize a ~ 170-kD receptor. (*B*) Detection of tyrosine phosphorylation of HER1 and HER4 in HUC in response to no growth factor (−), 100 ng/ml EGF or 100 ng/ml HB-EGF. Immunoprecipitation was with anti-HER1 and anti-HER4 Abs, detected by antiphosphotyrosine Western blot. MW markers in kD.

compete with ^{125}I-EGF for cell surface binding (Fig. 7 *A*). However, EGF only displaced ~ 35% of ^{125}HB-EGF binding (Fig. 7 *B*), suggesting the presence of a distinct class of binding sites for HB-EGF on HUC surfaces. Previous studies of HB-EGF binding to cell lines (40, 56) suggest that these alternative binding sites may be cell-surface HSPGs.

Normal human bladder tissue was examined for possible sites of HB-EGF synthesis in vivo by immunohistochemistry using anti–HB-EGF antisera raised against a synthetic peptide corresponding to 16 amino acids at the carboxyl terminus of human proHB-EGF (Ab 3100 [46]). Because the 3100 antisera recognizes the cytoplasmic domain of unprocessed proHB-EGF, immunostaining with this Ab identifies sites of HB-EGF synthesis. The proHB-EGF Ab decorated the suprabasal layers of the urothelium as well as the detrusor and vascular smooth muscle, but did not decorate inflammatory cell infiltrates or connective tissue elements in the submucosa (Fig. 8). While vascular and detrusor muscle cells were uniformly positive in all specimens (n = 4), urothelial staining ranged in intensity within the same specimens from strongly to weakly positive. These data suggest that HB-EGF expression may be dynamically regulated in the urothelial compartment. The variable expression of proHB-EGF could not be attributed to histologically evident local inflammation or injury in the specimens examined. In contrast, proHB-EGF appeared uniform in the two muscle compartments. These data indicate that the membrane-anchored form of HB-EGF is synthesized and accumulates in the urothelium and in two smooth muscle cell types that play distinct functional roles in the human bladder.

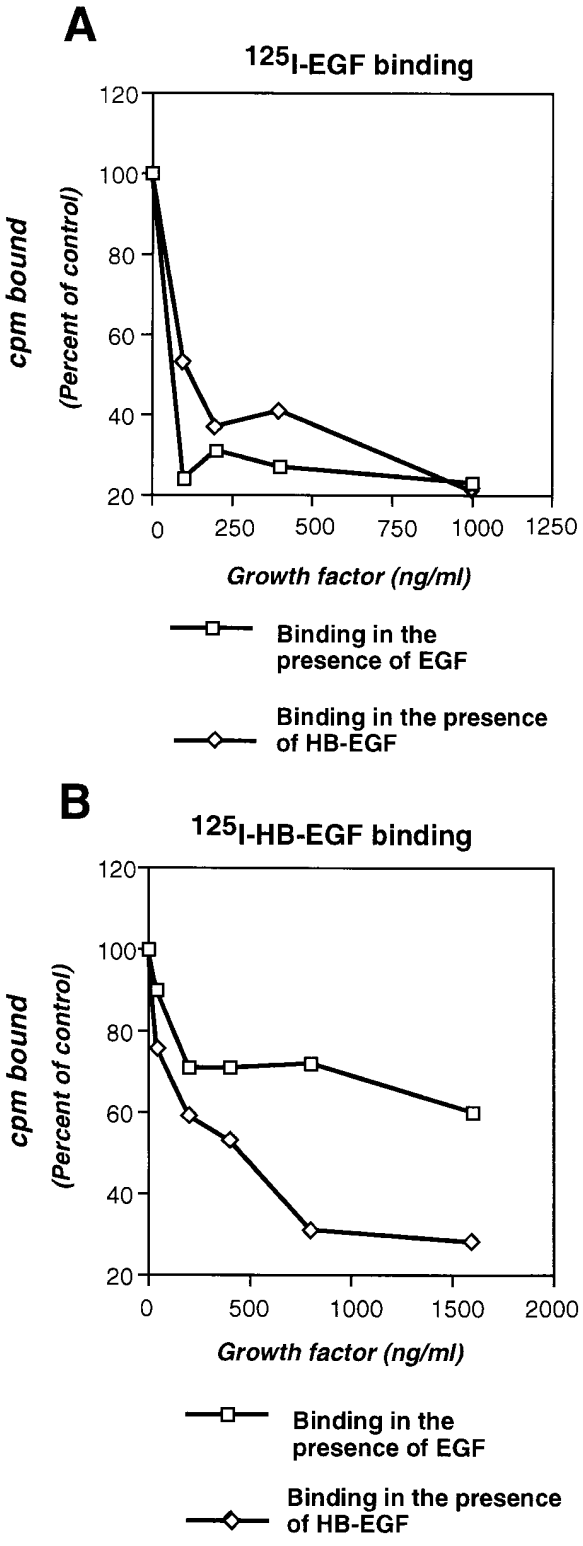

Figure 7. Binding of ^{125}I-EGF and ^{125}I–HB-EGF to HUC monolayers in the presence of excess unlabeled EGF and HB-EGF. (*A*) Displacement of ^{125}I-EGF was similar for EGF and HB-EGF, however (*B*) EGF only displaced ~ 35% of ^{125}I-HB-EGF binding.

Discussion

In this study, we have identified HB-EGF as an autocrine growth factor for normal HUC. This is the first demonstration of a functional role for a HER1 ligand in the normal human bladder. Our conclusion is based on: (*a*) demonstration of HB-EGF synthesis by primary culture HUC, (*b*) stimulation of HUC clonal growth by recombinant HB-EGF, (*c*) inhibition of log-phase HUC growth by CRM 197, a specific HB-EGF competitive antagonist, (*d*) identification of functional HB-EGF

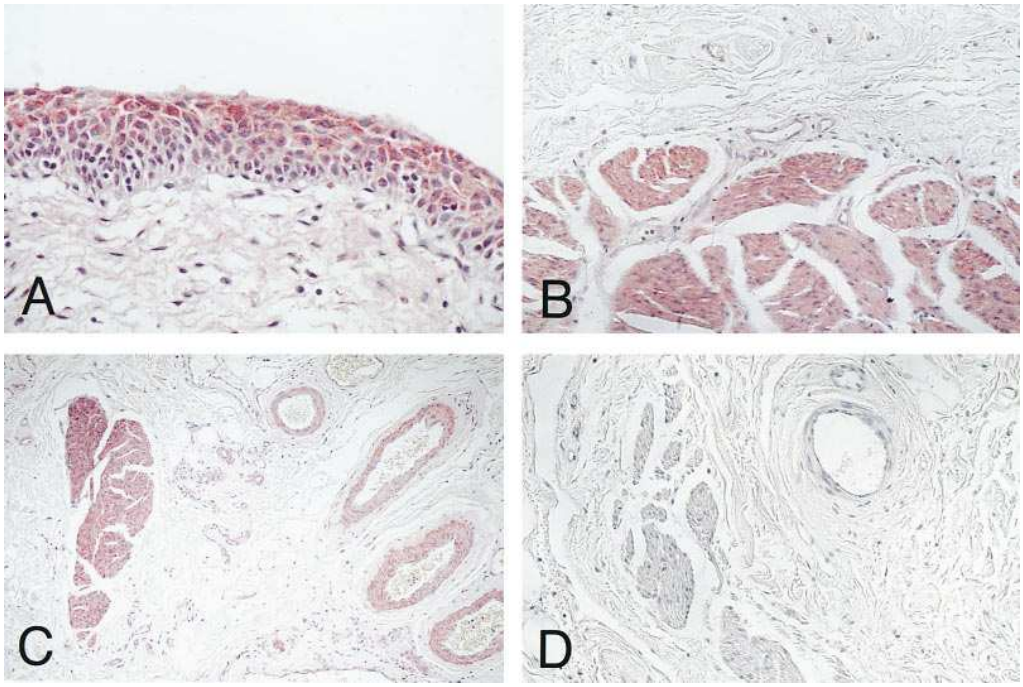

Figure 8. Immunolocalization of proHB-EGF in human bladder tissue. The affinity purified antisera recognizes the carboxy terminus of proHB-EGF. The sections were counterstained with hematoxylin (*blue*). Specific labeling (*red*) was found in many segments of the urothelium (*A*), and in detrusor (*B* and *C*) and vascular smooth muscle (*C*). (*D*) Negative control using preimmune sera in place of the primary antibody. In the urothelium, the suprabasal layers stained more intensely, suggesting an upregulation during urothelial cell differentiation.

receptors on HUC surfaces, and (*e*) localization of proHB-EGF to human urothelium in situ. Interestingly, human bladder detrusor muscle (muscularis propria) and vascular smooth muscle cells in vivo were specifically labeled with an anti–proHB-EGF antibody, indicating that HB-EGF is synthesized within multiple, functionally distinct cellular compartments in the bladder. Our identification of HB-EGF synthesis in the urothelium in situ is consistent with our in vitro findings and suggests that HB-EGF synthesized in the bladder plays a physiologic role. Based on the results described herein, a reasonable function for HB-EGF within the urothelium is as a regulator of cell cycling or regeneration in response to mucosal injury. The ability of cell surface heparin-like molecules to coordinate the functional activity of soluble and membrane-bound HB-EGF suggests that HB-EGF may act in concert with proteoglycans and glycosaminoglycan components of bladder mucins to mediate cellular responses in vivo.

Several EGF-R ligands have been reported to be expressed by rodent or human urothelial cells in vivo or in primary culture, including EGF, TGFα, and amphiregulin (13, 14, 24). Of these, none has yet been reported to stimulate growth of normal human urothelial cells in primary culture. EGF has been shown in previous studies not to be a potent mitogen for normal HUC in primary culture (22, 23). However, EGF has been reported to stimulate the growth of bladder carcinoma cells in vitro and in vivo (4, 23). In our study, EGF was not a consistent stimulator of HUC growth (not shown), despite the presence of high-affinity EGF binding sites as noted by others. Although the five known HER1 ligands expressed by human cells activate the same high-affinity receptor tyrosine kinase, their biological activities are frequently distinct. The mechanism of differential control of biological functions by signaling through the same receptor is generally not known; however, for the EGF-R–related molecules it appears to involve ligand-specific patterns of receptor heterodimerization, in which ligand-activated HER1 couples to other HER/ErbB receptors after ligand binding (54). In our study we showed that HB-EGF binds to cell-surface sites from which it cannot be displaced by excess EGF. In contrast, EGF was fully displaceable by HB-EGF. Although the identity of these alternative HB-EGF binding sites is unknown, they may consist of cell surface HSPGs, which have been implicated in HER1/ErbB1 activation by HB-EGF. Interaction with these cell surface sites might be relevant to the potency of HB-EGF as an HUC growth factor. The bladder stores significant levels of EGF in urine, and it is possible that HER1 activation in vivo occurs through such a coreceptor mechanism. This might protect the urothelium from the growth-inducing properties of high local concentrations of urinary EGF. In bladder cancer, TCC cells might lose this requirement for EGF-R activation, thereby permitting the urine to stimulate proliferation of malignant cells. This could explain the rapid local growth of superficial TCC seen clinically in humans as well as the demonstrated capability for urinary EGF to act as a tumor promoter in animal models of bladder cancer (11).

Although HB-EGF is known to stimulate the growth of fibroblasts, smooth muscle cells and some carcinoma cells (57), an autocrine role for HB-EGF in a normal human epithelial cell type has been demonstrated previously for keratinocytes only (36). Notably, the HB-EGF concentration found to be optimal for most HUC strains tested was 1 ng/ml, the same concentration previously reported to be optimal for the stimulation of growth of subconfluent keratinocytes (36). The urothelium, while comprised of epithelial cells that are physiologically and morphologically distinct from those of the epidermis, nevertheless resembles epidermal epithelium in several respects. The urothelial mucosa is a multilayered epithelium that forms a permeability barrier confining urine to the bladder lumen, protecting the submucosal tissues from cytotoxic damage. As with skin, the urothelium is capable of rapid regenerative growth after injury. In addition, HUC proliferate in defined medium optimized for human keratinocytes. HB-EGF

has been implicated in healing of the epidermis; it is the major heparin-binding growth factor identifiable in fluid obtained from partial thickness skin lesions (58). In light of these intriguing similarities and the data presented in this report, it is likely that activation of HER1 is an important component of growth regulation for transitional urothelial cells as well as keratinocytes and that HB-EGF is a physiologic regulator of both cell types.

The finding that proHB-EGF can be immunolocalized to vascular and detrusor smooth muscle compartments in the bladder was unexpected; however, it is consistent with previous reports of immunocalization of HB-EGF to arterial smooth muscle (31). HB-EGF has also been identified as a secreted product of human fetal vascular smooth muscle cells (29) and it is a potent smooth muscle cell mitogen. We have recently shown that proHB-EGF can also be localized specifically to the smooth muscle compartment of the human prostate (59). These data are consistent with the recent demonstration that HB-EGF synthesis and promoter activity are upregulated with differentiation of myoblast-like C_2C_{12} cells into myotubes in vitro (46). ProHB-EGF was localized to discrete structures along the myotube surface after induction of the muscle phenotype in C_2C_{12} cells, suggesting that once muscle differentiation occurs, the HB-EGF precursor resides within a protein complex at the cell surface. While HB-EGF expression is not restricted to muscle cells, a cell-specific association of HB-EGF synthesis with muscle differentiation, as suggested by analysis of the HB-EGF promoter (46), would distinguish HB-EGF from the other known HER1 ligands. It is possible that in muscle cells, soluble HB-EGF might not be secreted at significant rates but might be retained in the pro- form at the cell surface. Our immunolocalization data suggest that steady-state levels of proHB-EGF in the detrusor and vascular smooth muscle cells are likely to be constitutive, while synthesis of proHB-EGF in the urothelium may be more dynamic. The functional significance of a diverse pattern of regulation of HB-EGF within the epithelial and muscular compartments in the bladder awaits further study, and a function for proHB-EGF expression within bladder muscle cells can only be surmised at this point. It is interesting, however, that proHB-EGF in monkey kidney Vero cells associates with the $\alpha_3\beta_1$ integrin and the integrin-associated molecule CD9/DRAP-27 (60, 61), suggesting a role for the membrane-anchored form in cell adhesion.

Finally, there may be clinical implications to the identification of HB-EGF as a product of the bladder mucosal epithelium. The demonstrated role of cell surface proHB-EGF as the high affinity receptor for DT, and our present identification of proHB-EGF/DT receptor synthesis in urothelium, suggest the possibility of intraluminal targeting of this molecule at the urothelium. This is conceivable because the receptor binding region of DT, which is distinct from the enzymatically active A fragment responsible for toxicity, is functional as an independent binding domain (62). The bladder is a relatively privileged site for the introduction of locally acting pharmacologic agents because of the relative ease of delivering them into the bladder lumen and of retaining them there for significant lengths of time. Novel strategies for targeting proHB-EGF/DT receptors at the bladder mucosa using DT-based pharmacotherapies might conceivably be applied to diverse inflammatory diseases of the urothelium, such as interstitial and bacterial cystitis, and possibly bladder cancer.

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