A Nuclear Form of the Heparin-biding Epidermal Growth Factor-Like Growth Factor Precursor Is a Feature of Aggressive Transitional Cell Carcinoma


ABSTRACT

Heparin-binding epidermal growth factor-like growth factor (HB-EGF), one of the ErbB receptor ligand family, exists in distinct molecular forms with disparate biological activities. Previous studies have shown that the HB-EGF precursor, proHB-EGF, localizes to the cytoplasm of transitional cells of the human bladder urothelium and that the soluble form of the growth factor is an autocrine urothelial cell mitogen. In this study, we identify a potential role for proHB-EGF in transitional cell carcinoma (TCC) of the bladder. In an analysis of 33 TCC specimens and 8 normal controls, proHB-EGF, identified using an antibody directed against the cytoplasmic tail domain, localized to cell nuclei in a manner that correlated positively with tumor stage and grade (P < 0.001). The ability of proHB-EGF to localize to the nucleus was independently confirmed in a TCC cell line (TCCSUP), in which ~40% of transfected proHB-EGF was found to reside in the nuclear compartment. In Kaplan-Meier survival analysis, TCC patients with >20% proHB-EGF-positive cell nuclei demonstrated markedly reduced survival compared with patients with <20% proHB-EGF-positive nuclei (P < 0.005, log-rank test). In multivariate analysis, nuclear localization of proHB-EGF of >20% was an independent prognostic indicator of disease-specific mortality. This is the first report in any cell type that HB-EGF is capable of translocating to the cell nucleus. In addition, our findings suggest that nuclear proHB-EGF may play a role in disease progression in bladder cancer and possibly other cancers.

INTRODUCTION

HB-EGF is a multifunctional member of the EGFR-like growth factor family and elicits diverse effects in cells. The pleiotropic nature of HB-EGF arises, in part, from the existence of different molecular forms. HB-EGF is initially synthesized as a membrane-anchored precursor, proHB-EGF, which can participate in juxtacrine interactions with the EGFR and/or the ErbB4 receptor tyrosine kinase expressed on adjacent cells, leading to receptor activation and increased cell-cell adhesion (1, 2). Membrane-anchored HB-EGF also serves as the source of soluble HB-EGF, which is released after a regulated, metalloproteinase cleavage step (3, 4). Soluble HB-EGF mediates paracrine and autocrine activation of the EGFR and ErbB4 and thereby promotes survival, proliferation, and migration in different cell types.

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2 To whom requests for reprints should be addressed, at John F. Enders Research Laboratories, Room 1161, Children’s Hospital Boston, 300 Longwood Avenue, Boston, MA 02115. Phone: (617) 355-6054; Fax: (617) 355-7760; E-mail: Michael.Freeman@ch.harvard.edu.

3 The abbreviations used are: HB-EGF, heparin-binding epidermal growth factor-like growth factor; CI, confidence interval; EGFR, epidermal growth factor receptor; EGFR, epidermal growth factor receptor; FGF-2, fibroblast growth factor 2; IC, interstitial cystitis; IHC, immunohistochemistry; IQR, interquartile range; proHB-EGF-AP, proHB-EGF-alkaline phosphatase fusion protein; PTHrP, parathyroid hormone-related peptide; ROC, receiver-operating characteristic; TCC, transitional cell carcinoma; TGF-α, transforming growth factor α.

There are now substantial data to suggest that the actions of soluble HB-EGF and proHB-EGF are not equivalent and that several biological activities of HB-EGF are exclusive to the precursor alone (5–7). In contrast to soluble HB-EGF, proHB-EGF can achieve some of its effects independently of EGFR/ErbB receptor activation. Uniquely among the EGFR-like growth factor family, proHB-EGF is the cell surface receptor for diphtheria toxin (8), a function that cannot be replicated by soluble HB-EGF and that does not involve the EGFR. A recent study from our laboratory demonstrated that proHB-EGF can also interact with coagulation protein, BAG-1, to promote cell survival (7). This interaction is mediated by the proHB-EGF cytoplasmic tail domain and, in contrast to the activity of soluble HB-EGF, occurs intracellularly and independently of the EGFR.

HB-EGF has recently been identified as a physiologically relevant growth factor in a number of studies of the urinary bladder. We have shown previously that the smooth muscle and urothelial compartments of the normal human bladder are sites of synthesis of proHB-EGF and that soluble HB-EGF, acting through the EGFR, is an autocrine factor for urothelial cells in vitro (9). HB-EGF has also been linked to several pathological bladder conditions such as outlet obstruction (10, 11) and IC (12–15), in which it is presumed to function as a mediator of “outside-in” signaling through activation of ErbB receptors. Although soluble HB-EGF has been described as an autocrine factor for bladder cancer cell lines in culture (16), HB-EGF mRNA levels have been evaluated in bladder cancer (17), the role of HB-EGF in bladder cancer has not been extensively studied. In the current study, we present evidence that a nuclear form of proHB-EGF is a marker of disease progression in TCC.

MATERIALS AND METHODS

Localization of proHB-EGF to the Nucleus in Vitro. To evaluate nuclear localization of proHB-EGF in vitro, two approaches were used. Firstly, the bladder cancer cell line TCCSUP was engineered to express pcDNA3.1/proHB-EGF, an expression construct under the control of the cytomegalovirus promoter that encodes the HB-EGF precursor. Cells expressing pcDNA3.1/LacZ served as control. Cells were transfected using FuGENE6 reagent (Roche Molecular Biochemicals, Minneapolis, MN), and stable clones were selected in G418-containing medium. TCCSUP/LacZ and TCCSUP/proHB-EGF were subjected to nuclear/cytoplasmic fractionation as described previously (18). Cytoplasmic and nuclear fractions were desalted using Micro Biogel 6 spin columns (Bio-Rad Laboratories, Hercules, CA), and the protein content was determined using the Bio-Rad DC protein assay. Equal amounts of nuclear and cytoplasmic extracts were fractionated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membrane, and immunoblotted with antibodies to nuclear lamin A/C (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), the proHB-EGF cytoplasmic tail (QCB, Hopkinton, MA), and the HB-EGF ectodomain (R&D Systems Inc., Minneapolis, MN). Secondly, Chinese hamster ovary or COS7 kidney cells were transiently transfected with a vector encoding proHB-EGF under control of the cytomegalovirus promoter. Cells were transfected and stained for proHB-EGF as described previously (7).

Patient Population. Bladder biopsies were obtained by transurethral resection or during open surgery at a single urologic practice (The Freeman Hospital, University of Newcastle-upon-Tyne, England, United Kingdom) during the period 1989–1998. The tissues under study were obtained before or
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HB-EGF was previously shown to localize to the cell nucleus in a small series of TCC specimens by IHC using an antibody raised to the proHB-EGF cytoplasmic tail domain. Before its use in IHC, the ability of the antibody to recognize the target protein was confirmed in vitro using HEK293 cells transiently transfected with proHB-EGF-AP (2). ProHB-EGF-AP is ~84 kDa, composed with 22–30 kDa for wild-type proHB-EGF, such that proHB-EGF-AP can be readily distinguished from the IgG light chain after immunoprecipitation and immunoblotting. HEK293 cells were transfected with proHB-EGF-AP plasmid using LipofectAMINE 2000 (Invitrogen). The ability of the antibody to recognize the target was evaluated by immunoprecipitation of HEK293 lysates and immunoblot analysis (Fig. 1). Specific staining for proHB-EGF in tissue was determined using the Vectastain Elite ABC kit as described previously (9). Sections receiving nonspecific antisera served as negative control, and immunoprecipitates were resolved by SDS-PAGE. Membranes were probed with rabbit antibody against the same epitope but was not identified as significant transfected with empty vector (Ctrl) or a proHB-EGF-AP fusion protein construct (HB-AP). Cell lysates (~500 µg) were immunoprecipitated using a rabbit anti-HB-EGF tail antibody, and immunoprecipitates were resolved by SDS-PAGE. Membranes were blotted separately with chicken preimmune IgY, chicken anti-HB-EGF tail IgY, or rabbit anti-HB-EGF tail antibody. The positions of the HB-EGF-AP fusion protein and the IgG heavy and light chains are indicated on the right, and molecular masses are shown on the left.

RESULTS

Expression of proHB-EGF in TCC. To define a potential role for HB-EGF in bladder cancer, we initially evaluated its expression in a small series of TCC specimens by IHC using an antibody raised to the proHB-EGF cytoplasmic tail domain. Before its use in IHC, the ability of the antibody to recognize the target protein was confirmed in vitro, using HEK293 cells transiently transfected with a proHB-EGF-AP fusion protein (2). As shown in Fig. 1, the chicken anti-HB-EGF tail antibody recognized a single band corresponding to HB-AP (Fig. 1). Correlation between the two observers, with respect to scoring of immunohistochemical staining data averaged across three random fields, was determined using the interclass correlation coefficient (20) and found to be excellent (interclass correlation = 0.916; P < 0.001). ROC curve analysis was performed to identify the optimal cutoff point giving maximal sensitivity and specificity (21). This was determined to be 20% proHB-EGF-positive nuclei (sensitivity = 88%; specificity = 80%), with the area under the ROC curve showing excellent discrimination (area under the curve = 0.858; P = 0.004, Wilcoxon statistic). The Kaplan-Meier product-limit method was used to determine survival probability for subgroups based on the 20% cutoff. Survival curves were compared by log-rank tests (22), and error bars constructed around the survival curves denote 95% CIs, as calculated by Greenwood’s formula (23). Univariate and multivariate analysis of risk factors predicting TCC-specific death was performed using the Cox proportional hazards regression model. The hazard ratio and 95% CI were used as the measures of risk, and the assumption of proportional hazards was examined using log-log plots (24). The Pearson χ² test was used to test the association between categorical variables. A two-sided P of <0.05 was considered statistically significant. Statistical analysis was performed using the SAS (Version 6.12; SAS Institute, Inc., Cary, NC) and SPSS (Version 11.0; SPSS Inc., Chicago, IL) software packages.

Table 1 Patient characteristics

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Fig. 2. proHB-EGF localizes to the nucleus of bladder cancer cells in vitro. The bladder cancer cell line TCCSUP, engineered to express control vector (LacZ) or proHB-EGF, was subjected to cytoplasmic-nuclear fractionation. Ten μg of cytoplasmic (C) or nuclear (N) extracts were resolved by SDS-PAGE and immunoblotted for HB-EGF using rabbit anti-HB-EGF tail or goat anti-HB-EGF ectodomain antibodies; membranes were also probed for nuclear lamins A/C to confirm effective fractionation. The positions of nuclear lamins and the differently processed forms of proHB-EGF are shown on the right. Molecular mass markers are on the left.

HB-EGF localizes to the nucleus of bladder cancer cells. The cell-associated fragment that remains after cleavage of the pro-HB-EGF precursor includes a nuclear localization sequence that directs the fragment to the nucleus (26–28). The predominant forms of proHB-EGF were detected as two major bands of approximately 40% of the transfected proHB-EGF was present in the nuclear fraction, which confirmed the effective fractionation procedure. Robust expression of proHB-EGF was detected in the TCCSUP/proHB-EGF nuclear fraction using antibodies directed against either the HB-EGF ectodomain (Fig. 2, right panel) or the cytoplasmic tail region (Fig. 2, left panel); approximately 40% of the transfected proHB-EGF was present in the nuclear compartment. The predominant forms of proHB-EGF were ~19 and 23 kDa, consistent with previous studies reporting the migration pattern of proHB-EGF in SDS-PAGE gels (31). A smaller form of proHB-EGF, detectable only with the anti-tail antibody, was present only in the cytoplasmic fragment (Fig. 2, left panel) and likely represents the cell-associated fragment that remains after cleavage of the HB-EGF ectodomain. Nuclear localization of HB-EGF was also confirmed by immunofluorescent imaging of COS and Chinese hamster ovary proHB-EGF transfectants (data not shown). Together, these data demonstrated that proHB-EGF is able to localize to cell nuclei, including nuclei of human bladder cancer cells, lending support to our preliminary observations in TCC specimens.

Nuclear Staining for proHB-EGF Correlates Inversely with Patient Survival. To extend our initial findings with TCC specimens and to determine whether nuclear localization of proHB-EGF correlated with a disease phenotype, we evaluated proHB-EGF expression by IHC in a cohort of 33 TCC patients and 8 normal controls. As shown in representative examples in Fig. 3, increased frequency of nuclear staining for proHB-EGF in TCC was markedly associated with tumor aggressiveness. Normal bladder tissue showed little or no nuclear proHB-EGF staining (see Fig. 4), and in tumors of low stage and/or grade (T1/T2 and grade 1/2), proHB-EGF staining was predominantly cytoplasmic (Fig. 3, a–d), with relatively few positive nuclei. However, with increased stage or grade, there was a striking increase in the number of proHB-EGF-positive nuclei per field (Fig. 3, e–h).

The correlation between proHB-EGF-positive nuclei frequency and tumor grade was significant (Pearson coefficient, $\sigma = 0.79$; $P < 0.001$). Positive nuclear staining for proHB-EGF did not conform to a normal distribution, therefore the percentage of proHB-EGF-positive nuclei/field was expressed in terms of median and IQR. Controls and patients with TCC grade 1, 2, or 3 were then compared using the Mann-Whitney $t$ test. The median percentage of proHB-EGF-positive nuclei was 3.2% (IQR 0.8–6.3) for the control group; 7.7% (IQR 3.4–13.2) for patients with grade 1 disease; 8.9% (IQR 5.9–19.1) for patients with grade 2 disease; and 34.6% (IQR 22.0–47.1) for patients with grade 3 disease. The empirical data and group comparisons are summarized in Fig. 4. Positive nuclear staining for proHB-EGF also correlated significantly with tumor stage; patients with stage T3–4 TCC had a median value of proHB-EGF-positive nuclei of 33.9% (IQR 19.3–45.8) compared with 9.6% (IQR 5.1–13.6) in patients with superficial (Ta–T1) disease.

To determine whether nuclear proHB-EGF correlated with disease outcome, patients were stratified into two groups with less than or greater than 20% proHB-EGF-positive nuclei. The cutoff value of 20% was determined by ROC curve analysis, as described in “Materials and Methods.” Kaplan-Meier survival analysis (Fig. 5) revealed a statistically significant difference between the two groups (log-rank test, $P = 0.0005$). When all 33 patients were considered (Fig. 5A), patients with $\geqslant 20\%$ proHB-EGF-positive nuclei showed markedly reduced survival compared with those with $<20\%$ positive nuclei. A similar pattern was observed for disease-specific survival (Fig. 5B, log-rank test, $P = 0.0018$). When all 33 patients were analyzed, the estimated 5-year survival rate for patients with $<20\%$ proHB-EGF-positive nuclei was 95% (95% CI, 90–100%) compared with 48% (95% CI, 34–62%) for patients with $>20\%$ proHB-EGF-positive nuclei. The 5-year disease-specific survival rate was estimated to be 93% (95% CI, 87–100%) for patients with $<20\%$ proHB-EGF-positive nuclei versus 42% (95% CI, 26–58%) for those with $>20\%$ proHB-EGF-positive nuclei.

To identify the significant prognostic factors associated with TCC-specific death, univariate and multivariate risk factor analysis was performed using the Cox proportional hazards regression model. Tumor stage ($P = 0.017$), tumor grade ($P < 0.001$), and nuclear proHB-EGF positivity $\geqslant 20\%$ ($P = 0.002$) were found to be significant univariate predictors of death from TCC, whereas age and gender were not. In a multivariate analysis, only tumor grade and $>20\%$ proHB-EGF-positive nuclei were independent predictors of TCC-specific death ($P < 0.01$). The Cox regression model indicated that the monthly risk of death from TCC was more than 13 times greater in patients with $>20\%$ pro-HB-EGF-positive nuclei (risk ratio = 13.5; CI = 2.5–80.0; $P < 0.001$).
DISCUSSION

In this study, we demonstrate that proHB-EGF can reside in the nucleus of bladder cancer cells, and we have identified nuclear-localized proHB-EGF to be a feature of clinically aggressive TCC. Quantitative evaluation of nuclear proHB-EGF in a series of tumor specimens indicated that this cellular characteristic was highly correlated with tumor grade, tumor stage, and a significantly increased risk of disease-specific mortality. This study provides the first evidence linking nuclear localization of proHB-EGF to disease progression in any cancer; it is also the first report to our knowledge that an HB-EGF isoform can be present in the nucleus in significant amounts.

In contrast to TGF-α, another direct activating ligand for the EGFR and a well-known autocrine growth factor overexpressed in many cancers, there are presently very few reports linking HB-EGF to either carcinogenesis or tumor progression. Where a cancer link has been made, increased HB-EGF expression tends to be associated inversely with aggressiveness (32–34). In the human tissues we examined, nuclear proHB-EGF in high-grade tumors appeared to coincide with clearance of staining from the tumor cell cytoplasm (see Fig. 3), suggesting that alterations in intracellular protein trafficking rather than in absolute expression of the protein are likely to account for our observations. Interestingly, a recent study reported that the TGF-α expression.

Fig. 3. Immunohistochemical localization of proHB-EGF in TCC. The figure illustrates examples of the proHB-EGF staining pattern in TCC. Staining for proHB-EGF in low-grade tumors was predominantly cytoplasmic (a–d), whereas higher grade tumors (e–h) showed increasing amounts of proHB-EGF in the nucleus. Pathological stage and grade of tumors is as follows: a, T1, grade 1/2; b, T1, grade 2; c, T1, grade 2; d, T1, grade 1/2; e, T1, grade 2; f, T1, grade 2; g, T3, grade 3; and h, T3, grade 3.

Fig. 4. Nuclear staining for proHB-EGF correlates with tumor grade in TCC. The extent of positive staining for proHB-EGF in the nucleus was determined for 8 controls and for each of the 33 patients grouped according to tumor grade. The median percentage of proHB-EGF-positive nuclei is represented by the horizontal line. This value is 3.2% (IQR = 0.8–6.3) for controls, 7.7% (IQR = 3.4–13.2) for patients with grade 1 disease, 8.9% (IQR = 5.9–19.1) for patients with grade 2 disease, and 34.6% (IQR = 22.0–47.1) for patients with grade 3 disease.
nuclear proHB-EGF may interact with binding partners other than the EGFR to elicit functional effects. We recently reported that proHB-EGF binds to the co-chaperone, BAG-1, through the proHB-EGF cytoplasmic tail domain, leading to increased cell survival, adhesion, and alterations in regulated secretion of the soluble growth factor (7). Association with BAG-1 is exclusive to the pro form of HB-EGF because the soluble protein does not possess the cytoplasmic tail. Furthermore, we observed the interaction in yeast and in mammalian cells lacking EGFR, indicating that proHB-EGF is capable of binding BAG-1 in an EGFR-independent manner. Notably, the HB-EGF homologue amphiregulin has been shown to bind directly to DNA (27), suggesting that these factors may effect gene expression changes directly at the level of transcription.

Our results indicate that analysis of nuclear proHB-EGF may have prognostic value in bladder cancer, and we believe this warrants further investigation. A number of proteins have been evaluated as prognostic indicators of survival in TCC, including cyclin D1, cyclin E, p21, p27, and p53, as well as other molecules associated with growth control (44–50). Our study identified the appearance in the nucleus of a protein not normally present in this cellular compartment. A similar phenomenon has been observed for β-catenin in several tumor types. β-Catenin acts as a component of a cell-cell adhesion complex in epithelial cells, but upon down-regulation of its binding partner, E-cadherin, it can translocate to the nucleus, where it functions as part of a transcription complex. Increased nuclear localization of β-catenin is correlated with poor patient outcome in a range of tumor types (51–54). Like β-catenin, proHB-EGF is capable of functioning at the level of the plasma membrane as a cell adhesion factor via a juxta-activation signaling mechanism.

The identification of nuclear as well as cytoplasmic forms of HB-EGF is consistent with previous reports of distinct subcellular locations for other regulatory peptides such as basic FGF/FGF-2 (55) and PTHrP. Both proteins exist as isoforms that differ in biological activity depending on subcellular location. Nuclear isoforms of FGF-2 have been associated with a more aggressive phenotype compared with cells expressing secreted FGF-2 (56–59). Selective expression of the 24-kDa nuclear FGF-2 isoform in rat bladder cancer cells increased their tumorigenicity and metastatic potential compared with cells expressing 18-kDa secreted FGF-2, which showed comparable tumor-forming ability to parental cells (60). Nuclear-localized PTHrP has also been associated with increased proliferation (61), decreased apoptosis, and suppression of differentiation (62, 63), in contrast to the activity of exogenous PTHrP (64, 65). These observations provide additional evidence that localization of secreted mitogens/survival factors to the nucleus can be associated with a more aggressive phenotype.

Several studies have linked HB-EGF to urinary bladder function (9–11, 16), although the precise role of this factor in bladder physiology is still incompletely understood. HB-EGF levels in the urine of patients with IC were markedly decreased compared with controls (12). Urine from IC patients was also shown to contain high levels of so-called antiproliferative factor, a peptide that selectively inhibited the production and mitogenic activity of HB-EGF (13), but not EGF or IGF-1. IC is characterized by damage to the urothelium and loss of urothelial integrity, which would be consistent with a decreased proliferative index in the tissue. These and other data indicating that soluble HB-EGF is an autocrine urothelial cell mitogen (9) suggest that HB-EGF functions in the normal bladder to maintain and repair the urothelium. Our present findings now extend these observations and suggest that the HB-EGF precursor may play a specialized role, distinct from that of the soluble form of the growth factor, in disease progression in bladder cancer.
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References


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Rosalyn M. Adam, Theodora Danciu, Dawn L. McLellan, et al.


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