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Heparin-Binding EGF-Like Growth Factor in the Human Prostate: Synthesis Predominantly by Interstitial and Vascular Smooth Muscle Cells and Action as a Carcinoma Cell Mitogen

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Abstract Heparin-binding epidermal growth factor-like growth factor (HB-EGF) is an activating ligand for the EGF receptor (HER1/ErbB1) and the high-affinity receptor for diphtheria toxin (DT) in its transmembrane form (proHB-EGF). HB-EGF was immunolocalized within human benign and malignant prostatic tissues, using monospecific antibodies directed against the mature protein and against the cytoplasmic domain of proHB-EGF. Prostate carcinoma cells, normal glandular epithelial cells, undifferentiated fibroblasts, and inflammatory cells were not decorated by the anti-HB-EGF antibodies; however, interstitial and vascular smooth muscle cells were highly reactive, indicating that the smooth muscle compartments are the major sites of synthesis and localization of HB-EGF within the prostate. In marked contrast to prostatic epithelium, proHB-EGF was immunolocalized to seminal vesicle epithelium, indicating differential regulation of HB-EGF synthesis within various epithelia of the reproductive tract. HB-EGF was not overexpressed in this series of cancer tissues, in comparison to the benign tissues. In experiments with LNCaP human prostate carcinoma cells, HB-EGF was similar in potency to epidermal growth factor (EGF) in stimulating cell growth. Exogenous HB-EGF and EGF each activated HER1 and HER3 receptor tyrosine kinases and induced tyrosine phosphorylation of cellular proteins to a similar extent. LNCaP cells expressed detectable but low levels of HB-EGF mRNA; however, proHB-EGF was detected at the cell surface indirectly by demonstration of specific sensitivity to DT. HB-EGF is the first HER1 ligand to be identified predominantly as a smooth muscle cell product in the human prostate. Further, the observation that HB-EGF is similar to EGF in mitogenic potency for human prostate carcinoma cells suggests that it may be one of the hypothesized stromal mediators of prostate cancer growth. J. Cell. Biochem. 68:328-338, 1998.

Key words: cell proliferation; tumor progression; EGF receptor; ErbB; HER1

The intrinsic tyrosine kinase activity of the HER1 (EGF-R/ErbB1) growth factor receptor can be activated by a number of structurally related ligands. Human cells express six known HER1 ligands: epidermal growth factor (EGF), transforming growth factor-α (TGFα), amphiregulin, heparin-binding EGF-like growth factor (HB-EGF), epiregulin and betacellulin [reviewed in Prigent and Lemoine, 1992; Mason and Gullick, 1995]. Cell signaling through HER1 has been implicated in normal development, tissue homeostasis, human cancer, and a variety of pathophysiologic processes such as atherosclerosis and normal wound healing. HER3 (ErbB3) and HER4 (ErbB4), two receptor tyro-

Abbreviations: HB-EGF, heparin-binding epidermal growth factor-like growth factor; EGF, epidermal growth factor; EGF-R, epidermal growth factor receptor; HER1–HER4, human epidermal growth factor receptor types 1–4 (also ErbB1–ErbB4); TGF-α, transforming growth factor-α; DT, diphtheria toxin; ^{3}HTdR, tritiated thymidine; BPH, benign prostatic hyperplasia; TURP, transurethral resection of the prostate; PSA, prostate-specific antigen; FBS, fetal bovine serum; PBS, phosphate-buffered saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT-PCR, reverse transcription-polymerase chain reaction

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sine kinases related to HER1, are receptors for a complex class of EGF-like molecules known as the heregulins, which are expressed from a single gene, but which contain a diverse array of structural motifs [Mason and Gullick, 1995; Pinkas-Kramarski et al., 1996]. Soluble heregulin isoforms do not activate HER1 or the orphan receptor HER2 by direct ligand binding.

The physiological significance of multiple soluble regulators of HER/ErbB-family tyrosine kinases is still unclear, although each of the HER1 ligands found in higher vertebrates is expressed in a distinctive manner in vivo and sometimes can be shown to exhibit unique biological activities in bioassays. Cell signaling through HER1 is also complicated by the fact that activation of this receptor can result in the coordinate activation of other HER-type receptors expressed within the same cell. This "transactivation" results from heterodimerization of the heterotypic ErbB receptors [reviewed by Lemmon and Schlessinger, 1994]. The various HER1 ligands do not show identical patterns of receptor transactivation [Beerli and Hynes, 1996]. Further, ligand-mediated signaling and subsequent biological responses through HER-family receptors are dependent on such factors as (1) which receptors are expressed in the target cell, (2) the relative expression levels of the various receptors, and (3) the availability and identity of the major activating ligand(s) in the extracellular space. This level of complexity suggests that the various ligands and HER/ErbB receptors serve nonoverlapping functions in vivo and therefore may play context-dependent roles in human diseases.

From studies of prostatic cells and tissues, signaling through HER1/ErbB1 has been linked to benign and malignant prostatic growth. EGF, TGF-α, and amphiregulin are synthesized by cultured prostatic cells and several prostate carcinoma lines exhibit functional autocrine loops involving activation of HER1 by one or more ligands [Ching et al., 1993; Sehgal et al., 1994; Connolly and Rose, 1991; Liu et al., 1993; Limonta et al., 1995]. Androgenic steroids, which regulate prostatic function and growth in vivo, mediate synthesis or availability of HER1 in prostatic cells [Liu et al., 1993; Brass et al., 1995]. Serum-free culture of normal prostate epithelial cells requires EGF in combination with several peptide and steroid hormones, but notably not androgens, suggesting that the growth-promoting effects of androgens in the prostate are mediated by peptide growth factors [McKeehan et al., 1987]. TGF-α, EGF, HER1/ErbB1, and the HER2/ErbB2 receptor kinase, which can be activated by HER1 [Wada et al., 1990; Beerli and Hynes, 1996], can be expressed within hyperplastic and malignant prostate tissue [Myers et al., 1993; Harper et al., 1993; Zhau et al., 1992]. These observations suggest that alterations in pathways of growth control in prostatic disease are likely to involve ligand-HER/ErbB interactions of several types.

HB-EGF is a 14- to 20-kDa HER1 ligand that was originally identified as a heparin-binding fibroblast mitogen secreted by macrophages and was subsequently cloned from U937 macrophage-like cells [Higashiyama et al., 1991, 1992]. HB-EGF is notable among the various HER1 ligands because of its potency as a smooth muscle cell growth factor and because the plasma membrane-associated, precursor form of the molecule (proHB-EGF) is the high-affinity receptor for diphtheria toxin (DT) in human cells [reviewed by Mekada, 1995]. Certain species, notably mouse and rat, are resistant to DT because of amino acid sequence substitutions within the EGF-like motif. After binding of the DT holotoxin to the proHB-EGF ectodomain in DT-sensitive cells, the growth factor precursor mediates entry of the toxin into cells by an endocytotic mechanism. HB-EGF is of potential interest in prostatic disease because of its role as a connective tissue and tumor cell growth factor [Peoples et al., 1995], and also because of the on-going searches for cell-surface molecules that might potentially be used as targets for drug delivery in therapy for prostatic diseases. In this study, we have examined the potential role of HB-EGF in human prostate pathophysiology.

MATERIALS AND METHODS

Human Tissues and Immunohistochemistry

Histologic specimens fixed in 10% formalin and embedded in paraffin were from BPH specimens obtained by TURP and prostate cancer specimens obtained by biopsy. Sections were previewed for normal histomorphology by a genitourinary pathologist (A.A.R.) before staining. Immunostaining for expression of proHB-EGF was carried out using a streptavidin-biotin detection system as previously described [Freeman et al., 1995]. The 3100 antibody to the proHB-EGF cytoplasmic tail domain and the 2911 antibody, which recognizes both

proHB-EGF and mature HB-EGF [Chen et al., 1995], were both used at a 1:500 dilution.

Morphometry

Six random high power (40×) fields of each stained tissue section (4 benign prostatic hyperplastic specimens and 4 prostate cancer specimens, Gleason grade 5–7) and a representative pre-immune negative control were photographed by an investigator not familiar with the study. Images were stored in Adobe Photoshop (version 2.5.1 for Macintosh) and area and intensity of staining, expressed as integrated density of each pixel, were quantified using NIH Image (version 1.56). Final values for integrated density of all fields on a given slide were evaluated by subtracting the integrated density measured for the preimmune control slide. Statistical analysis was carried out with Instat (version 2.0) for Macintosh (GraphPad Software, Inc., San Diego, CA), using Welch's alternate t-test for distributions with unequal variances.

Cell Growth Assays

LNCaP human prostate carcinoma cells were obtained from the American Type Culture Collection. For growth experiments, LNCaP cells were seeded in poly-L-lysine-coated 96-well tissue culture plates at a density of 1×10^4 cells/well in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), and allowed to plate overnight at 37°C in a humidified atmosphere of 5% CO_2. Plating medium was removed and replaced with 100 µl/well of appropriately diluted EGF or HB-EGF in RPMI/1% FBS. Cells were incubated at 37°C for 7 days, and the extent of proliferation at this time was evaluated using the modified MTT assay [Denizot and Lang, 1986]. Briefly, 10 µl (5 mg/ml) MTT was added to each well and the cells allowed to metabolize the dye for 4 h at 37°C, protected from light. Converted dye was solubilized by addition of 100 µl/well 10% sodium dodecyl sulfate (SDS) containing 0.01 N HCl, and cells were incubated in the dark for a further 16 hr at 37°C. Absorbance at 550 nm was read on a Molecular Devices UV MAX microplate reader with a baseline correction at 650 nm. For DNA synthesis assays, approximately 5,000 cells/well were plated in a 96-well plate, allowed to adhere for 24 h, and then serum starved for 72 h before addition of growth factor. Cells were incubated with growth factor for 24 h before the addition of 0.1 µCi ^{3}HTdR per well for 5 h. The cells were then harvested with trypsin and the extent of radiolabel incorporation was measured by Beta-counter (Wallac, Gaithersburg, MD).

Affinity Cross-Linking

^{125}I-Iodinated HB-EGF was prepared using IodoBeads (Pierce, Rockford, IL) and Na^{125}I (Amersham; Arlington Heights, IL) according to the manufacturer's instructions to a specific activity of 100,000 cpm/ng. Iodinated HB-EGF was partially purified from free label, using size exclusion columns (NAP5, Pharmacia, Kalamazoo, MI). Cross-linking was performed as described previously [Elenius et al., 1997]. Briefly, labeled growth factor was incubated with cells on ice in DMEM with 25 mM HEPES (pH 7.4), and 0.2% gelatin for 2 h. Cells were then cross-linked with 20 mM disuccinimidyl suberate (DSS) (Pierce) in DMSO for 15 min. The cross-linking reaction was terminated using 250 mM glycine, 10 mM Tris–HCl (pH 7.5), and 2 mM EDTA buffer for 1 min. Cells were harvested with scraping in phosphate-buffered saline (PBS) containing 2.5 mM EDTA and 2 mM PMSF (Pierce) and lysed with a 10 mM Tris–HCl (pH 7.0), 1% NP-40, 1 mM EDTA, 2 nM PMSF, 10 µg/ ml aprotinin, and 10 µg/ml leupeptin containing buffer. The lysates were then run on 6% SDS polyacrylamide gels, dried, and exposed to X-ray film.

Immunoprecipitations and Phosphotyrosine Western Blotting

Confluent 10-cm^2 dishes of LNCaP cells were starved for 24 h in serum-free media and stimulated with 100 ng/ml HB-EGF and EGF on ice for 10 min and then 5 min at 37°C. Lysates were prepared in 1 ml of 10 mM Tris–HCl (pH 7.0), 1% NP-40, 1 mM EDTA, 2 mM PMSF, 10 µg/ ml aprotinin, 10 µg/ml leupeptin, 1 mM sodium orthovanadate, 10 mM sodium fluoride, and 10 mM sodium pyrophosphate containing buffer. Cell lysates were centrifuged at 10,000 rpm for 10 min at 4°C and the supernatant was preincubated with Sepharose (Sephadex, Pharmacia) for 1 h at 4°C with end-over-end rotation. Lysates were centrifuged, and 300 µl of lysate was added to 4 µl 100 ng/ml rabbit polyclonal antireceptor antibodies (anti-HER1, Upstate Biotechnology, New York; anti-HER3, anti-HER2, and anti-HER4, Santa Cruz Biotechnology, Santa Cruz, CA) and incubated for

1 h at 4°C with end-over-end rotation. Twenty µl of 50% protein G–Sepharose in lysis buffer was then added to each lysate/antibody mixture and incubated for 1 h at 4°C with end-over-end rotation. Lysates were centrifuged and protein G–Sepharose complexes were washed three times in lysis buffer, resuspended in 25 µl 6× SDS-PAGE sample buffer (0.5 M Tris–HCl (pH 6.8), 1% SDS, 100 mM β-mercaptoethanol, and 0.01% bromophenol blue), heated at 100°C for 5 min, run on 6% SDS-PAGE gels, and transferred to 0.1 µm nitrocelluose (Schleicher and Schuell; Keene, NH). Blots were blocked in 5% milk in TBS-T (10 mM Tris–HCl (pH 7.5), 150 mM NaCl, and 0.05% Tween-20 (USB; Cleveland, OH) for 2 h at room temperature, incubated with 1:10,000 mouse monoclonal anti-phosphotyrosine antibodies in 5% milk in TBS-T for 4 h at room temperature and washed 4 times with TBS-T for 40 min. Blots were then incubated with 1:40,000 rabbit polyclonal anti-mouse HRP antibodies in 5% milk in TBS-T for 2 h at room temperature, and washed 4 times with TBS-T for 40 min. Reactivity was observed by development with ECL reagents (Amersham) for 1 min followed by exposure to X-ray film.

RNA Expression Analysis

Northern blot hybridization and reverse transcription-polymerase chain reaction (RT-PCR) were performed as described [Freeman et al., 1995, 1997].

Diphtheria Toxin Sensitivity

Approximately 10,000 cells per well were plated in a 96-well plate and exposed to diphtheria toxin (5–120 ng/ml) for 90 min. The medium was removed and the cells were incubated with ^{3}H-Leu in RPMI 1640 leucine-free media (Gibco) (0.005 mCi/ml) for 60 min. The cells were harvested with trypsin and the extent of radiolabel incorporation was measured by Beta-counter (Wallac).

RESULTS

Synthesis of HB-EGF in the Human Prostate

HB-EGF mRNA was detected in adult human prostatic tissue by Northern blot hybridization (data not shown), suggesting that HB-EGF is synthesized within prostatic tissue. To identify cellular sites of HB-EGF synthesis, we immunohistochemically stained formalin-fixed, paraffin-embedded prostate tissues from patients with benign prostatic hyperplasia (BPH, n = 5) and prostate adenocarcinoma (n = 6) with two monospecific anti-HB-EGF antibodies. One antibody (2911) recognizes soluble HB-EGF and the ectodomain of the HB-EGF precursor (proHB-EGF). The second antibody (3100) recognizes the cytoplasmic domain of proHB-EGF, and not the mature form of the growth factor. Because the 3100 antibody recognizes the unprocessed form of the molecule exclusively, immunoreactivity shown with this antibody identifies sites of synthesis of the growth factor. The prostate cancer specimens had Gleason sum scores of 5–7.

The major classes of anti-HB-EGF immunoreactive cells were interstitial and vascular smooth muscle cells of the fibromuscular stroma (Fig. 1A–D). Staining patterns seen with both antibodies in serial sections of the same tissue were similar in every specimen, indicating that HB-EGF does not accumulate at significant levels at locations distant from the sites of synthesis. In contrast to strong staining in the muscle cell types, normal glandular epithelium and carcinoma cells were either negative or faintly positive in a manner suggesting a non-specific reaction (Figs. 1B, 2A). However, in marked contrast to prostatic epithelium, normal seminal vesicle epithelium, evident in some of the specimens, was strongly positive (Fig. 2B). Inflammatory infiltrates, undifferentiated fibroblasts, and nerve bundles in the stroma were uniformly negative. These results suggest that HB-EGF synthesis in the prostate is regulated in a cell-specific manner and occurs primarily within smooth muscle cells. Normal prostatic epithelium and organ-confined cancerous epithelium apparently do not synthesize high levels of HB-EGF in vivo. In addition, the contrast between prostatic and seminal vesicle staining patterns suggests cell-specific regulation of HB-EGF synthesis within various epithelial compartments of the male accessory sex glands.

To determine the relative levels of HB-EGF expression in this series of benign and cancerous tissues, we performed morphometric analysis of four BPH tissues and four PCa specimens stained with the 3100 antibody. A total of 24 randomly chosen, nonoverlapping fields distributed equally among the specimens were measured in each group. Microscopic fields were chosen and digitally imaged by computer by an

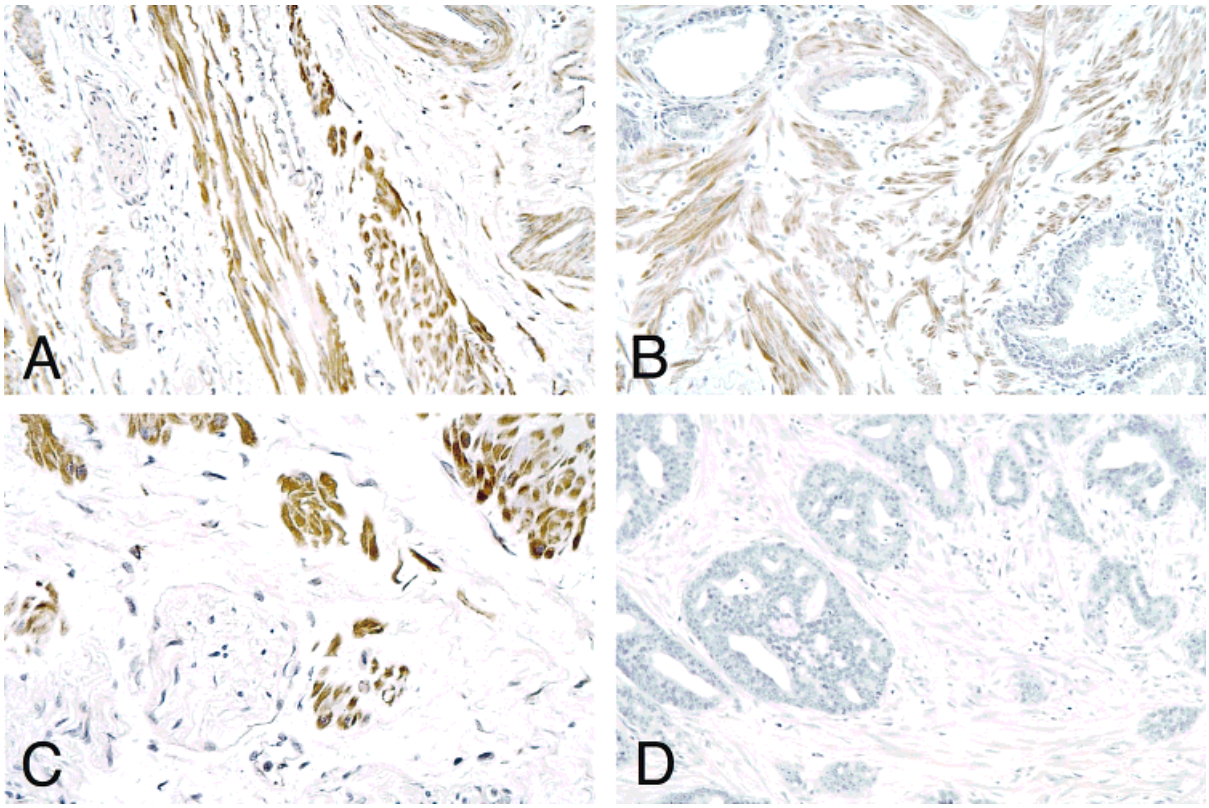

Fig. 1. Immunohistochemical staining for HB-EGF in the human prostate. Antibodies against the HB-EGF ectodomain and the cytoplasmic tail domain of the proHB-EGF precursor were used and showed similar staining patterns, in which the interstitial and vascular smooth muscle cells are the only definitively positive cell types. **A–C:** Staining within the smooth muscle bundles of the fibromuscular stroma. A: Staining within vascular smooth muscle. C: Smooth muscle cell staining and the absence of staining in a nerve bundle (center left). **D:** Representative staining seen with pre-immune serum.

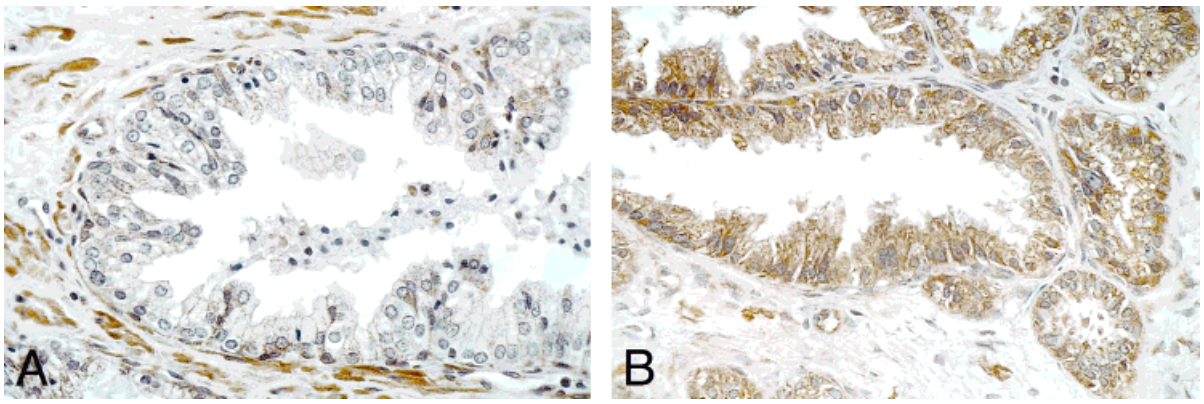

Fig. 2. Comparison of immunostaining for HB-EGF within prostatic and seminal vesicle epithelium. **A:** Absence of HB-EGF staining by epithelia within a benign epithelial duct. Similar patterns were seen for prostate cancer cells and for prostatic intraepithelial neoplasia (PIN), thought to be a precursor to prostate adenocarcinoma. **B:** In contrast to prostatic epithelium, intense cytoplasmic staining is seen in seminal vesicle epithelium.

observer unfamiliar with the study. Digital morphometry was performed using a computer algorithm that takes into account focal staining intensity as well as percentage area stained. Average staining levels in these two groups were 22.7 ± 4.6 vs 10.6 ± 3.1 integrated density units for BPH and prostate cancer, respectively. This difference was found to be statistically significant ($P < 0.05$), using Welch's alternate t-test. These data indicate that, in this series of

specimens, HB-EGF was not overexpressed in the prostate cancers in comparison to the benign tissues.

Responses of Prostatic Carcinoma Cells to HB-EGF and EGF

EGF has previously been demonstrated to be a growth factor for prostate carcinoma cells. To determine whether HB-EGF is also a growth factor for human prostate cancer cells and to evaluate its potency relative to EGF, HB-EGF and EGF were tested in mitogenic assays using the LNCaP human prostate adenocarcinoma cell line. LNCaP is considered the most faithful in vitro model of human prostate cancer because it is the only cell line to display most of the phenotypes associated with the in vivo disease: synthesis of the organ-specific marker protein, prostate-specific antigen (PSA), expression of functional androgen receptors, and stimulation of cell proliferation by androgens [reviewed by Chung, 1996]. In addition, HER1 mediates androgen-dependent growth in LNCaP cells [Liu et al., 1993; Sehgal et al., 1994]. In mitogenic assays, HB-EGF and EGF were similarly potent at inducing cell proliferation (Fig. 3). Similar results were obtained when ^{3}HTdR incorporation into DNA was used as an experimental end point (data not shown). Affinity cross-linking of ^{125}I-HB-EGF to LNCaP monolayers confirmed the presence of high affinity HB-EGF receptors in these cells (Fig. 4A). Consistent with the cell growth analysis, HB-EGF and EGF were quantitatively and qualitatively similar in inducing tyrosine phosphorylation of cellular proteins (Fig. 4B).

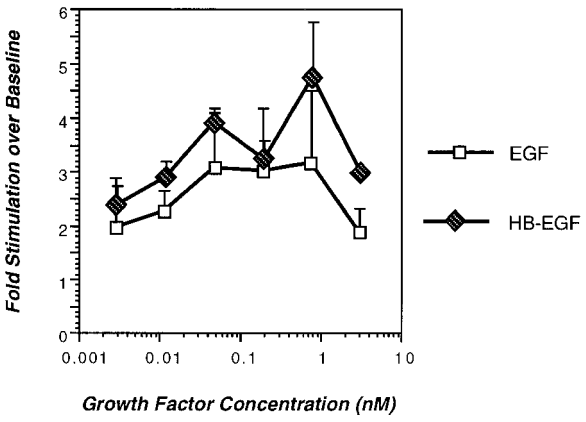

Fig. 3. Proliferation of LNCaP human prostate carcinoma cells in response to EGF and HB-EGF. LNCaP proliferation in response to the HER1 ligands, EGF and HB-EGF, was evaluated by examining the uptake of MTT following incubation with ligand for 7 days in reduced serum medium. Data are presented as fold stimulation over baseline in response to the indicated concentrations of recombinant human EGF or HB-EGF.

Although HB-EGF was originally identified as a ligand for HER1 [Higashiyama et al., 1991], at least one isoform of HER4 can be activated by HB-EGF by direct ligand binding [Elenius et al., 1997]. Therefore, to confirm the identity of the LNCaP HB-EGF receptor, cells were treated with HB-EGF and EGF and HER receptors 1–4 were immunoprecipitated using monospecific antibodies. The precipitates were tested in Western blots for receptor activation, using an anti-phosphotyrosine antibody. Activation of HER1 and HER3 was evident with both ligands (Fig. 4C). In contrast, activation of HER2 or HER4 was not detected. These data indicate that the cognate HB-EGF receptor in LNCaP cells is HER1, since HB-EGF does not bind HER3 [Beerli and Hynes, 1996]. Phosphorylation of HER3 is known to occur as a result of HER1 activation by ligand [Sliwkowski et al., 1994]; therefore, HER3 activation in this case can be concluded to be the result of HB-EGF-HER1 binding. RT-PCR analysis of LNCaP total RNA, using oligonucleotide probes for HER1–4 mRNAs, was also carried out. PCR products corresponding to HER1, HER2, and HER3 mRNAs were detected, while HER4 mRNA was not detected by this method (not shown). Thus, low levels of HER2 activation might have occured in the tyrosine phosphorylation experiment but might have gone undetected. Our data suggest that LNCaP cells do not express HER4.

proHB-EGF Expression in LNCaP Cells

HB-EGF mRNA was not detected in LNCaP cells by Northern blot hybridization of total cellular RNA; however, it was detected by RT-PCR (not shown). Comparison of steady-state expression levels of HB-EGF mRNA with other continuous tumor cell lines in our laboratory suggests that HB-EGF synthesis in LNCaP is relatively low. This interpretation is consistent with Western blot analysis of cell lysates (not shown). However, LNCaP cells showed specific sensitivity to diphtheria toxin (Fig. 5), indicating that proHB-EGF, the DT receptor in human cells [Mekada, 1995], is localized to the cell surface and available for DT-binding and toxin internalization.

DISCUSSION

A number of investigators have proposed that HER1 ligands (generally EGF and TGF-α), de-

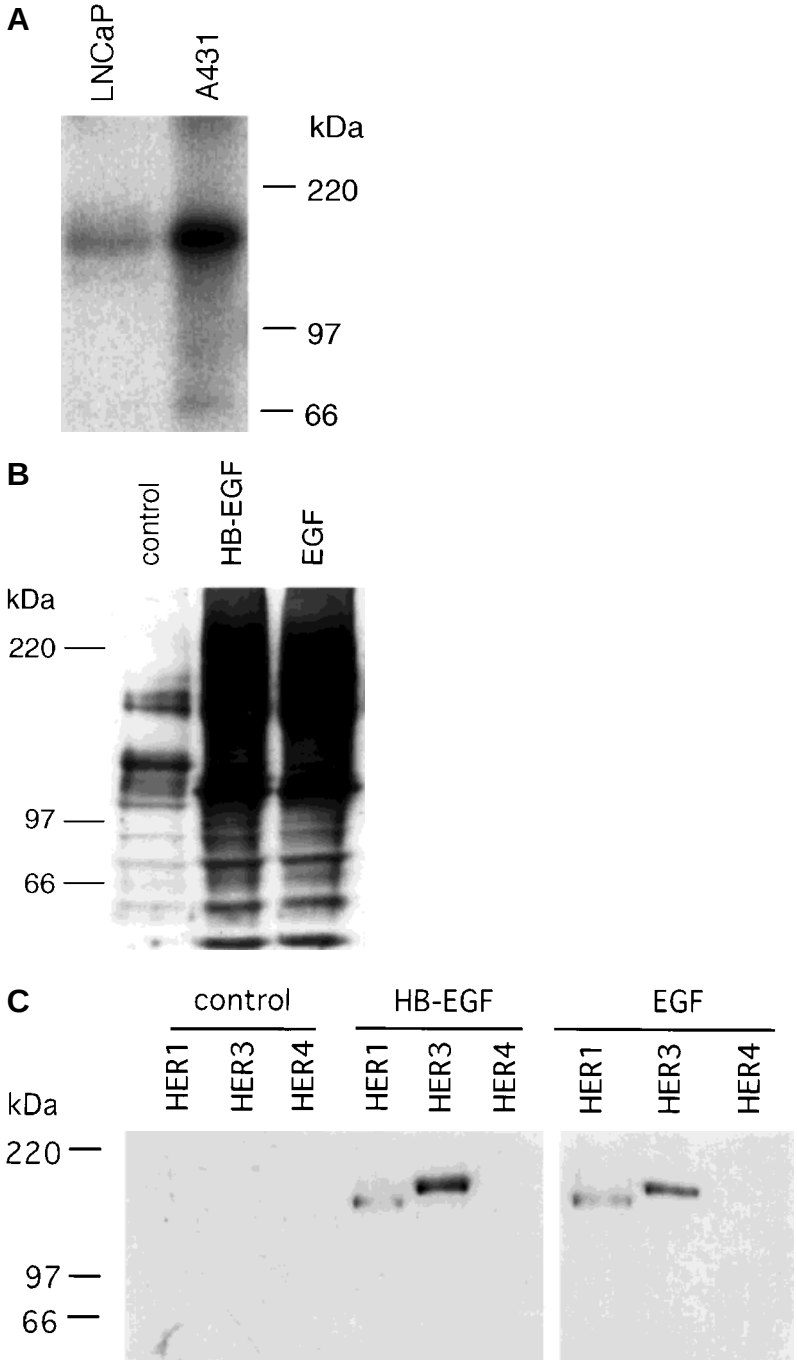

Fig. 4. HB-EGF activation of HER1 in LNCaP cells. **A:** Expression of HB-EGF receptors by LNCaP cells demonstrated by affinity cross-linking of ^{125}I-HB-EGF to cell monolayers. HER1-expressing A431 cells were used as a positive control. **B:** Anti-phosphotyrosine western blot of total cell protein prepared from LNCaP cells after stimulation with HB-EGF and EGF. **C:** Activation of HER receptors after stimulation of LNCaP cells with HB-EGF and EGF. After challenge with the growth factors, HER1, -2, -3, and -4 were immunoprecipitated with monospecific antibodies, and the precipitated material was examined by antiphosphotyrosine Western blot. There was no evidence of HER2 or HER4 activation in response to either EGF or HB-EGF (HER2 data not shown). The negative result for HER4 is shown because a HER4 isoform has recently been shown to be activated by HB-EGF by direct ligand binding [Elenius et al., 1997].

rived either from the epithelial or the stromal compartments, are involved in benign and malignant prostatic growth [Connolly and Rose, 1991; Myers et al., 1993; Harper et al., 1993; Brass et al., 1995; Sehgal et al., 1994; Marengo and Chung, 1994]. In this study, we have provided the first evidence that the HER1 ligand, HB-EGF, may also be involved in prostatic growth regulation. Using two monospecific anti-HB-EGF antibodies, one of which specifically recognizes the cytoplasmic domain of the membrane-anchored proHB-EGF form, we have shown that HB-EGF is synthesized and accumulates in benign and malignant human prostate tissues, primarily within the interstitial and vascular smooth muscle cells of the fibromuscular stroma. Significant levels of HB-EGF synthesis or localization were not detected in prostatic epithelia, even in malignant prostate cancer cells, and no elevation of HB-EGF expression in

prostate adenocarcinoma was detected in a morphometric analysis of a limited number prostate cancers and benign prostatic tissues. This expression pattern is in marked contrast with that reported for TGF-α, which is produced by malignant cells in human prostate cancer [Myers et al., 1993] and possibly at low levels by ductal epithelial cells in BPH [Harper et al., 1993]. Collectively, these data suggest that TGF-α and HB-EGF perform distinct physiologic roles within the prostate and possibly in prostatic disease.

Using androgen-dependent LNCaP human prostate carcinoma cells as a model system, HB-EGF and EGF were shown to qualitatively and quantitatively induce similar mitogenic effects, similar patterns of tyrosine phosphorylation of cellular proteins and identical patterns of HER/ErbB receptor co-activation. These experiments also identified HER1 as the HB-EGF receptor, and they confirmed the expression of HER3 in LNCaP cells. The presence of cell-surface HB-EGF was detected indirectly in LNCaP cells by DT-sensitivity tests; however, Western and Northern blot analysis suggested that synthesis of HB-EGF by LNCaP cells is likely to be low, relative to a number of other carcinoma lines we have studied. The HB-EGF mRNA and protein level determinations are consistent with our ability to inhibit protein synthesis in LNCaP cells with exogenous DT because of the extreme efficiency by which DT is internalized, even by low numbers of cell surface DT receptors [Mekada, 1995]. Low levels of HB-EGF expression by LNCaP may reflect the normal in vivo phenotype of prostatic epithelia and prostate carcinoma as described in this study. Notably, significant levels of HB-EGF synthesis were detected in seminal vesicle epithelium in vivo, indicating that HB-EGF expression is regulated in a cell type-specific manner within various epithelial tissues of the reproductive tract, and is therefore likely to perform a specialized function when present. Our findings also suggest that soluble HB-EGF produced locally by the seminal vesicle might promote growth of invasive prostate cancer cells, thereby contributing to further progression of the cancer.

HB-EGF and EGF both induced co-activation of the HER3 membrane tyrosine kinase in LNCaP cells. We did not detect HER2 or HER4 activation after challenge with both ligands. Based on RT-PCR analysis, LNCaP expresses HER2 but not HER4 mRNA. Interestingly, HER4 mRNA was also not detected in a recent tyrosine kinase expression survey of human prostate tissues by RT-PCR, suggesting that the absence of HER4 in LNCaP reflects the normal prostatic phenotype [Robinson et al., 1996]. The physiological significance of HER3 co-activation in response to a HER1 ligand has been seen previously in model systems [Soltoff et al., 1994; Beeri and Hynes, 1996], and its significance is unknown. Presumably, distinctive patterns of HER kinase activation reflect specific mechanisms of downstream signaling, based on the recognition that a diverse set of cytoplasmic proteins is recruited to the activation complex of each receptor dimer after ligand binding. Because HER3 is a high-affinity receptor for heregulins, HER3 expression in LNCaP also suggests that LNCaP responds to heregulin isoforms. We are now examining this possibility in our laboratory. Aberrant HER3 expression has been associated with prostatic intraepithelial neoplasia and invasive prostate cancer [Myers et al., 1994]. Because a number of studies have implicated HER1/ErbB1 in the regulation of prostate growth, and because HER1 acts coordinately with the related ErbB receptors when co-expressed with them, the consequences and mechanisms of signaling through the HER/ErbB receptors in prostate disease deserve further investigation.

Results from a variety of model systems developed over a number of years have led to the suggestion of an important role for diffusible mediators produced by prostatic mesenchymal cells, fibroblasts, smooth muscle cells, and other interstitial stromal cells in the control of growth and cytodifferentiation of the normal glandular epithelium as well as that of prostate cancer cells [reviewed by Cunha, 1994; Chung, 1995]. In the normal prostate, HER1 is predominantly expressed by basal cells of the prostatic ducts [Myers et al., 1993], presumably in a location where it is able to mediate epithelial cell signaling by ligands produced by the periductal stroma. In prostate tumor cells, HER1 can be overexpressed and can mediate androgen-dependent growth signals. Mostly uncharacterized stromal or mesenchymal factors have been shown to alter cell growth and cytodifferentiation of transplantable prostatic adenocarcinoma tumor implanted as tissue in rodent hosts [Chung et al., 1990; Hayashi and Cunha, 1991; Wong et al., 1992] and to promote malignant growth of human prostatic tumors implanted in rodents as single cells

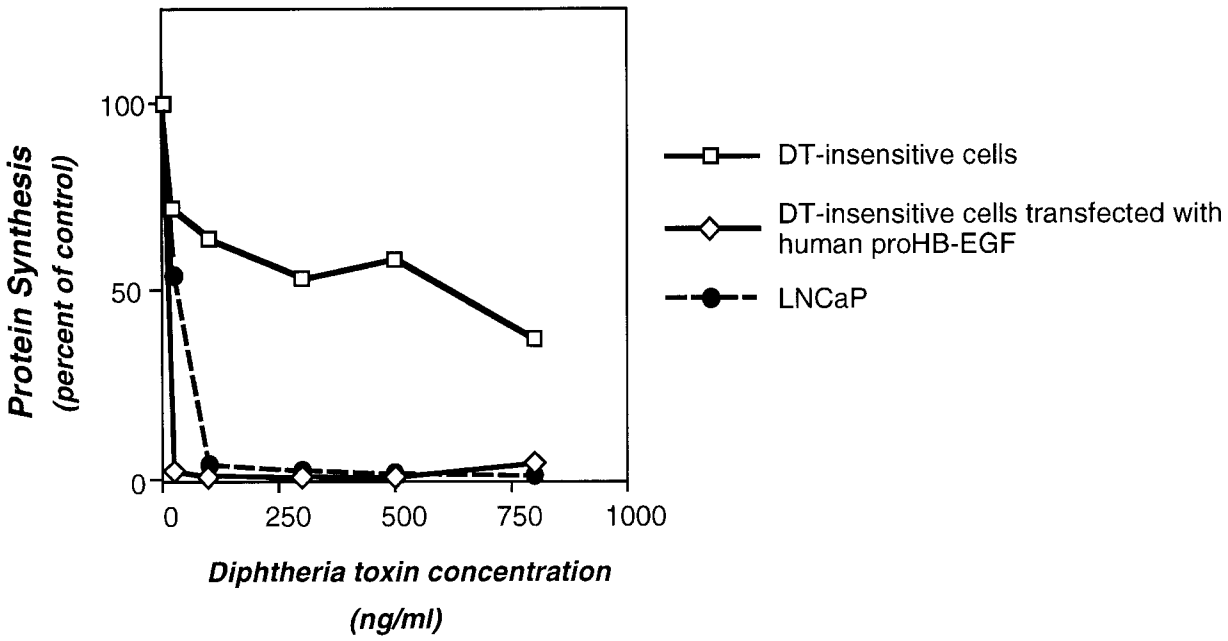

Fig. 5. LNCaP cells express DT receptors/proHB-EGF at the cell surface. Diphtheria toxin (DT) specifically inhibited protein synthesis in LNCaP cells, indicating the presence of functioning DT receptors/proHB-EGF at the plasma membrane. Negative control, DT-insensitive cells were NbMC-2 rat prostate tumor cells [Freeman et al., 1994] that are resistant to DT because of their rat origin. Positive control, DT-sensitive cells were NbMC-2 cells that express a transfected human proHB-EGF fusion protein [Chen et al., 1995]. LNCaP cells also express HB-EGF mRNA, as evaluated by RT-PCR, and HB-EGF-immunoreactive protein, as evaluated by Western blot analysis (not shown).

[Camps et al., 1990; Gleave et al., 1991]. Our present results suggest that HB-EGF originating from smooth muscle bundles, which ramify throughout the interstitial stroma and are in close apposition to the ductal epithelium [Hayward et al., 1995], might regulate glandular epithelia in the normal state. In addition, in prostate cancer HB-EGF might promote the growth of carcinoma cells within pseudoacini surrounded by stroma.

Importantly, HB-EGF is also a potent fibroblast and vascular smooth muscle cell mitogen and motility factor [Higashiyama et al., 1991, 1993], suggesting that its synthesis within prostatic stroma may also point to a specialized role specifically within the muscle cell types. Expansion of the stromal compartment can occur in BPH [Claus et al., 1993] and growth of a prostatic adenoma has been proposed to be the result of aberrant expression or activity of unknown prostatic growth factors [McKeehan, 1991; Cunha, 1994; Freeman et al, 1996]. An interesting possibility is that HB-EGF plays a role in hyperplastic growth of interstitial smooth muscle and undifferentiated fibroblasts. It is interesting to note that the demonstration of proHB-EGF synthesis and localization in prostatic muscle may also reflect a biological role distinct from growth regulation. The predominant form of HB-EGF present in prostatic smooth muscle cells is likely to be the transmembrane, pro- form. Experiments with a number of model systems have provided evidence that membrane proHB-EGF may play a number of diverse biological roles, independently of the mitogenic function of the secreted form. Plausible cellular activities for proHB-EGF based on in vitro data include juxtacrine intercellular signaling, cell-cell adhesion, and regulation of apoptosis [Raab et al., 1996; Higashiyama et al., 1995; Miyoshi et al., 1997]. The interstitial smooth muscle bundles in the prostate mediate the contractile response required to expel prostatic fluid into the ejaculate. The reported segregation of proHB-EGF into membraneous plaques during overt muscle differntiation in cell culture [Chen et al., 1995] suggests the interesting possibility that proHB-EGF is a component of the contractile machinery of the prostate, playing a role in muscle function that is unrelated to mitogenesis.

In summary, the results reported here have identified HB-EGF as a human prostate cancer cell growth factor that is a produced in vivo

primarily by the interstitial and vascular smooth muscle cells of the human prostate. These data suggest that HB-EGF is one of the hypothesized stromal mediators of prostate cancer growth. In addition, the diverse cellular activities of the secreted and membrane-bound forms of this growth factor also suggest the possibility that HB-EGF serves other functions within the prostate, distinct from a role as a diffusible mitogen.

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