Amphiregulin Is coordinately Expressed with Heparin-Binding Epidermal Growth Factor-Like Growth Factor in the Interstitial Smooth Muscle of the Human Prostate*

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ABSTRACT

Peptide growth factors have been proposed as mediators of smooth muscle-epithelial cell interactions in the human prostate; however, the identity of these molecules has not been established. In this study, we compared expression levels of messenger RNAs (mRNAs) encoding the epidermal growth factor (EGF) receptor-related receptor tyrosine kinases (ErbB1 through 4), the six EGF receptor ligands, EGF, transforming growth factor (TG)F-α, amphiregulin (ARG), HB-EGF, betacellulin, and epiregulin, and the related molecule heregulin-α, in a series of 10 prostate tissue specimens. Only EGF showed a disease-specific association, with increased mRNA levels in four of five PCA specimens in comparison to matched normal tissue from the same subject. In contrast, ARG and HB-EGF mRNAs showed a coordinate pattern of expression in 7/10 specimens that was distinct from all other growth factor or receptor genes examined and from mRNAs for prostate specific antigen, the androgen receptor and GAPDH, a housekeeping enzyme. Analysis of an additional series of benign prostatic hyperplasia and prostate cancer specimens from 60 individuals confirmed that ARG and HB-EGF mRNA levels varied in a highly coordinate manner (r = 0.93; P < 0.0001) but showed no association with disease. ARG was immunolocalized largely to interstitial smooth muscle cells (SMC), previously identified as the site of synthesis of HB-EGF in the prostate, while the cognate ARG and HB-EGF receptor, ErbB1, was localized exclusively to ductal epithelial cells and carcinoma cells. Although ARG was a relatively poor mitogen for Balb/c3T3 cells in comparison to HB-EGF, it was similar in potency to HB-EGF in stimulating human prostate epithelial cell growth, suggesting that prostate epithelium may be a physiologic target for ARG in vivo. Expression of both ARG and HB-EGF mRNAs was induced in cultured prostate SMC by fibroblast growth factor-2, a human prostate SMC mitogen linked to prostate disease. These findings indicate that ARG and HB-EGF are likely to be key mediators of directional signaling between SMC and epithelial cells in the human prostate and appear to be coordinately regulated. (Endocrinology 140: 5866–5871, 1999)
of carcinomas include transforming growth factor (TGF)-β, stromelysins (16–19) and vascular endothelial growth factor (20). Functional effects of stromal cell products on carcinoma growth in vivo have also been demonstrated. For example, aromatase activity produced locally by the tumor stroma is capable of promoting estrogen-dependent growth of human breast carcinomas in vivo (21).

In a series of recent reviews (22–24), Cunha and colleagues have pointed out that the physiologically relevant stromal-epithelial interaction in the prostate under normal conditions is likely to be an epithelial-SMC interaction. This argument derives primarily from correlative data in which epithelial and SMC differentiation occur coordinately within epithelial-mesenchymal tissue recombinants studied in model systems (22–24) and from the close apposition between SMC and ducal epithelial cells observed microscopically (25) in tissue sections. SMC are thus anatomically positioned to secrete paracrine factors capable of activating signaling cascades within the epithelial cells of the prostatic ducts. Although a few candidate stroma-derived soluble factors have been identified, which may act as mediators of epithelial cell growth and phenotypic differentiation in the prostate (26–28), persuasive evidence for the hypothetical paracrine factors that mediate epithelial-SMC interactions specifically has not been reported.

We recently identified heparin-binding epidermal growth factor (EGF)-like growth factor (HB-EGF) as a product predominantly of interstitial and vascular SMC of the human prostate (29). HB-EGF is one of six known activating ligands for the epidermal growth factor receptor (EGF-R)/ErbB1 receptor tyrosine kinase expressed by human cells (30). In the present study we demonstrate that a structurally related ErbB1 ligand, amphiregulin (ARG), is also expressed predominantly in the SMC of the prostatic stroma and, on the basis of an analysis of a large series of human prostate tissue specimens, appears to be coordinately expressed with HB-EGF. ErbB1 has been identified as a positive regulator of normal and transformed prostate epithelial cells and is expressed predominantly by basal epithelial cells in the ducal network and by prostate carcinoma cells (31, 32). The basal location of ErbB1 in the prostatic ducts, combined with the observation that HB-EGF and ARG are synthesized by the SMC of the interstitial stroma, suggests that these growth factors form a unique class of mediators of directional SMC → epithelial signaling in the prostate.

Materials and Methods

Materials

All chemicals were obtained from Sigma (St. Louis, MO), unless otherwise indicated. Penicillin-streptomycin solution, TRizol reagent, human recombinant epidermal growth factor (EGF), oligo(dT) 2-10 primer, custom PCR primers, 0.1 mM dithiothreitol (DTT), 5′ first-strand-α-nucleotide mix was from Roche Molecular Biochemicals (Indianapolis, IN). Recombinant TGF-α, heparin-binding EGF-like growth factor (HB-EGF), amphiregulin (ARG), betacellulin (BTC), and heregulin-α (HRG-α) were obtained from R+D Systems (Minneapolis, MN). Antibodies against CD44, anti-erbB1 antibody, Ab-1, and anti-erbB1 antibody were obtained from Neomarkers (Fremont, CA). Antibodies against CD44, anti-α-smooth muscle actin and antidesmin antibodies were from Sigma Immunochemicals, and antibody against high molecular weight (HMW) cytokeratin (34BE12), was from DAKO Corp., (Carpenteria, CA). The Vectastain ABC kit was from Vector Laboratories, Inc. (Burlingame, CA). PBS was from HyClone Laboratories, Inc. (Logan, UT). Normal prostate epithelial cells, dissociation reagents, serum-free medium and supplements were obtained from Clonetics Corp. (San Diego, CA).

Methods

Processing and RNA extraction of human prostate tissue samples. Prostate tissue biopsy specimens comprising normal prostate, tissue from benign prostatic hyperplasia, and tissue from prostate carcinoma were obtained at the time of surgery (Department of Urology, Brigham and Women’s Hospital, Boston, MA; and Department of Urology, Louisiana State University Medical Center, Shreveport, LA) as per IRB approval. Tissue specimens were snap frozen in liquid nitrogen and stored at −80°C until required. Tissue samples were thawed in TRIzol reagent and minced finely with razor blades to disperse tissue fragments, before proceeding with the extraction procedure, according to the manufacturer’s protocol. Microcarrier gel was added to samples at the start of the procedure to assist in extraction of small amounts of RNA and maximize yield. RNA pellets were reconstituted in dH2O rendered RNase-free by diethylpyrocarbonate treatment and RNA yield and purity were determined following following measurement of absorbance at 260 and 280 nm.

Complementary DNA (cDNA) preparation and amplification. Expression of messenger RNAs (mRNAs) of interest was assessed by RT-PCR following first-strand synthesis and precipitation of cDNA. Briefly, 3 μg total RNA was reverse-transcribed using oligo-dT primer and the reverse transcriptase Superscript II, according to the manufacturer’s protocol. First-strand synthesis was allowed to proceed at 42°C for 50 min, followed by denaturation of reverse transcriptase at 70°C for 15 min. cDNA was precipitated from the reaction mix by addition of 0.1 vol linear acrylamide, 1 vol 4 mM ammonium acetate, and 4 vol absolute ethanol at room temperature. cDNA was pelleted by centrifugation, pellets washed with 80% ethanol and reconstituted in 40 μl 10 mM Tris-Cl, 0.1 mM EDTA, pH 8. For subsequent amplification, 2 μl of cDNA and 0.4 μM each primer pair were used with 23 μl PCR Supermix, in the presence of 0.05 μl/reaction of 32P-dCTP. PCR cycling parameters were as follows: 1 cycle of 94°C for 5 min, 30 cycles of 94°C for 30 sec, denaturation, 50–58°C for 30 sec (annealing; see Table 1 for specific annealing temperature for primer pair), 72°C for 60 sec (extension), and 1 cycle of 72°C for 7 min. To ensure integrity and equivalence of RNA, separate reactions were performed using primers specific for the housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). All PCR products were analyzed by gel electrophoresis through a 5.1% acrylamide gel in 1 × Tris-borate-EDTA (TBE) buffer and bands were identified following exposure of x-ray film to dried gels. Densitometric analysis was performed using an IS1000 Digital Imaging System (Alpha Innotech Corp.) and band densities were normalized to that of GAPDH. Specific primer sequences are presented in Table 1.

Immunohistochemical analysis of ARG expression in human prostate. Expression of ARG protein in the prostate was assessed by immunohistochemical staining using three primary antibodies raised to distinct epitopes of the ARG protein. Serial sections of paraffin-embedded prostate tissue, comprising normal and tumor tissue, were deparaffinized in xylene and rehydrated by successive incubations in 100% and 95% ethanol and distilled water. Endogenous peroxidase activity was removed by incubation in 1% hydrogen peroxide and nonspecific binding was blocked by incubation in normal blocking serum for 1 h at room temperature. Primary antibodies were prepared at a dilution of 1/100 in PBS containing 1.5% normal blocking serum and incubated with tissue sections for 30 min, at room temperature. Biotinylated secondary antibody was added to slides for 30 min at room temperature, followed by incubation with avidin-biotin complex (ABC) reagent. Specific staining was identified following incubation with a solution of the chromogenic peroxidase substrate, diaminobenzidine (DAB)/hydrogen peroxide. Slides were counterstained with Gill’s hematoxylin and 1% ammonia, followed by successive dehydration in 95% ethanol, 100% ethanol and xylene before placement of coverslips.
Culture and characterization of primary prostate epithelial and smooth muscle cells. Normal human prostate epithelial cells (PrEC, strain 4428) were obtained at first passage (Clonetics Corp.) and propagated in serum-free growth medium supplemented with bovine pituitary extract (BPE), recombinant human EGF, hydrocortisone, gentamicin-amphotericin (GA), and three other growth-promoting factors to promote tritiated thymidine (3H TdR) incorporation into DNA. To evaluate the mitogenic potency of recombinant ARG and HB-EGF, the ability of these growth factors to promote Balb/c 3T3 thymidine incorporation assay. Briefly, Balb/c 3T3 fibroblasts were seeded in DMEM/10% Colorado calf serum (CCS) at a density of 3 x 10^4 cells/well in microtitre plates and grown to confluence. Receptor and ligand expression in the human prostate, we employed semiquantitative RT-PCR primer sequences

### RT-PCR primer sequences

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<th>Primer</th>
<th>Sequence (5′-3′)</th>
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Clonal growth assay of primary prostate epithelial cells. The response of prostate epithelial cells to exogenous ARG and HB-EGF was determined in a clonal growth assay. Cells were seeded in 25-mm dishes at a density of 70 cells per dish in PrEGM lacking hEGF and allowed to plate down overnight. Recombinant ARG or HB-EGF was added to the medium at the indicated concentrations and cells were incubated at 37°C for 10 days. At the end of the incubation period, growth medium was aspirated and cells were fixed in 3.7% formaldehyde for 15 min. To visualize colony formation, fixed cells were stained with 0.1% crystal violet for 15 min and excess dye was removed by thorough washing in tap water.

ARG and HB-EGF gene expression in prostate smooth muscle cells. Regulation of ARG and HB-EGF mRNA expression in response to stimulation with fibroblast growth factor (FGF)-2 or TGF-β1 was assessed in primary cultures of human prostate smooth muscle cells. Briefly, PrSMC were maintained in a humidified atmosphere at 37°C, 5% CO2 and used between passages 5 and 7.

### Results

**ErbB receptor and ligand expression in the human prostate**

To evaluate the potential role of the ErbB signaling axis in the human prostate, we employed semiquantitative RT-PCR to evaluate the relative mRNA expression levels of the four ErbB receptor subtypes and seven principal members of the EGF-like growth factor family in a panel of ten tissue specimens, comprising matched normal and tumor tissue from five individuals. Expression of androgen receptor and prostate-specific antigen (PSA) mRNAs was also evaluated (Fig. 1). All ten samples were found to express ErbB1, ErbB2, and ErbB3, with little or no expression of ErbB4 evident. Expression of ARG, betacellulin (BTC), EGF, HB-EGF,
Heregulin-α (HRG-α), and TGF-α, was demonstrated to a variable extent in all specimens, however none of the samples expressed epiregulin (EPR), consistent with its identity as a growth factor expressed primarily in early development (33, 34).

Most of the receptors or ligands appeared not to be expressed in a disease-specific manner, with the exception of EGF mRNA, which was demonstrably higher in the tumor tissue in four of five subjects in comparison to the matched controls. We also noted that the expression patterns for ARG and HB-EGF varied in a coordinate manner in a majority (7/10) of the tissue specimens. The epithelial cell component of the tissue specimens was likely to be similar, based on comparable PSA mRNA expression levels, suggesting that the apparent coordinate expression pattern of ARG and HB-EGF mRNAs was not a reflection of variable cellular content, but rather may be of biological significance. Quantitative evaluation of densitometric data indicated that HB-EGF and ARG mRNA levels only exhibited a positive correlation with each other. BTC and EGF mRNA levels also exhibited a positive correlation with each other (r = 0.72, with a positive slope of 0.94); however, a disease-specific association for BTC was not evident as was the case with EGF. EGF is known to be a secretory product of prostate epithelial cells and is present at high levels in prostatic fluid (35). Consequently, correlation of BTC and EGF mRNA levels suggests that BTC is likely to be an epithelial cell product in the prostate. Consistent with this possibility, we did not detect BTC mRNA in primary human prostate SMC cultured in vitro, whereas expression was detected in primary human prostate epithelial cells (PrEC) (data not shown). Positive correlations were also observed between BTC and HRG-α (r = 0.47) and BTC and TGF-α (r = 0.5). HRG-α and TGF-α are typically epithelial cell products (30).

These results suggest that ARG and HB-EGF may represent a unique class of prostatic ErbB1 ligand in that their expression appears to be coordinately regulated in a manner not observed with other members of the family. To further examine this possibility, ARG and HB-EGF mRNA expression analysis was performed on an additional sixty human prostate specimens comprising tissue from benign prostatic hyperplasia (BPH) and from prostate carcinoma (PCa). Expression of mRNAs encoding ARG and HB-EGF was normalized to that of the housekeeping gene, GAPDH, following densitometric analysis of PCR products. The data are presented in graphical form in Fig. 2. As seen with the first set of specimens, the level of expression of the ARG gene varied when plotted against the level of GAPDH mRNA, which remained relatively constant (Fig. 2A); the line of best fit through the data points exhibited a shallow slope, with a numerical value of 0.18. A similar plot was obtained for HB-EGF vs. GAPDH (data not shown). In marked contrast, however, the expression patterns for ARG and HB-EGF varied in a highly coordinate manner; when the band densities for ARG and HB-EGF were plotted against each other, the line of best fit displayed a much steeper slope, with a numerical value of 0.8 and a high degree of correlation (r = 0.930; P < 0.0001). When plotted separately, both BPH and PCa specimens displayed similarly steep positive slopes, of numerical values 0.786 (r = 0.935) and 0.790 (r = 0.925), respectively (Fig. 2B). No significant difference was detected between the BPH and PCa specimens with regard to the level or range of ARG and HB-EGF band intensities.

Localization of ARG in the human prostate

ARG protein expression in human prostate tissue was confirmed by immunohistochemical staining using three unrelated primary antibodies, each recognizing a distinct epitope within the mature ARG sequence. Immunostaining for ErbB1, the cognate receptor for ARG, was also performed using an antibody raised to the extracellular domain of the receptor. As shown in Fig. 3C, light positive staining for ARG was evident in epithelial cells comprising the prostatic ducts, with more intense staining in the adjacent stroma (Fig. 3D). Significantly, stromal staining for ARG was localized primarily to the interstitial and vascular smooth muscle cells and displayed a pattern similar to that previously observed by us for HB-EGF (29). Consistent with previous reports (32, 36–39), ErbB1 staining was localized to the basal epithelial cells of normal prostatic ducts (Fig. 3B), with less intense staining for ErbB1 evident in the pseudoacini characteristic of normal prostatic ducts.
of prostate carcinoma. Little or no stromal staining for ErbB1 was demonstrated, also consistent with previous observations.

Mitogenic activity of ARG and HB-EGF on prostate epithelial cells

Localization of ARG and HB-EGF to the SMC compartment, and ErbB1 to the basal epithelial cells, suggested the possibility of paracrine activation of epithelial ErbB1 by smooth muscle-derived ARG and HB-EGF. ErbB1 expression was confirmed in primary culture PrEC by RT-PCR (Fig. 4a) and Western blot (data not shown). ErbB2 and ErbB3 mRNAs were also detected in these cells, suggesting the possibility for receptor coactivation following ligand binding to ErbB1. Mitogenic activity of ARG and HB-EGF was assessed using PrEC as a target cell. Both growth factors demonstrated similar potency as PrEC mitogens (Fig. 4B). This result was somewhat surprising because ARG has been described in the literature as being significantly less potent as an epithelial or fibroblast cell mitogen in comparison to EGF (40) or to HB-EGF (41). To verify this, we examined the relative potency of EGF, ARG, and HB-EGF in a standard thymidine incorporation assay using Balb/c 3T3 fibroblasts as the target cell, as described in Materials and Methods. As shown in Fig. 4C, whereas HB-EGF was found to be as potent as EGF in promoting DNA synthesis in Balb/c 3T3 cells, ARG was 100-fold less active, consistent with its activity described previously (40, 41).

Coordinate regulation of ARG and HB-EGF in prostate smooth muscle cells

From our mRNA expression analysis of a large number of prostate tissue specimens, described above, ARG and HB-EGF mRNAs were found to be expressed in a highly coordinate manner, suggesting that these genes are coordinately regulated in the SMC compartment in vivo. ARG and HB-EGF were expressed by human prostate SMC (PrSMC) in primary culture (Fig. 5A). To determine whether independent evidence for coordinate regulation of these growth factors might be obtained, we analyzed the expression of ARG and HB-EGF mRNAs following treatment of primary cultures of PrSMC with FGF-2 or TGF-β1, as described in Materials and Methods. Both FGF-2 and TGF-β1 are known to be expressed in the prostatic stroma and altered expression of these molecules has been implicated in benign and malignant prostate pathologies (42, 43). Expression levels for ARG and HB-EGF mRNAs were normalized to that of GAPDH following densitometric analysis of autoradiographs. Figure 5, B and C, illustrates that the expression of both ARG and

Fig. 2. Coordinate expression of ARG and HB-EGF mRNAs in human prostate tissue. Following PCR to amplify products specific for ARG, HB-EGF or GAPDH, densitometry of bands was performed; band density for ARG and HB-EGF was normalized to that for GAPDH. In (A), GAPDH band density (arbitrary units) is plotted against ARG band density (arbitrary units) (n = 60); in (B) HB-EGF band density is plotted against ARG band density for BPH specimens (left panel; n = 20) and PCa specimens (right panel; n = 40). In each case, the line of best fit was computed and the numerical value of the slope is displayed. The correlation coefficient, r, and P values for the linear correlation are also shown.
HB-EGF could be up-regulated in response to treatment with FGF-2 and/or TGF-β1. In response to treatment with FGF-2, ARG expression increased up to 3.5-fold, with peak expression observed at 8 h following treatment; in contrast, no difference in ARG expression in response to TGF-β1 stimulation, was observed relative to control. HB-EGF expression in PrSMC was found to increase up to 2.7-fold and 2.8-fold in response to treatment with FGF-2 and TGF-β1, respectively; peak expression of HB-EGF mRNA in response to FGF-2 occurred at 24 h following treatment, whereas HB-EGF mRNA expression in response to TGF-β1 stimulation was maximal at 4–8 h and decreased thereafter.

**Discussion**

In this study, we have presented evidence for a directional paracrine signaling axis between the SMC compartment and the ductal epithelial cells of the adult human prostate. This signaling mechanism involves the synthesis of the soluble ErbB1 ligands, ARG and HB-EGF, by prostate SMC and the interaction of these growth factors with cells expressing their cognate receptor, ErbB1, which is present exclusively in the epithelial tissue compartments (the normal prostatic ducts and pseudoacini and foci of prostate adenocarcinoma). A SMC → epithelial paracrine mechanism has been hypothesized to exist within the prostate by Cunha and co-workers (22–24). These investigators have suggested that SMC-epithelial signaling may be the predominant means of stromal-epithelial interaction in the fully differentiated organ. This hypothesis was based on the anatomical relationship between SMC and the ductal network in rodent prostate, on results from other organ systems, such as the uterus, and on experimental models in which urogenital mesenchyme and epithelia (typically from rodents) are recombined and their differentiation state evaluated after engraftment under the renal capsule. However, before this report, evidence that this paracrine system actually exists in the adult human prostate was indirect and limited, although several candidate growth factors have been proposed as potential mediators of such an interaction.

We now present a variety of independent lines of evidence in support of the existence of this paracrine signaling mechanism in humans. Using immunohistochemistry, we have identified the SMC compartment of the human prostate as a site of expression of amphiregulin, an activating ligand for ErbB1. A series of previous reports have localized ErbB1 ligands, ARG and HB-EGF, by prostate SMC and the interaction of these growth factors with cells expressing their cognate receptor, ErbB1, which is present exclusively in the epithelial tissue compartments (the normal prostatic ducts and pseudoacini and foci of prostate adenocarcinoma). A SMC → epithelial paracrine mechanism has been hypothesized to exist within the prostate by Cunha and co-workers (22–24). These investigators have suggested that SMC-epithelial signaling may be the predominant means of stromal-epithelial interaction in the fully differentiated organ. This hypothesis was based on the anatomical relationship between SMC and the ductal network in rodent prostate, on results from other organ systems, such as the uterus, and on experimental models in which urogenital mesenchyme and epithelia (typically from rodents) are recombined and their differentiation state evaluated after engraftment under the renal capsule. However, before this report, evidence that this paracrine system actually exists in the adult human prostate was indirect and limited, although several candidate growth factors have been proposed as potential mediators of such an interaction.

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We now present a variety of independent lines of evidence in support of the existence of this paracrine signaling mechanism in humans. Using immunohistochemistry, we have identified the SMC compartment of the human prostate as a site of expression of amphiregulin, an activating ligand for ErbB1. A series of previous reports have localized ErbB1 ligands, ARG and HB-EGF, by prostate SMC and the interaction of these growth factors with cells expressing their cognate receptor, ErbB1, which is present exclusively in the epithelial tissue compartments (the normal prostatic ducts and pseudoacini and foci of prostate adenocarcinoma). A SMC → epithelial paracrine mechanism has been hypothesized to exist within the prostate by Cunha and co-workers (22–24). These investigators have suggested that SMC-epithelial signaling may be the predominant means of stromal-epithelial interaction in the fully differentiated organ. This hypothesis was based on the anatomical relationship between SMC and the ductal network in rodent prostate, on results from other organ systems, such as the uterus, and on experimental models in which urogenital mesenchyme and epithelia (typically from rodents) are recombined and their differentiation state evaluated after engraftment under the renal capsule. However, before this report, evidence that this paracrine system actually exists in the adult human prostate was indirect and limited, although several candidate growth factors have been proposed as potential mediators of such an interaction.
immunolocalization in this study is consistent with the conclusion that ErbB1 expression is restricted to the epithelial tissue compartments. This cellular location indicates that ErbB1 is present at a privileged site, i.e. in the basal layer of the ductal epithelium, for regulatory control by one or more of the receptor’s cognate ligands were they to originate from the stroma. Our finding that ARG is expressed by prostate SMC in vitro is consistent with an earlier report from our laboratory in which we demonstrated that a growth factor that is structurally related to ARG, HB-EGF, is also synthesized predominantly by SMC in the prostate (29). Therefore, ARG and HB-EGF expression in vivo is likely to result in diffusion of the soluble forms of the growth factors from the stromal to the epithelial compartments.

Using in vitro clonal growth assays, we demonstrated that both ARG and HB-EGF are potent mitogens for normal human prostate epithelial cells, consistent with a potential paracrine role for both growth factors. Notably, the epithelial cells we used for the mitogenic assays express what appears to be a basal cell phenotype, based on their expression of CD44 and high molecular weight cytokeratins (45–47). A basal cell phenotype has also been ascribed to cultured prostatic epithelial cells by others (48–50). Therefore, although the limitations of in vitro models should be considered, this experiment may be physiologically relevant, in that normal basal PrEC, which express ErbB1 in vivo, are shown to be highly responsive to both mitogens. ARG has been described in the literature as a growth factor typically exhibiting one or two orders of magnitude lower potency than either EGF or HB-EGF (40, 41). This observation was confirmed by us using a standard Balb/c 3T3 thymidine incorporation assay, in which we showed ARG to be significantly less mitogenic, on a molar basis, in comparison to HB-EGF and EGF. In light of these findings, the equivalent molar potency of ARG and HB-EGF in the PrEC clonal growth assay was unexpected but may identify prostate epithelial cells as a specific physiologic target for the actions of ARG.

In an analysis of a large series of benign and malignant human prostate tissues, HB-EGF and ARG mRNAs were found to exhibit highly coordinate expression, a result that is consistent with the conclusion that they are synthesized predominantly within the same tissue compartments. Moreover, we also observed that both HB-EGF and ARG mRNAs could be induced in human prostate SMC by FGF-2 (basic FGF), a growth factor synthesized within the stromal compartment that has been linked to pathologic cell growth in the prostate (42). FGF-2 has been shown previously to be a human prostate SMC mitogen (51). HB-EGF mRNA expression was also induced by TGF-β1, which has been linked to both prostate cancer and BPH (43). These data indicate that the HB-EGF and ARG genes are under partial coordinate regulation in prostate SMC, a finding that is also likely to account, at least in part, for the high degree of coordinate expression we observed in vivo. Consistent with this interpretation, coordinate expression of ARG and HB-EGF has been demonstrated previously in several published studies employing cultured cells (52–54). EGF mRNA levels were increased in four of five prostate carcinoma specimens in comparison to matched normal tissue from the same patient, and EGF mRNA levels were positively correlated with mRNA for BTC. EGF is principally a secretory product of epithelial cells in the prostate (35). ARG and HB-EGF mRNA levels, in contrast, did not show a disease-specific association and were observed only to correlate with each other. These data suggest a distinct mechanism of regulation and/or cellular localization for ARG and HB-EGF in comparison to other members of the ligand family. The most recently identified ErbB1 ligand, epiregulin, was not detected in the prostate, consistent with previous reports of a restricted pattern of expression in placenta, uterus, peripheral blood cells and

FIG. 4. Mitogenic activity of ARG and HB-EGF on prostate epithelial cells. A, ErbB receptor expression in normal human prostate epithelial cells (PrEC), as determined by RT-PCR. PCR products of 729 bp (ErbB1), 824 bp (ErbB2), 1131 bp (ErbB3) and 1051 bp (ErbB4) were expected. B, Colony formation in duplicate wells of primary human (ErbB1), 1131 bp (ErbB3) and 1051 bp (ErbB4) were expected. B, Colony formation in duplicate wells of primary human prostate epithelial cells (PrEC) in vitro, in response to no added growth factor (control), 0.16 nM or 2.5 nM recombinant ARG or HB-EGF. C, Relative mitogenic potency of recombinant ARG and HB-EGF as determined in a standard Balb/c 3T3 thymidine incorporation assay. The graph shows uptake of tritiated thymidine (cpm) in Balb/c 3T3 fibroblasts, in response to increasing doses of EGF (●), HB-EGF (○) or ARG (●). Data points represent the mean of duplicate determinations and the data are representative of at least three experiments.
during early development (33, 34). ErbB2 and ErbB3 receptor mRNAs, and the ErbB3/ErbB4 ligand, HRG-α, were detectable, but their expression pattern did not resemble that seen for HB-EGF and ARG. ErbB4 mRNAs were faint or undetectable, consistent with previous reports that this receptor is not expressed in the prostate (55).

Early studies on the role of ErbB1 in paracrine and autocrine growth regulation in the prostate assessed the possible function of the ErbB1 activating ligand, transforming growth factor-α (TGF-α). TGF-α is known to be expressed in the prostate (28) and potential sites of TGF-α synthesis have been identified in the epithelial and stromal compartments by in situ hybridization (44, 56) and immunohistochemical analyses (32, 36, 38). In the normal or benign adult prostatic tissues, TGF-α protein expression has typically been observed to increase in prostate carcinoma cells in comparison to normal epithelial cells (32, 37, 39), reminiscent of the expression pattern in early development (32). Co-expression of TGF-α and ErbB1 within epithelial cells has been proposed to enable the “switch” from paracrine to autocrine ErbB1 activation and to promote the uncontrolled carcinoma cell proliferation characteristic of tumor progression (32, 39).

From our mRNA expression analysis, TGF-α mRNA was present in normal and tumor tissue, however, it was not coordinately expressed with HB-EGF and ARG, suggesting that these molecules are subject to different regulatory mechanisms in vivo. Furthermore, because TGF-α has been identified in the prostatic epithelium, as well as the stroma, our data suggest that this molecule acts primarily as an autocrine growth factor in the prostate and less so as a mediator of stromal-epithelial interactions.

Taken together, we conclude from these observations that ARG and HB-EGF act as physiologic SMC-derived paracrine regulators of prostatic epithelium and may play a similar functional role in vivo. The significance of the apparent coordinate regulation of these factors in the prostate is unknown; however, from their defined biological activities, ARG and HB-EGF may function as mediators of epithelial cell growth, differentiation or survival. In addition, their roles in regulation of cancer cell behavior may differ from those performed in normal tissue. Lin et al. (57) recently identified several ErbB1 ligands, including HB-EGF, as cell
survival factors, distinct from their role as mitogens, for human prostate carcinoma cells. These investigators also observed that the cell survival pathways operating in prostate cancer cells were distinct from those in normal PrECs. HB-EGF and ARG have a similar domain structure and exhibit similar biochemical affinities for immobilized heparin. In addition, their membrane-anchored, precursor forms have been shown to interact functionally with CD9 (58), a membrane protein that belongs to the tetraspanin protein family. CD9 is capable of altering the juxtaocrine activities of membrane HB-EGF and ARG. Interestingly, however, the membrane form of TGF-α appears not to interact with CD9, suggesting that HB-EGF and ARG may play similar functional roles distinct from those performed by TGF-α (58). The localization of ARG and HB-EGF synthesis in prostate SMC is also consistent with a role for these growth factors as “andromedins,” hypothetical peptide mediators of the androgenic signaling characteristic of the prostate gland. ARG synthesis has been reported previously to be under the control of androgens in the anaplastic human prostate cell line, LNCaP (59). Notably, Prins et al. (60) found that although stromal fibroblasts and basal epithelial cells in the prostate were generally found to lack the androgen receptor (AR), strong AR staining was evident in SMC, supporting the proposal that AR-positive smooth muscle cells could mediate stromal-epithelial interactions. Whether the AR lies upstream of ARG and HB-EGF synthesis in vivo remains to be examined; however, it is conceivable that these growth factors play a role in the maintenance of the functional integrity of the gland in the normal physiologic state. Recently, Levine et al. (61) reported that androgen stimulated increased gene expression and synthesis of VEGF by isolated prostate stromal cells, consistent with the hypothesis that androgen-dependent growth factor signaling can be stroma-mediated.

A number of other growth factors have been localized to the prostate and have been suggested as mediators of stromal-epithelial interactions, including members of the fibroblast growth factor (FGF) (42, 62), TGF-β (43, 63) and insulin-like growth factor (IGF)-families (64, 65). Of these, only the fibroblast growth factor, FGF-7/keratinocyte growth factor (KGF) has emerged as an unambiguous candidate, based on localization of the mRNA and protein, the expression pattern for the receptor and regulation by androgen. KGF mRNA has been localized to stromal cells by in situ hybridization analysis in the human (66, 67) and rat (68, 69) prostate, whereas the high-affinity KGF receptor is localized exclusively to prostatic epithelial cells (66–69), thus presenting the opportunity for directional stromal-epithelial paracrine interactions. Furthermore, KGF expression has been shown to be androgen-regulated in isolated prostatic stromal and epithelial cells in co-culture (70). Also KGF was found to partially substitute for androgen in branching morphogenesis of the rat ventral prostate (68) and in development of the rodent seminal vesicle (71), strongly implicating KGF action in the androgen-stimulated phenotype and behavior. However, more recent evidence suggests that KGF expression in the rat prostate is unresponsive to changing androgen levels (68, 72) in vivo. This suggests further studies are required to confirm the identity of KGF as a true andromedin.

In summary, we have presented the first evidence that two structurally similar ligands for the ErbB1 receptor tyrosine kinase, ARG and HB-EGF, are directional mediators of ErbB1-dependent signaling between the SMC and epithelial compartments of the human prostate and may be regulated in a coordinate manner in vivo. These findings are potentially relevant to the normal function of the prostate gland as a secretory organ as well as to several prostate diseases, including prostate cancer and benign prostatic hyperplasia (BPH), a condition of aberrant prostatic enlargement and urethral obstruction that afflicts most men with age. Our results provide further evidence for the critical importance of the stroma in regulating epithelial cell growth and behavior.

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