Activation of the Erk Mitogen-activated Protein Kinase Pathway Stimulates Neuroendocrine Differentiation in LNCaP Cells Independently of Cell Cycle Withdrawal and STAT3 Phosphorylation

Jayoung Kim, Rosalyn M. Adam, and Michael R. Freeman

The Urologic Laboratory, Department of Urology, Children’s Hospital, and the Department of Surgery, Harvard Medical School, Boston, Massachusetts 02115

ABSTRACT

Neuroendocrine (NE) differentiation in prostate cancer (PCa) has been found in some studies to correlate with unfavorable clinical outcome. The mechanisms by which PCa acquires NE properties are poorly understood. In this study, we demonstrate that heparin-binding epidermal growth factor-like growth factor (HB-EGF), a prostate smooth muscle-derived mitogen and survival factor, can evoke NE differentiation in LNCaP human PCa cells. HB-EGF induction of NE differentiation was mediated by a mitogen-activated protein kinase (MAPK) kinase-dependent mechanism, and this process was blocked by p38 MAPK signaling. NE differentiation induced by HB-EGF occurred independently of STAT3 phosphorylation and coincided with continued cell cycle transit. These findings suggest that endogenous stroma-derived factors, acting through MAPK signaling pathways, may play a significant role in the acquisition of NE properties by PCa cells. They also demonstrate that withdrawal from the cell cycle is not a prerequisite for expression of NE characteristics by PCa.

INTRODUCTION

Epithelial cells of the human prostate are generally classified as either basal, secretary, or NE, based on their morphology, location, and expression of marker proteins. NE cells comprise a minor subpopulation of the normal prostatic epithelium, and PCa exhibiting predominantly NE characteristics are rare, comprising only 0.5–2% of clinical specimens. Nevertheless, NE differentiation in PCa is of interest because tumors with a prominent NE component are typically androgen independent and highly aggressive and because many PCa are infiltrated with NE-like cells (1–3). Although reports vary on the extent of NE differentiation in PCa (4–9), evaluation of NE cell number has been correlated with androgen-independent disease and poor prognosis in some studies (4–7). Several authors have argued that even a small NE component has the potential to stimulate growth and progression of PCa, primarily through paracrine mechanisms.

NE-like cells can secrete a variety of cytokines and neurohormones linked to growth and survival in prostate and other cancer cells (8). Secretion of these molecules into the extracellular space by NE cells may affect adjacent adenocarcinoma (9–13). Furthermore, the NE differentiation process itself has been proposed to involve cell cycle arrest (14, 15), thus rendering differentiated tumor cells resistant to radiation and chemotherapy.

The molecular basis by which NE cells arise in prostatic tumors is not understood; however, studies have demonstrated that significant plasticity exists between the adenocarcinoma and NE phenotypes. This suggests that NE cells arise from adenocarcinoma cells in response to epigenetic stimuli. NE differentiation can be induced in PCa cells by several means, including IL-6, agents that increase levels of intracellular cyclic AMP, and culture in steroid-depleted medium (14–19). Although the transcription factor, STAT3, and the tyrosine kinase, Erk, have been implicated in NE differentiation in LNCaP human PCa cells (17, 20, 21), the signaling mechanisms underlying acquisition of NE properties by PCa cells remain undefined. Moreover, the potential role of the prostatic microenvironment, e.g., the influence of the prostatic stroma, in control of NE differentiation in PCa has not been established.

HB-EGF is an EGFR/ErbB1 ligand that is synthesized primarily by interstitial and vascular SMCs of the prostatic stroma (22). HB-EGF is a potent mitogen and survival factor for prostate epithelial and carcinoma cells (22–24). EGFR activation by HB-EGF and other ErbB1 ligands triggers signaling through the Erk MAPK pathway, resulting in diverse biological responses (25). The stromal location of HB-EGF synthesis, coupled with the observation that EGFR is expressed principally by prostatic epithelial and carcinoma cells (24), suggests that HB-EGF mediates directional paracrine signaling from the SMCs to the epithelial compartment.

In this study, we demonstrate that HB-EGF is a mediator of NE differentiation in PCa cells. Our results suggest that endogenous stroma-derived factors, acting through the Erk-MAPK signaling pathway, may play a significant role in the acquisition of NE properties by PCa cells.

MATERIALS AND METHODS

Reagents. Human recombinant HB-EGF and neutralizing anti-HB-EGF antibody were purchased from R&D Systems, Inc. (Minneapolis, MN). Anti-NSE antibody was obtained from Neomarkers (Fremont, CA). IL-6 was from Upstate Biotechnology, Inc. (Lake Placid, NY), and charcoal dextran-treated serum was from HyClone Laboratories, Inc. (Logan, UT). SB203580, PD153035, and PD098059 were from Calbiochem (San Diego, CA). Antiphospho-Erk, anti-Erk, antiphospho-p38, anti-p38, antiphospho-STAT3 (Tyr705), antiphospho-STAT3 (Ser277), and anti-STAT3 antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antiactin antibody was from Sigma Chemical Co. (St. Louis, MO).

Cultured Cells. The human PCa cell line LNCaP was purchased from American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (Life Technologies, Inc., Grand Island, NY). These supplements were used in all of the media unless otherwise indicated. Cells were cultured in a humidified atmosphere of 5% CO₂ at 37°C.

HB-EGF Expression Construct and Generation of Stable Transfectants. To generate siHB-EGF, a fragment encoding the mature soluble form of HB-EGF was amplified by PCR from a previously engineered HB-EGF construct (26) using the primers: 5’-GGATCCCTAGAAACGCTGGTCGAGTGGTGTG-3’ and 5’-AAGTCGGGGCCTTCCACTGGGAGCTCAG-3’.

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Supported by NIH Grants R37 DK47556, ROI CA77386, and ROI DK57691 (to M. R. F.) R. M. A. is an American Foundation for Urological Disease Research Scholar. 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.

1 The abbreviations used are: NE, neuroendocrine; PCa, prostatic adenocarcinoma; Erk, extracellular signal regulated; EGFR, epidermal growth factor receptor; HB-EGF, heparin-binding EGFR-like growth factor; MAPK, mitogen-activated protein kinase; SMC, smooth muscle cell; FBS, fetal bovine serum; siHB-EGF, constitutively secreted mutant of HB-EGF; NSE, neuron-specific enolase; HB-EGF, soluble HB-EGF; cFBS, charcoal-stripped fetal bovine serum; sHB-EGF, constitutively secreted mutant of HB-EGF; Statistical Analysis System; IL-6, interleukin-6; PD153035, PD098059, PD098059, CYp, cyclin-dependent kinase inhibitor; MDR1, multidrug resistance gene; KG015, tunicamycin; HHMEC, human dermal microvascular endothelial cells; PLA2, phospholipase A2.

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which incorporated recognition sites for BamHI and Apal, respectively. The resulting PCR product, corresponding to soluble HB-EGF, in which the COOH-terminal residue was Glu (151) as described previously (27), was ligated into pcDNA3.1 MycHis (Invitrogen) and subcloned into pIRES/Hyg (Clontech, Palo Alto, CA) to create pIRES/sHB-EGF. pIRES/sHB-EGF was transfected into LNCaP using FuGENE 6 transfection reagent (Roche Molecular Biochemicals, Indianapolis, IN), and stable populations isolated after selection in 200 µg/ml hygromycin. Cells transfected with empty vector (pIRES/Hyg) served as controls (vector only). Expression of soluble HB-EGF was confirmed by immunoblot analysis of heparin-binding proteins in conditioned medium from vector-only and sHB-EGF cell populations. Detection of sHB-EGF was performed using an antibody against the HB-EGF ectodomain (R&D Systems, Inc).

The MKK3b(EE) construct was kindly provided by Dr. Anning Lin at the University of Chicago and was transfected into subconfluent LNCaP cells by calcium phosphate-DNA precipitation. Stable transfectants were selected in RPMI 1640 containing 100 µg/ml G418.

**Western Blot Analysis.** Cell lysates were prepared in lysis buffer containing 62.5 mM Tris-Cl (pH 6.8), 2% SDS, and 10% glycerol. Proteins were electrophoresed through SDS-PAGE gels and electroblotted onto nitrocellulose membranes. Western blotting was performed as described previously (22).

**Cell Cycle Analysis.** Subconfluent LNCaP cells were serum starved for 12 h. Cells were treated with 10 µM nocodazole for 30 min, after which medium was replaced with RPMI 1640 containing 10% FBS, nocodazole, and the agents described in the text (50 ng/ml IL-6, 10 µM SB203580, or 50 ng/ml HB-EGF). After treatment (48 h), cells were trypsinized, washed, and resuspended in 70% ethanol for 20 min at 4°C. Cells were then washed and resuspended in staining solution containing 10 µg/ml propidium iodide and 50 µg/ml RNase A in PBS, incubated for 30 min at 37°C, and analyzed with fluorescence activated cell sorting.

**RESULTS**

**HB-EGF Stimulates NE Differentiation in LNCaP Cells.** The effect of recombinant HB-EGF on LNCaP cell morphology was examined. In comparison with cells cultured in RPMI/10% FBS, LNCaP cells treated with HB-EGF under serum-free conditions extended neuron-like processes and adhered more tightly to culture plates (Fig. 1A). These morphological changes were similar to descriptions in the literature of LNCaP cells undergoing differentiation toward an NE phenotype (14, 17, 21). To evaluate NE differentiation more objectively, expression of NSE was examined by Western blot. This marker has been used previously to assess the extent of NE differentiation in LNCaP cells (14, 17, 21). LNCaP cells were serum starved and treated with varying doses of HB-EGF (0–50 ng/ml) for 6 days or with a single dose (50 ng/ml) for varying times (0–6 days). NSE expression was dramatically increased in a dose- and time-dependent manner under these conditions compared with controls and reached a maximum level 6 days after a single treatment with HB-EGF (Fig. 1B, C). NSE levels in cells treated with HB-EGF were suppressed by the addition of a neutralizing anti-HB-EGF antibody (Fig. 1D). These findings identify HB-EGF as a factor capable of stimulating NE differentiation in LNCaP cells.

To explore this effect in greater depth, LNCaP cells were engineered to express sHB-EGF constitutively. Endogenous HB-EGF levels are extremely low in LNCaP cells (data not shown); therefore, the transfected form is the major source of HB-EGF produced by the cells in these experiments. Consistent with the above experiments, LNCaP/sHB-EGF transfectants expressed high levels of NSE after culture in serum-free medium for 6 days. NSE levels in LNCaP/sHB-EGF transfectants were higher than those seen when control cells were treated with IL-6, a known inducer of NE differentiation in LNCaP cells and a positive control in the experiment (Fig. 2A).

Culture of control cells in steroid-depleted medium also induced NSE expression (data not shown), consistent with published data (18). Interestingly, we found that an inhibitor of the p38 MAPK pathway, SB203580, also induced NSE expression. In several independent trials, a one-time treatment with 10 µM SB203580 induced higher NSE levels than IL-6 (Fig. 2B).

**Erk-MAPK Activation Mediates NE Differentiation in LNCaP Cells.** HB-EGF, an activating ligand for the EGFR (22), triggers the Erk-MAPK phosphorylation cascade (25), which typically mediates signaling events distinct from those regulated by the p38 MAPK pathway. Collectively, our data suggested that NE differentiation can be triggered by activation of the Erk-MAPK cascade and that this is antagonized by p38 MAPK pathway activation. To test this hypothesis, we performed the following experiments. The extent of Erk-MAPK phosphorylation was determined after treatment with four agents capable of inducing NE differentiation. As expected, HB-EGF rapidly (in ≤10 min) activated Erk phosphorylation in serum-free medium, whereas IL-6 was a weak Erk-MAPK activator (Fig. 3A); in contrast, SB203580 (Fig. 3B) and cFBS (data not shown) did not activate Erk-MAPK signaling. Erk-MAPK activation by HB-EGF was decreased significantly by PD098059, an inhibitor of the Erk-activating kinase, MEK/MEK1, and by PD153035, an inhibitor of the EGFR (Fig. 3C). Several other drugs, including inhibitors of PI3k, protein kinase C, and ErbB2, had no inhibitory effect on Erk-MAPK signaling (data not shown). These data indicate that HB-EGF triggers the
MAPK were analyzed by Western blot. In addition, NSE expression and phosphorylation of Erk-MAPK activation) or 2 days (for NSE expression) after 30 min (for Erk-MAPK phosphorylation, and NE differentiation was evaluated at 1–3 days after treatment (Fig. 3–D). In these experiments, Erk-MAPK phosphorylation at each time point was determined by Western blotting. LNCaP cells were treated with 50 ng/ml IL-6 or HB-EGF. The EGFR inhibitor, PD153035, and MEK1 inhibitor, PD098059, reduced NSE expression and Erk-MAPK phosphorylation levels in the presence of HB-EGF (Fig. 3C), whereas SB203580 resulted in slightly increased levels, consistent with the result shown in Fig. 2. Cells treated with PD098059 1 h before HB-EGF treatment exhibited lower NSE than control cells when the extent of NE differentiation was evaluated at 1–3 days after treatment (Fig. 3D). In these experiments, Erk-MAPK phosphorylation at each time point was suppressed by PD098059 (data not shown). To confirm that the inhibitory effects of PD098059 on NSE levels were not the result of cytotoxicity, DNA ladder and poly(ADP-ribose) polymerase cleavage assays were performed, and cell morphology was observed. No cytotoxic effects attributable to PD098059 were detected in these experiments (data not shown), suggesting that the PD098059-induced decrease in NSE results from specific inhibition of MEK, not from a nonspecific effect.

The p38 MAPK Pathway Antagonizes Erk-MAPK Regulation of NE Differentiation in LNCaP Cells. The ability of SB203580 to induce NE differentiation in LNCaP cells suggests that positive signaling through the p38 pathway antagonizes mediation of this process by the Erk-MAPK pathway. To examine this possibility, LNCaP cells were stably transfected with a construct expressing MKK3b(EE) (28), a direct and selective upstream activator of p38α (29). Transfected cells demonstrated enhanced p38 activation in response to H2O2 (Fig. 4A), demonstrating that this construct was functional in vivo. Notably, MKK3b(EE)-transfected cells exhibited significantly reduced NSE expression in response to NE differentiation induced by either IL-6 or HB-EGF (Fig. 4B). Treatment of LNCaP cells with HB-EGF and the p38 pathway inhibitor, SB203580, in combination resulted in enhanced phosphorylation of Erk (Fig. 4C), indicating that p38 MAPK directly opposes signaling through Erk-MAPK. Interestingly, whereas PD098059 and the EGFR inhibitor PD153035 independently suppressed Erk activation by HB-EGF (Fig. 3C), Erk-MAPK activation was observed in the presence of SB203580, PD098059, and HB-EGF (Fig. 4D). In addition, suppression of Erk activation by PD153035 was not reversed by SB203580, implying that p38 MAPK negatively regulates the EGFR→Erk-MAPK pathway downstream from the EGFR. Collectively, the findings shown in Fig. 4 indicate that p38 MAPK activation antagonizes NE differentiation stimulated by multiple agents.

HB-EGF Stimulation of NE Differentiation Occurs by a Novel Mechanism that Is Not Dependent on STAT3 Activation or Cell Cycle Withdrawal. Data published previously indicate that IL-6 induces NE differentiation in LNCaP cells by a mechanism dependent on constitutive activation of the transcription factor, STAT3, and coincides with withdrawal from the cell cycle (14). To determine whether NE differentiation induced by either p38 pathway inhibition or HB-EGF treatment involves either of these processes, LNCaP cells
were evaluated for evidence of STAT3 phosphorylation after treatment with IL-6, HB-EGF, and SB203580. Consistent with published findings (20, 21), IL-6 induced phosphorylation of STAT3 on Tyr705, a residue implicated previously in STAT3 activation in LNCaP and other cells after treatment with IL-6 (Fig. 5A). In contrast, phosphorylation of STAT3 on Tyr705 was not observed after either HB-EGF or SB203580 treatment (Fig. 5A and B) or by culture in cFBS (data not shown). Phosphorylation at an alternative site, Ser727, after either HB-EGF or SB203580 treatment was similarly not observed (data not shown). These findings indicate that the mechanism of NE differentiation induced by HB-EGF, SB203580, or cFBS is unlikely to involve sustained STAT3 activation.

Cells treated with IL-6, HB-EGF, or SB203580 were analyzed by flow cytometry to determine the effect of these treatments on cell cycle progression. Cultures of cells treated with IL-6 or SB203580 demonstrated an increase in the proportion of the culture in G0-G1 in comparison with control cells. In contrast, cells treated with HB-EGF did not show an increase in the G0-G1 fraction (Fig. 5C). These results indicate that HB-EGF-induced NE differentiation appears not to require a cell cycle block. Consistent with this conclusion, growth of the LNCaP/SF Hyundai Cell line in vector-only transfected cells (data not shown). These results indicate that NE differentiation is not obligatorily coupled to withdrawal from the cell cycle.

DISCUSSION

Mechanisms that evoke the multiple cell phenotypes encountered in human tumors are poorly understood. Cell and tissue recombination experiments in model systems have demonstrated, however, that the microenvironment is likely to play a critical role in growth and control of phenotypic properties by prostatic tumors (30, 31). This study, we demonstrated that an endogenous activator of the Erk-MAPK pathway, HB-EGF, a growth factor expressed primarily in the smooth muscle compartment of the prostate stroma in humans, can stimulate NE differentiation in the LNCaP human PCa cell line. Our findings are novel because they: (a) identify a new and unanticipated role for HB-EGF, a mitogen and survival factor for PCa and other cell types; (b) demonstrate that NE differentiation in human PCa cells can occur by a MEK-dependent mechanism; (c) provide a new role for the p38 MAPK pathway as a direct antagonist of Erk-MAPK signaling in PCa cells; (d) indicate that NE differentiation can occur independently of cell cycle arrest and STAT3 phosphorylation, both identified as aspects of IL-6-stimulated NE differentiation in PCa cells (14, 15); and (e) suggest a role for stroma-derived Erk-activating growth factors in the control of NE differentiation in prostatic tumors. Although the clinical significance of NE differentiation in PCa is still uncertain, these findings identify a new mechanism of altering a PCa cell differentiation pathway in a manner likely to have an impact on the disease course. They also highlight an important biological role for the p38 MAPK pathway in PCa cells.

Peptide growth factors and cytokines, operating either within the context of androgen-dependent signaling mechanisms or in an androgen-independent context, are likely to be critical to the process of PCa progression. HB-EGF, an M1, 14,000–20,000 ErbB1 ligand originally identified as a secretory product of macrophage-like cells (32), appears to play a role as a directional SMC→epithelial cell signaling molecule in the prostate, based on its site of synthesis and that of its primary cognate receptor, the EGFR tyrosine kinase (24). HB-EGF was shown previously to be equipotent to EGF in stimulating LNCaP cell growth and protecting cells from apoptotic signals triggered by PI3K inhibition (22, 23). Like other ErbB1 ligands, HB-EGF activates the Ras→Raf→MEK→Erk cascade. The role of signaling through the Erk-MAPK pathway in PCa is still undefined, although various prostate cell mitogens, at least five of which act through this pathway, have been studied, mostly using in vitro systems. Erk1 overexpression was demonstrated recently in human prostate intraepithelial neoplasia lesions and adenocarcinoma (33). In one study, Erk activation was associated with PCa cell proliferation induced by EGF and lysophosphatic acid but, significantly, not androgen, suggesting the potential for paracrine factors to be the predominant contributor to activation of this pathway in PCa cells (34). We demonstrate that HB-EGF activates Erk-MAPK signaling under the experimental conditions used.
cells. Elevated levels of p38 MAPK protein have been reported previously in prostate intraepithelial neoplasia (33). Inhibition of Erk-MAPK pathway signaling by p38 MAPK pathway activation has been demonstrated previously (29, 35, 36). The inhibitory effect of activated MKK3b on IL-6-induced differentiation suggests that p38 activation may suppress the STAT3-dependent pathway of NE differentiation, possibly through a downstream target shared with the Erk-MAPK pathway. This possibility remains to be investigated.

The role of a proliferating NE component of prostatic tumors deserves additional study, given the potential for enhanced paracrine signaling originating from these cell types. Model systems have suggested a relationship between NE differentiation and tumor progression (5, 37). Neuropeptides have been shown to stimulate androgen-independent growth, as well as resistance to apoptosis in human PCa cell lines (10, 12). Frequent NE differentiation has also been shown to correlate with disease progression, including metastases to distant organ sites, in a transgenic model of PCa (37).

In conclusion, in this study, we identify a role for Erk-MAPK signaling as a mediator of NE differentiation in human PCa cells and a role for the p38 MAPK pathway as an antagonist of this differentiation mechanism. Our findings suggest a previously unrecognized function for the stroma-derived growth factor HB-EGF and, potentially, for other endogenous Erk-MAPK activators as inducers of the NE phenotype. These results provide insight into how the prostatic stroma might alter the clinical behavior of PCa cells, as well as an underlying mechanism that might potentially be manipulated therapeutically.

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