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c-jun-NH2JNK Mediates Invasive Potential and EGFR Activation by Regulating the Expression of HB-EGF in a Urokinase-Stimulated Pathway

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Abstract In this study, we demonstrated that tyrosine phosphorylation of EGFR and the autocrine expression of uPA and HB-EGF depend on the activity of c-jun amino-terminal kinase (JNK) in human prostatic DU-145 cells. These cells overexpress EGFR and produce a high amount of uPA. Treatment with either SP600125, a specific chemical inhibitor of JNK, or the expression of a dominant-negative JNK form inhibited autocrine production of uPA and HB-EGF, which block EGFR phosphorylation and mitigates invasive capacity. Our data provided evidence that in DU-145 cells, the maintenance of the activation level of EGFR, which determines the cellular invasive potential, operates through an autocrine loop involving the JNK-dependent production of uPA and HB-EGF activity. Moreover, we found that exogenously added uPA stimulates autocrine production of HB-EGF, and that blocking HB-EGF activity curbed DU-145 cell invasive potential. J. Cell. Biochem. 103: 986–993, 2008.

Key words: JNK; EFGR; uPA; invasiveness

Activation of c-jun amino-terminal kinase (JNK) is a mechanism facilitating acquisition of migratory cancer cell properties stimulating an array of phenotypic changes including the expression of urokinase (uPA) in the cell [Santibañez, 2006]. Urokinase, which in cancer cells is a highly expressed serine protease, plays a central role by binding to its membrane receptor (uPAR) in the degradation of extracellular matrix and cell signaling [Blasi and Carmeliet, 2002]. uPA is also able to transactivate the Epidermal Growth Factor Receptor (EGFR) a mechanism tumor cells use to maintain pathways involved in cell proliferation and migration activated [Liu et al., 2002; Jo et al., 2003; Guerrero et al., 2004]. Moreover, the integrity of EGFR-dependent signaling system determines uPA-dependent activation and finally mitogen-activated protein kinases (MAPK), which is a key element in the stimulation of cell migration [Guerrero et al., 2004].

MAPK modules include the ERK, JNK, and p38 cascades. Constitutive activation of the ERK pathway is functionally related to autocrine production of molecules such as uPA [Mandell et al., 1998; Ma et al., 2001]. ERK activation is often mediated by a metalloproteinase-processed EGF-like ligand binding to an EGFR [Guerrero et al., 2004; Kansra et al., 2004].

Traditionally, in tumorigenesis the JNK pathway has been linked to the induction of cellular apoptosis [Curtin and Cotter, 2004]. Nevertheless, a recent study in brain tumors showed that JNK is basally activated, while ERK is not. EGF-dependent activation of JNK was also found to be a common feature of brain tumor cells; in normal cells this stimulus was absent [Antonyak et al., 2002]. In mice embryos a functional linkage between JNK pathway and EGFR activation was proposed by Zenz et al. in which the eyelid protruding epithelium is expressed simultaneously with enhanced c-Jun expression and EGFR activation. c-jun-deficient keratinocytes in mice prevents the

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EGF-induced expression of HB-EGF and the subsequent activation of EGFR resulted in mice born with open eyes. This suggests that c-Jun activation-related HB-EGF production provides a positive feedback loop for sustaining EGFR activation [Zenz et al., 2003].

In the present study, using the DU-145 prostate cancer cells that overexpress EGFR [Yates et al., 2005] we investigated the effects of JNK inhibition on different aspects of cell behavior: autocrine production of uPA and HB-EGF, basal activation of EGFR and on the invasive potential of prostatic cells.

MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal antibodies to uPA were from American Diagnostica (Stanford, CT), to β-Actin was from Sigma (St. Louis, MO). 4G10 anti phosphotyrosine monoclonal antibody was a gift by Dr. M.R. Bono (University of Chile). Polyclonal antibodies against peptides of residues 984–996 (EGFR984) and 1176–1186 (anti C-EGFR) have been previously characterized [Faúndez et al., 1992; Salazar and Gonzalez, 2002]. Anti c-jun antibody was from Calbiochem (San Diego, CA). Peroxidase-conjugated goat anti mouse and anti rabbit were from Rockland (Gilbertsville, PA). Anti JNK2 monoclonal antibody was from Santa Cruz (Santa Cruz, CA). Neutralizing anti human HB-EGF used in invasion assays were purchased from R&D systems (Minneapolis, MN). A purified non-related rabbit IgG (Sigma) was used as a control.

The inhibitor of JNK SP600125 was from BioMol (Plymouth Meeting, PA); the ERK inhibitor PD98059, Tyrphostin (AG1478) and uPA were from Calbiochem. EGF was from Upstate Biotechnology (Lake Placid, NY).

Cell Culture and Transfection Methods

Prostate DU-145 cells were obtained from the ATCC (Manassas, VA) and were grown in phenol red-free DMEM/ F12 plus 10% fetal calf serum (FCS). cDNA that encodes for the dominant-negative version of c-jun-NH2JNK was kindly provided by Dr. Dan Mercola (Cancer Center, University of California at San Diego, La Jolla, California [Mandlekar et al., 2000]). To generate stable DU-145 cell clones that overexpress a dominant version of JNK (mutated Thr 183 and Tyr 185), cells were transfected with 2 μg of the DN-JNK expression construct using Lipofectamine Plus reagents (Invitrogen, Carlsbad, CA) as described previously [Cáceres et al., 2005]. JNK activity was evaluated by co-transfecting DU-145 cells using a pFA-Luc (5× Gal4 binding element) as has been described [Santibañez, 2006].

Immunodetection of EGFR, c-Jun/AP-1, and ERK

Serum starved DU-145 cells were subjected to a mild acid treatment to release endogenous ligands as previously described [Guerrero et al., 2004]. Afterwards, cells were allowed to restore their basal EGFR phosphorylation level by incubating them in serum-free medium for 16 h in the presence of SP600125 (10 μM) or PD98059 (10 μM). EGFR was immunoprecipitated using the anti-C as described [Guerrero et al., 2004]. ERK 1, 2 activation and total amount were assessed by immunoblots of cell lysates (30 μg) resolved by 10% SDS–PAGE using anti-ERK and anti-phosphotyrosine ERK, as previously described [Cáceres et al., 2005].

uPA Activity

The activity of soluble uPA was measured by immunobloting and zymography on a 10 times concentrated media conditioned for 24 h as previously described [Cáceres et al., 2005].

Cell Invasion Assay

The ability of control and DN JNK transfected DU-145 cells to migrate was assayed using Transwell chambers (Costar Corp., Cambridge, MA). Untreated or treated cells with 10 μM SP600125, PD98059 or with 10 μg/ml of a neutralizing HB-EGF antibody, were subjected to invasion assay for 24 h as described [Cáceres et al., 2005].

RT-PCR Analysis

Total RNA was isolated with Trizol (Gibco) and cDNA were synthesized for 1 h (42°C) with M-MLV reverse transcriptase (Promega) using oligo dT (Invitrogen). Primer sequences were as follows:

uPA, forward: 5′GCAGGAACCCAGACAACCG3′

reverse: 5′ GACCCAGGTAGACGATGTAG3′ (amplified a fragment of 357 bp at 26 cycles)
HB-EGF, forward: 5′GGACCCTCCCACTGTATC3′
reverse: 5′CCGTGCTCCTCCTTGTTT3′ (amplfied a fragment of 156 bp at 27 cycles)
GAPDH, forward: 5′ACCACAGTCCATGCCATCAC3′,
reverse: 5′TCCACCACCCTGTTGCTGTA3′ (amplified a fragment of 452 bp at 23 cycles)

In experiments where the mRNA for HB-EGF were stimulated by uPA, cells were maintained in serum-free conditions for 16 h and were then incubated with 10 nM uPA for 2 and 7 h. RNA was isolated after this incubation, as described above.

RESULTS

Basal Production of Urokinase in DU-145 Cells Depends on the JNK Pathway

Figure 1A shows that the c-jun Kinase inhibitor, SP600125, exerted more pronounced inhibition on basal uPA production than PD98059-treated cells and is clearest at an inhibitor concentration of 10 μM (48% vs. 17% inhibition). Assessing kinase activity using c-jun transactivation assays [Santibañez, 2006], JNK was indeed inhibited under such conditions. Expressed in relative luciferase units (RLU), compared to control and PD98059-treated cells, SP600125 significantly reduced JNK activity (Fig. 1B).

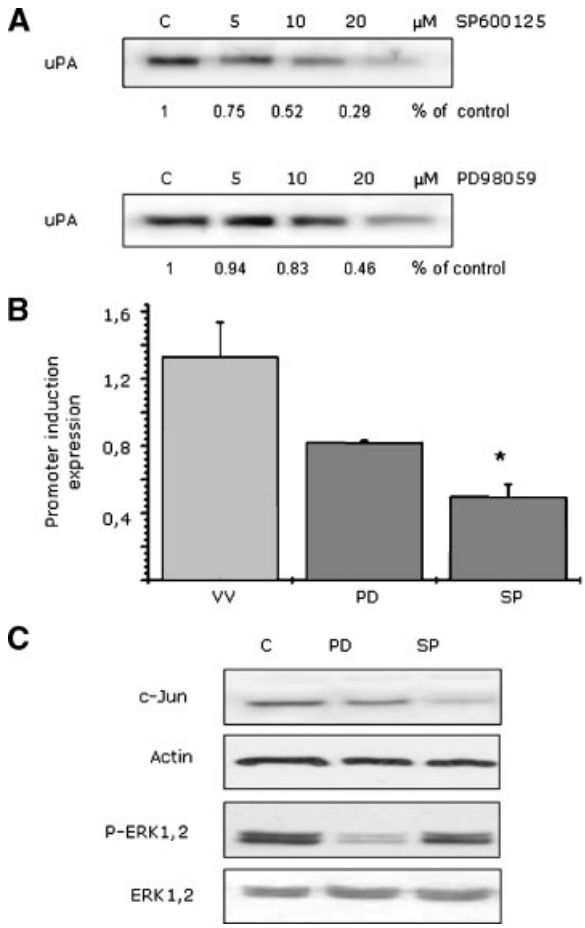

Fig. 1. Basal production of urokinase in DU-145 cells depends on the JNK pathway. **A**: Serum-starved DU-145 cells were treated for 24 h with increasing concentrations of the JNK inhibitor SP600125 and the MEK inhibitor PD98059. Autocrine uPA production was evaluated using Western blotting in 10-fold concentrated conditioned media. uPA production was quantified using immunoblot densitometric analysis and expressed as a percentage of the control. **B**: DU-145 cells (5×10^4) were transiently co-transfected with a GAL4-cJun/pRf-Luc as described above. After 16 h, the cells were treated with 10μM SP or 10 μM PD. To normalize, cells were tranfected with 50 ng/well of β-Gal. Data are expressed as means ± SE. **C**: DU-145 cell extract, treated for 24 h with 10 μM of each inhibitor, were subjected to immunoblot for c-Jun and P-ERK1,2. Actin and total ERK were blotted as loading reference.

No evidence of cross inhibition was found between the two inhibitors and they are, therefore, specific (Fig. 1C). Since active c-Jun, once phosphorylated, induces expression of a number of proteins, including itself, as previously reported [Curtin and Cotter, 2004], hence in long-term experiments (24 h), the scale of normal c-jun expression represents a satisfactory assay for JNK activity.

SP600125 Inhibits the Basal Activation of EGFR and the Invasive Capacity of DU-145 Cells

We analyzed the capacity of treated cells to activate the EGF receptor in an autocrine fashion and the subsequent invasive capacity of DU-145 cells, evaluating whether inhibition of the signaling routes that block endogenous uPA production plays a role in the two processes. Adding 10 μM SP600125 or PD98059 to the culture media had different blocking effects on autocrine EGFR autophosphorylation (56% vs. 33%) (Fig. 2A), and a cell invasion assay was performed to assess whether MAPK inhibitor modulation of the EGFR activation correlated with changes in the cellular invasive capacity. Figure 2B shows that cells treated with the JNK inhibitor have 40% of the invasive capacity of untreated cells while the invasive capacity of PD98059-treated cells reduced around 10%.

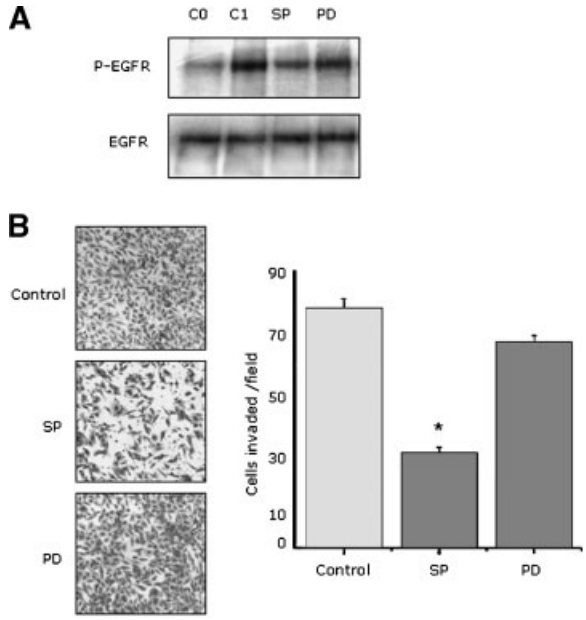

Fig. 2. JNK inhibition blocked basal EGFR activation and the invasive capacity of DU-145 cells. **A**: Serum-starved DU-145 cells subjected to mild acid treatment to eliminate endogenous ligands (C0) were then allowed to return to their basal EGFR phosphorylation levels through incubation in a serum-free medium for 16 h (C1) in the presence of either 10 μM SP600125 (SP) or 10 μM PD98059 (PD). Afterwards, cells were lysed and EGFR was immunoprecipitated with anti-EGFR antibody followed by immunoblotting with anti-phosphotyrosine antibody and EGFR antibody as the loading reference. The immunoblot shown is representative of three independent experiments. PY-EGFR fraction was quantified through densitometric analysis of the immunoblots and expressed as a percentage of the control (**B**) Invasive ability of JNK and MEK inhibitor treated DU-145 cells and untreated cells was assessed using Transwell chambers. Data are the mean values from three separate experiments ±SE. Differences were calculated against control cells and considered significant (*) at $P < 0.05$. Magnification = 20×.

DU-145 Cells Stably Transfected With a Dominant-Negative (DN) Form of JNK Expressed Low Basal EGFR Activation Levels and Decreased Invasive Capacity

Two clones expressing different amounts of c-Jun, the long term product of the JNK activity, displayed significantly lower EGFR activation in comparison to the control EV clone (Fig. 3A). The diminished amount of c-Jun cannot be explained by a differential expression of JNK because EV and DN clones expressed the enzyme in a similar level (Fig. 3B). Under Transwell assay, the DN JNK transfected cells expressed 36% and 24% respectively of the invasive activity of empty vector transfected cells (Fig. 3C).

DN JNK Transfected DU-145 Cells Expressed Decreased uPA and HB-EGF Levels. Effect of uPA on HB-EGF Expression

In DN transfected JNK cells, through semiquantitative RT-PCR, we analyzed expression of mRNA for uPA and HB-EGF (an EGF-like ligand) responsible for the androgen-independent behavior of malignant prostatic cells [Adam et al., 2002]. As Figure 4A shows, the two DN clones expressed significantly lower amounts of the HB-EGF transcript than empty-vector cells, which concurs with the lower amounts of uPA transcript. The result would suggest that a similar transcription control mechanism, that is dependent on the JNK pathway, is involved in both uPA and HB-EGF transcription. Then, investigating if uPA plays a role in the expression of HB-EGF, as Figure 4B shows, uPA induced far greater (a 2.39 ± 0.37 fold increase over control) expression of HB-EGF.

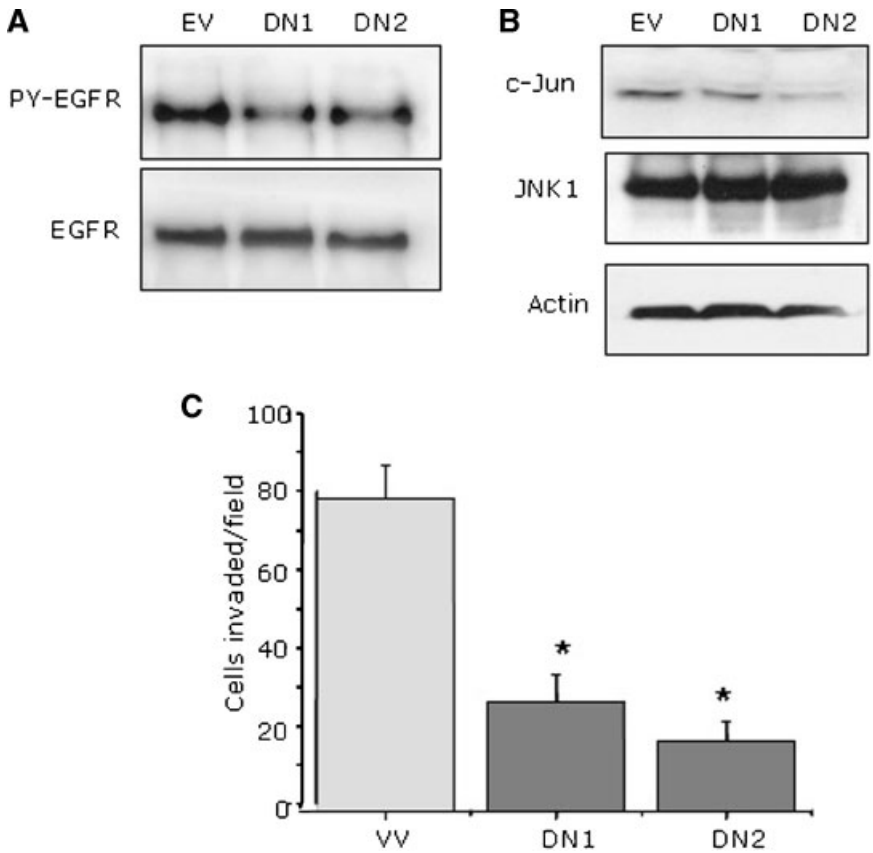

Fig. 3. DU-145 cells expressing dominant-negative JNK exhibited lower EGFR activation levels and diminished invasive capacity. **A**: Cell extracts from serum-starved empty vector (EV) and DN JNK were immunoprecipitated with anti-EGFR antibody followed by immunoblotting with anti-phosphotyrosine (PY) antibody and EGFR antibody as the loading reference. **B**: DU-145 cells expressing DN JNK were assessed for c-Jun, total JNK1 and actin as a loading control as described in Figure 1C. **C**: Invasive capacity of EV and two clones of DN JNK cells. Experiments were performed using the Transwell chamber method. Results are means ± SE. Differences were calculated against EV cells and considered significant at $*P < 0.05$ (DN1 $P < 0.0038$, DN2 $P < 0.0018$).

Blocking HB-EGF Activity Inhibited Basal DU-145 Cell Invasive Capacity

To identify whether autocrine production of HB-EGF modulates prostatic DU-145 cell motility, we performed an invasion assay in the presence of a neutralizing antibody that blocks HB-EGF activity. As Figure 5 shows, cells allowed to migrate in the presence of anti HB-EGF exhibited less invasive capacity than cells treated with either an anti rabbit unrelated IgG or with untreated cells.

DISCUSSION

In this study, using the prostatic DU-145 cell line, we provide evidence that the JNK pathway constitutes a key element in the autocrine activation of EGFR. We also identified uPA as a crucial factor stimulating endogenous expression of HB-EGF, which sustained phosphorylation of EGFR. Our results demonstrated that blockage of the JNK pathway considerably reduced the invasive capacity of DU-145 cells and correlated with inhibited autocrine production of uPA and HB-EGF (Figs. 2 and 4) and lower EGFR activation.

c-Jun protein is the best known substrate for JNK. In its activated form, the protein combines with members of the FOS, ATF, and MAF families constituting the transcription factor AP-1 that regulates uPA expression [De Cesare et al., 1995; Efer and Wagner, 2003]. In PC3 prostatic cells, the minimal promoter (MP), an Sp-1 transcription factor binding site, regulated uPA transcription. This activity was strongly affected by the dominant negative form of JNK and only minimally affected by the ERK1, 2 pathway [Benasciutti et al., 2004]. In addition,

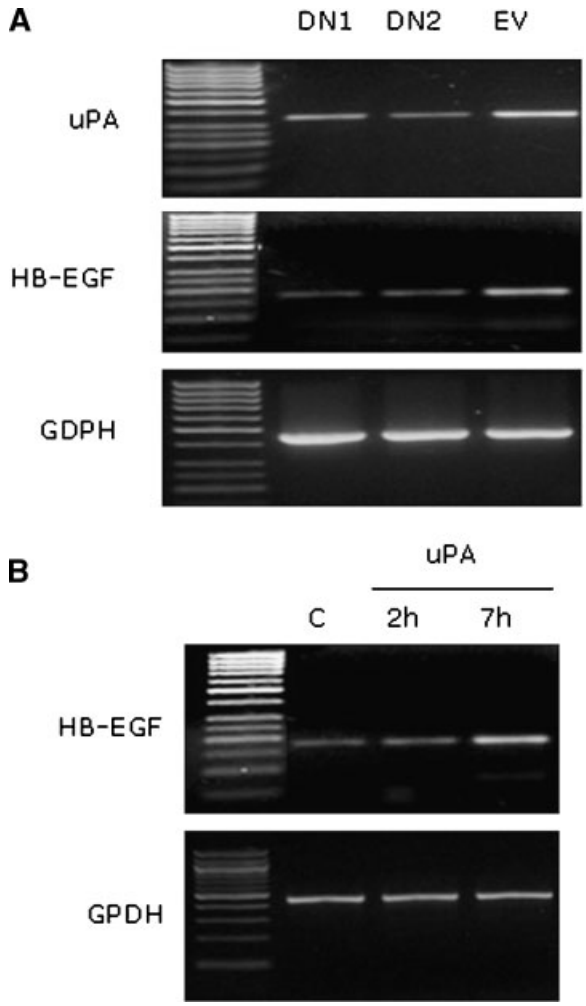

Fig. 4. DN JNK cells expressed decreased levels of uPA and HB-EGF. uPA stimulates the expression of HB-EGF. **A**: RT-PCR semi-quantitative analysis of the basal expression of uPA and HB-EGF in DU-145 cells transfected stably with empty vector (EV) and DN JNK. **B**: Serum-deprived DU-145 cells were treated with 10 nM of uPA for 2 and 7 h and evaluated for mRNA expression of HB-EGF following the same protocol used in (A). The left lane in each gel plate is a 50 bp DNA ladder.

studies on human keratinocytes have shown that estradiol-dependent wound re-epithelialization depends directly on HB-EGF transcription enhancement which in turn, also depends on AP-1 and Sp1 [Kanda and Watanabe, 2005]. These data strongly suggest that in our system uPA and HB-EGF could share a similar transcriptional mechanism with JNK playing a central role.

Compared to EGFR ligands, DU-145 cells expressed large amounts of uPA. We found no EGFR ligands as translated protein nor in media conditioned by DU-145 cells or in a membrane-enriched fraction (data not shown). In contrast, in conditioned media western blotting detected uPA easily (Fig. 1). This identification issue forced us to focus our study on the mRNA expression of HB-EGF that clearly showed the differences between the relative supplies of autocrine-produced molecules participating in EGFR activation. Our results also show that uPA, acting as a receptor ligand and at transcriptional level, modulated HB-EGF expression (Fig. 4B) establishing an uPA-dependent autocrine loop that perpetuates EGFR activation and maintains the invasive potential. This mechanism suggests that in cellular invasion of prostatic cells, rather than the traditional view that EGFR controls uPA/uPAR expression, the reverse appeared to occur [Mamoune et al., 2004; Festuccia et al., 2005]. We have demonstrated previously that

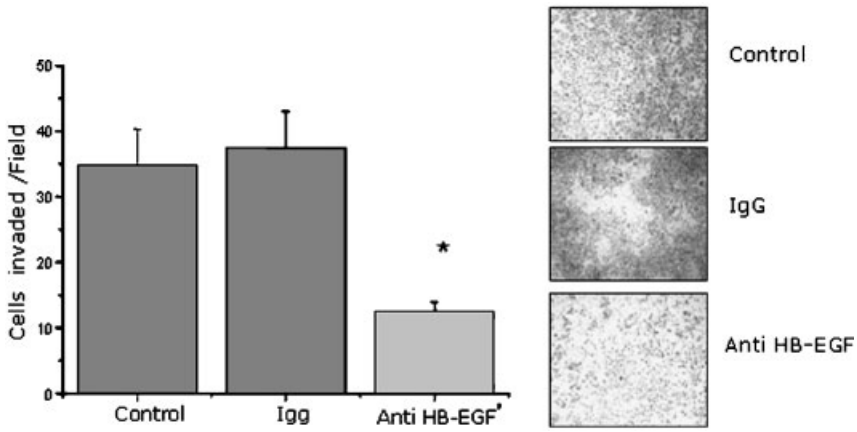

Fig. 5. Blocking HB-EGF activity inhibited the basal invasive capacity of DU-145 cells. Cells (5×10^3), were treated with 10 μg/ml of neutralizing anti HB-EGF or a rabbit not-related IgG or untreated. Results are means ± SE of three different experiments. Differences were calculated against untreated (control) cells and considered significant at $P < 0.05$. Magnification = 4×.

exogenously added uPA transactivates EGFR in MCF-7 breast cancer cells through a mechanism that involves metalloproteinase activity [Guerrero et al., 2004]. Whether long-term uPA-dependent activation of EGFR in DU-145 cells is a consequence of uPA-dependent transactivation or the result of enhanced HB-EGF expression through stimulation of uPA remains unclear yet it seems plausible that both processes occur simultaneously.

Elsewhere, in study of cells that respond to stressing stimulus activating EGFR, the authors proposed that p38 MAP kinase act as an upstream control for ligand-dependent EGFR activation in the autocrine supply of HB-EGF, while ERK1,2 activation occurs after EGFR phosphorylation [Fischer et al., 2004]. In a similar fashion, our results revealed that upstream JNK is responsible for signaling EGFR activation.

In ovarian cancer it has been demonstrated that HB-EGF plays a significant role in tumoral progression, because it is the only EGFR ligand enhanced at the mRNA and protein level contributing to tumor formation in nude mice [Miyamoto et al., 2004]. Using a blocking HB-EGF antibody we found strong inhibition of invasive capacity of DU-145 cells (Fig. 5), which provides additional support to the hypothesis that the autocrine production of HB-EGF modulates invasive potential of prostatic cells.

In this study, we advance a novel mechanism for sustaining cellular malignancy. In EGFR activation the JNK pathway controlled the endogenous supply of HB-EGF activating the receptor. Autocrine production of urokinase, which also depends on full activation of the JNK pathway, could also be responsible for the maintenance of the autocrine production of HB-EGF and, in consequence, for the activation of EGFR and the malignant properties of prostatic cells.

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