Clin. Exp. Metastasis, 1996, **14**, 409–418

EGF receptor signaling enhances *in vivo* invasiveness of DU-145 human prostate carcinoma cells

Timothy Turner[†], Philip Chen[†], Lyndon J. Goodly[†] and Alan Wells*[†]

**Pathology and Laboratory Medicine Service, Birmingham Veterans Administration Medical Center, Birmingham, AL, and [†]Department of Pathology, University of Alabama at Birmingham, Birmingham, AL, USA*

(Received 5 March 1996, accepted in revised form 20 May 1996)

Carcinomas of the prostate and other lineages often present an autocrine stimulatory loop acting via the EGF receptor (EGFR). We have recently shown that EGFR-mediated signals enhance DU-145 prostate carcinoma cell transmigration of an extracellular matrix *in vitro*, and that this increased invasiveness was independent of proteolytic degradation of the matrix (Xie *et al.*, 1995, *Clin Exp Metastasis*, 13, 407). To determine whether up-regulated EGFR signaling promotes tumor progression *in vivo* and to define the EGFR-induced cell property responsible, we inoculated athymic mice with genetically-engineered DU-145 cells. Parental DU-145 cells and those transduced to overexpress a full-length wild type (WT) EGFR formed tumors and metastasized to the lung when inoculated in the prostate and peritoneal cavity. The WT DU-145 tumors were more invasive. DU-145 cells expressing a mitogenically-active, but motility-deficient (c'973) EGFR formed small, non-invasive tumors without evidence of metastasis. All three sublines demonstrated identical, EGFR-dependent rates of cell growth *in vitro*, suggesting that the differential invasiveness was not due to altered growth rates. To determine whether cell motility may be, in part, responsible for tumor invasiveness, we treated WT DU-145 intraperitoneal tumors with a pharmacologic agent (U73122) which blocks EGFR-mediated cell motility but not mitogenesis. Under this treatment regimen, the WT DU-145 cells formed tumors of similar numbers and size to those formed without treatment; however, these tumors were much less invasive. These data suggest that EGFR-mediated cell motility is an important mechanism involved in tumor progression, and that this cell property may represent a novel target to limit the spread of tumors.

Keywords: epidermal growth factor receptor, phospholipase C, tumor progression

Introduction

Prostate carcinoma is the most widespread and pestilent malignancy encountered in the human male population. The frequency and mortality rate of prostate cancer has increased over the past 40 years and is expected to rise steadily in the impending years [1]. Androgen dependency of prostate carcinoma usually accompanies initial neoplastic growth, during which tumors respond favorably to hormonal therapy. However, androgen-independent tumors often emerge [2]. Deaths related to prostate cancer are invariably due to tumor invasion and metastasis to the lungs, skeleton, and lymph nodes [1, 3]. Once the tumor escapes it natural surroundings to invade and metastasize, none of the available treatments

Address correspondence to: Alan Wells, Department of Pathology, LHRB 531, University of Alabama at Birmingham, Birmingham, AL 35294–0007, USA. Tel: (+1) 205 934 0357; Fax: (+1) 205 975 9927; E-mail: wells@lh.path.uab.edu.

yield positive effects on patient survival [3–6]. Consequently, efforts to improve our understanding of the basic biology of this disease and particularly the progression to the invasive and metastatic stages should enhance the chances for developing meaningful therapeutic approaches.

Polypeptide hormones or growth factors play important roles in the normal and pathologic development of the prostate. Various growth factors promote cell proliferation, motility, and invasiveness of epithelial cells *in vitro*, all properties required for tumor invasiveness and metastasis. Growth of explanted cells is stimulated by non-steroidal growth factors, such as epidermal growth factor (EGF) [7] and not by steroids such as DHT [8, 9]. Prostatic fluid has the highest concentration of EGF in the human body [10]; numerous EGF-like factors are expressed by normal and neoplastic prostatic cells [9, 11–14]. Recent evidence suggests androgens stimulate prostate proliferation in the androgen-dependent cell line, ALVA-31, by upregulating an autocrine stimulatory growth loop involving the EGF receptor (EGFR) and one of its ligands, transforming growth factor-α (TGFα) [14]. However, the roles of EGFR and its ligands in tumor progression have not been defined.

EGFR, a transmembrane protein which possesses intrinsic tyrosine kinases activity, is the growth factor receptor found most often upregulated in human carcinomas [15]. Augmentation of the EGFR gene or the number of EGF binding sites directly correlates with tumor progression to the invasive and metastatic stages in a number of different tumors, most dramatically in glioblastomas [16, 17], gastric carcinoma [18], bladder carcinoma [19, 20], and breast cancer [21, 22]. In addition, in an animal model, a direct correlation was seen in the metastatic potential of human colon carcinoma cells and EGFR level and function [23]. Examination of normal prostate epithelial, benign prostatic hyperplasia (BPH) and carcinoma cells demonstrate increased levels of EGFR expression as one progresses through the different hyperproliferative states [9, 24], the highest levels of EGFR expression correlating with the loss of androgen-dependency by prostate carcinoma cells [25]. In prostate cancer one detects either an increase in the level of EGFR [25, 26] or in the production of its activating ligands, EGF and TGFα [9, 27], or both [28, 29]. In many cancers, the synchronous overexpression of EGF/TGFα and EGFR has been associated with more invasive phenotypes [30–33]. This autocrine stimulatory loop is often present in prostate carcinoma; an example of this autocrine system can be seen in the DU-145 human prostate carcinoma cell line [34], which produces TGFα and expresses EGFR [28, 29, 35, 36].

Previously, we genetically engineered DU-145 cells to overexpress a full-length, wild-type (WT) EGFR in order to delineate the role EGFR signaling plays in cell proliferation and invasion [36]. We demonstrated *in vitro* transmigration of a human extracellular matrix was increased for the cells overexpressing WT EGFR. Disruption of the TGFα-EGFR autocrine stimulatory loop by an EGFR antibody diminished DU-145 parental and WT EGFR-expressing cell invasion through the extracellular matrix *in vitro*, thus emphasizing the importance of EGFR signaling in cell migration and invasion. EGFR-dependent migration and invasion observed in DU-145 sublines expressing WT EGFR was not linked to increased proteolytic activity [36], but did correlate with signals which lead to increased cell motility [37, 38]. We have investigated whether EGFR-mediated signaling promotes tumor progression *in vivo*. Three DU-145 sublines were inoculated either into the prostate (to reflect an *in situ* lesion) or peritoneal cavity (to recapitulate the initial stages of localized invasiveness) of athymic mice. Tumor formation and invasiveness was assessed histologically. The mechanism by which EGFR signaling promotes tumor progression was probed by treatment with a pharmacologic agent which prevents EGFR-mediated cell motility.

Materials and methods

Animals

Male athymic BALB/c nu/nu mice were purchased from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research and Development Center (Frederick, MD, USA). The mice were housed in laminar flow cabinets under specific pathogen-free conditions. Mice were used at 6–8 weeks of age, and weighed 20–27 g. Animals were maintained in accordance with established institutional guidelines and approved protocols.

Pharmacologic agents

The pharmacological agent, U73122 (1-(6-((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-1H-pyrrole-2,5-dione) (BIOMOL, PA, USA) was used to inhibit phospholipase C (PLC) activity. The inhibitory effects on EGF-induced PLC activity by this compound were determined previously [38]; U73122 inhibits PLC activities but not phospho-

lipase A_2 or phospholipase D activities ([39, 40] and our unpublished observations). The inactive congener of U73122, U73343 (1-(6((17β-3-methoxyestra-1,3,5(10)-trien-17-yl)amino)hexyl)-2,5-pyrrolidine-dione), served as a control. Neomycin sulfate served as a second control. This biologically-active agent will bind to phosphoinositide bisphosphate (PIP_2) accessible on the cell surface. However, this agent does not inhibit PLC-dependent, EGF-induced fibroblast cell motility (unpublished observations), and is unlikely to disrupt this signaling pathway.

Cell culture and establishment of infectant cell lines

DU-145 cells, derived originally from a human prostate carcinoma brain metastasis [34], were grown in high glucose (4.5 g/l) Dulbecco's modified Eagle's medium (DMEM; Gibco, NY, USA) supplemented with fetal bovine serum (FBS; 7.5%), penicillin (100 U/ml), streptomycin (200 mg/ml), non-essential amino acids, sodium pyruvate (1 mM), and glutamine (2 mM) (37°C, 90% humidity, 5% CO_2). WT and c'973 EGFR transduced DU-145 were passaged in G418 (1000 μg/ml; Gibco) until subpassaging for testing; all cells were cultured in the absence of G418 for at least 3 days prior to testing. Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA).

Infection of DU-145 cells by retroviral-containing EGFR constructs was accomplished by established protocols [41] as described previously [36]. A SV40 early promoter-driven neomycin phosphotransferase gene in the *env* position served as the selectable marker. Polyclonal lines consisting of > 20 colonies were established and maintained in G418-containing media. DU-145 cells transduced with either the WT EGFR or the c'973 EGFR construct will hereafter be referred to as WT or c'973, respectively; uninfected DU-145 cells are referred to as parental cells. WT EGFR is a full-length cDNA [42] derived from a placental cDNA library [43]. The WT construct, expressed in appropriate cells, elicits all of the responses of WT EGFR. c'973 EGFR represents a carboxy-terminal-truncated EGFR in which a stop codon was introduced just distal to amino acid 973; although this construct fails to possess phosphotyrosine motifs or induce cell motility, it does present ligand-induced kinase and mitogenic activities. The presence of EGFR on the surface of infectant cell lines was determined by Scatchard analyses, immunohistochemistry and immunoblotting as described previously [36]. Expression of retrovirus-transduced EGFR is considered stable following passage both *in vitro* and *in vivo* as has been demonstrated previously for EGFR in fibroblastic tumors [41, 44]. A cell line, rescued from WT DU-145 intraprostate tumors, was found to present EGF binding and receptor levels and characteristics similar to the original WT DU-145 cells.

Tumor cell inoculations

For intraprostate injections, mice were anesthetized with ketamine hydrochloride/xylazine hydrochloride and placed in the supine position. Methoxyflurane (Pittman-Moore, NJ, USA) was used as an inhalation anesthetic during surgical procedures. The abdomen was cleaned with alcohol and betadyne; a lower midline incision was made and one lobe of the anterior prostate gland was exposed for injection. Tumor cells (2×10^6) resuspended in Ca^{2+}- and Mg^{2+}-free Hanks' balanced salt solution (total volume, 20 μl; HBSS, Gibco) were injected into one lobe of the anterior prostate gland using a 30-gauge needle, a 1-ml disposable syringe, and a calibrated push-button Hamilton dispensing device (Hamilton Syringe Co., NV, USA). A visual localized bleb within the injected prostate was accepted as the indicator of a satisfactory injection. The abdominal wound was initially closed utilizing discontinuous stitches; the skin was closed with stainless steel wound clips (Autoclip; Clay Adams, NJ, USA). Tumor cells (2×10^6) for intraperitoneal injections were suspended in HBSS (total volume, 200 μl) and injected (26.5-gauge needle, 1-ml disposable syringe) into the peritoneal cavity.

Pharmacologic treatments of tumors were on a q4d schedule starting 4 days after the mice were inoculated with the WT DU-145 tumor cells and continuing until day 44. Agents were dissolved in 10% DMSO in HBSS in a total volume of 100 μl. Neomycin sulfate was used at 150 μg/mouse and U73122 was used at 12 or 24 μg/mouse, doses below toxic levels but within therapeutic levels [45]. U73343 was injected at 12 μg/mouse, consistent with U73122 treatments.

Necropsy procedures and histologic studies

Mice were killed by CO_2-induced hypoxia at various times (intraprostate, 120 days; intraperitoneal, 50 days). All lobes of the mouse prostate (anterior, ventral and dorsal/lateral), regional lymph nodes (preaortic or axillary), kidneys, spleen, pancreas, liver, lungs and diaphragm (only taken from animals receiving intraperitoneal injections) were fixed in 10% buffered formalin, paraffin embedded, serially sectioned, and stained with hematoxylin and eosin.

Table 1. Prostate tumor progression by parental, WT and c'973 EGFR-expressing DU-145 cells

(A) Intraprostate

Subline	Tumor formation[a]	Invasiveness[b]	Lung metastases[a]
Parental	11/16	3.0 + (0–4)	8/16
WT EGFR	9/16	3.3 + (2–4)	7/16
c'973 EGFR	2/15	1.0 + (1,1)	0/15

(B) Intraperitoneal

Subline	Tumor formation[a]	Diaphragm tumors[a]	Diaphragm invasiveness[b]	Lung metastases[a]
Parental	5/10	5/10	1.6 + (0–3)[c]	3/10
WT EGFR	9/11	8/11	3.4 + (2–4)	4/11
c'973 EGFR	4/11	4/11	0.5 + (0–2)[c]	0/11

These numbers are a composite of three intraprostate (analysed after 120 days) or two intraperitoneal (after 50 days) inoculations of five to six mice per group.

[a] The number of mice with macroscopic tumors (confirmed by microscopic examination) in the prostate, peritoneal cavity, on the diaphragm surface or in the lungs over the number of mice challenged.

[b] Invasiveness was scored microscopically on a scale of 0 (non-invasive) to 4 (prostate: tumor invading through capsule into surrounding tissue; intraperitoneal: tumor obliterating the diaphragm); the number is the average invasiveness of all prostate or diaphragm tumors (not including mice which did not present tumors), the range of invasiveness is shown in parentheses.

[c] We did not detect invasion by one of the five parental DU-145 diaphragm tumors. One of the c'973 DU-145 diaphragm tumors showed 2+ invasiveness; the other three tumors did not invade the diaphragm.

In vitro *growth assays*

Cell proliferation was evaluated by assessing mitochondrial reduction of 3-(4,5-dimethythiazol-2yl)-2,5-diphenyl tetrazolium bromide (MTT) as described [46], with the following modifications. Cells were plated at 5000 cells/well in 96-well microtiter plates in 200 μl growth medium (7.5% FBS in DMEM) and allowed to attach for 24 h. Serum-containing medium was removed and cells were quiesced for 2 days in 0.5% dialysed FBS in DMEM. For all dose and time course studies using anti-EGFR, this medium was removed and replaced with 7.5% FBS in DMEM; in addition the following agents were evaluated: neomycin (0.01–1000 μM), U73122 (0.001–100 μM) (BIOMOL), and anti-EGFR antibody (0.001–4 μg) (AB-1, Oncogene Science, NY, USA). At harvest, medium was removed from the appropriate wells, replaced with 50 μl of MTT solution (2 mg MTT/ml PBS; Sigma, MO, USA) and incubated for 4 h at 37°C. After incubation, the MTT solution was carefully aspirated; 100 μl dimethylsulfoxide (DMSO) was added to each well. Data were analysed on plate reader using the Soft Max program (Molecular Devices Corp., Menlo Park, CA, USA).

Anchorage-independence was determined by growth in soft agar as described previously [41]. Briefly, 200 or 1000 cells were suspended in 3 ml of 0.35% agarose (Low-Melt Agarose, FMC, ME, USA), DMEM, 7.5% FBS, with antibiotics and supplements (in a 60 cm tissue culture dish), and cultured for 15 to 19 days. Cells were fed weekly by an overlay of 4 ml 0.70% agarose with complete media. Numbers of colonies were counted manually. Relative size of colonies was assessed using CCD camera (Sony, CA, USA), maximum cross-sectional area using microscopy (using NIH Image 1.47 software), with estimated volume calculated assuming a spherical colony shape.

Results

Infectant DU-145 cell lines form tumors in the prostate and peritoneal cavity

We examined the tumorigenicity of DU-145 sublines *in vivo* by injecting 2×10^6 cells either into one lobe of the anterior prostate (to reflect the *in situ* situation) or the peritoneal cavity (to recapitulate the initial stages of local invasion) of athymic mice. In intraprostate inoculated animals, an orthotopic site, tumor formation was observed in all DU-145 sublines, though c'973 DU-145 cells formed tumors only at a significantly lower rate. Distinct differences existed between the different cell lines when tumor invasiveness (local and distal) and metastasis (to lung) were examined (Table 1A). Only parental and WT sublines formed large tumors at the site of

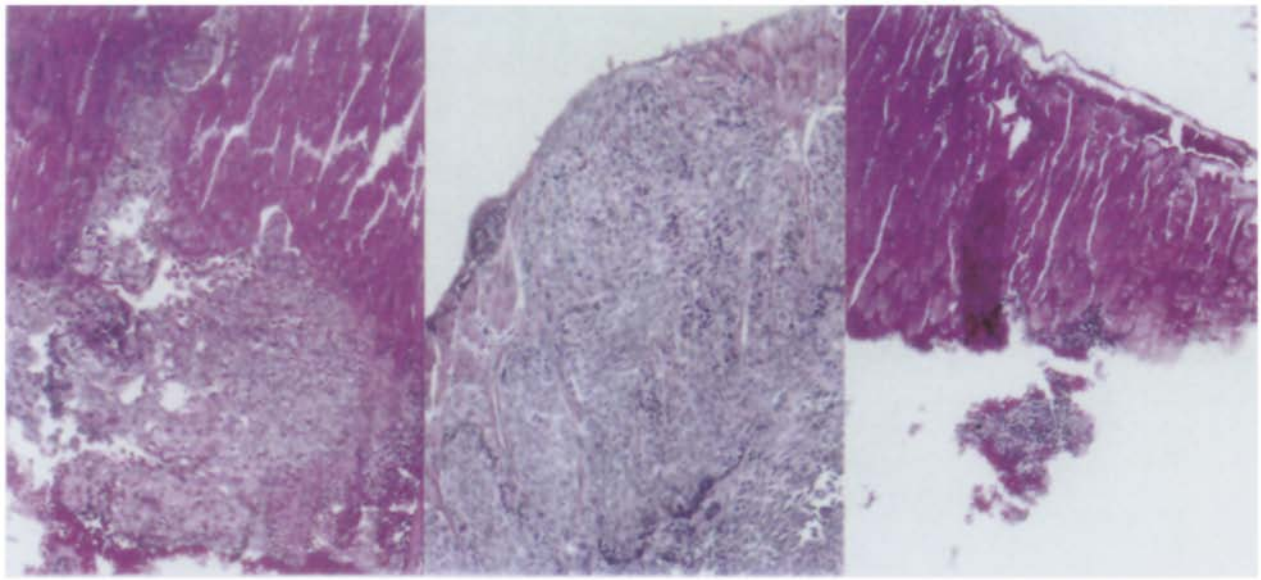

Figure 1. Invasion of parental (a), WT EGFR (b) and c'973 EGFR (c) DU-145 cells into the diaphragm. Mice were inoculated with 2×10^6 cells in the peritoneal cavity. Mice were euthanized 45 days later, and tumor growth, invasiveness and metastasis determined. Shown are representative tumors on the diaphragmatic surface as a measure of invasiveness. Parental tumors are fixed to the diaphragm with microscopic evidence of invasion, while WT EGFR cells have obliterated the diaphragm and have formed lung metastases. c'973 EGFR cells fail to attach firmly to the diaphragm with no evidence of invasion. The parental DU-145 tumor is shown at twice the magnification of the other tumors.

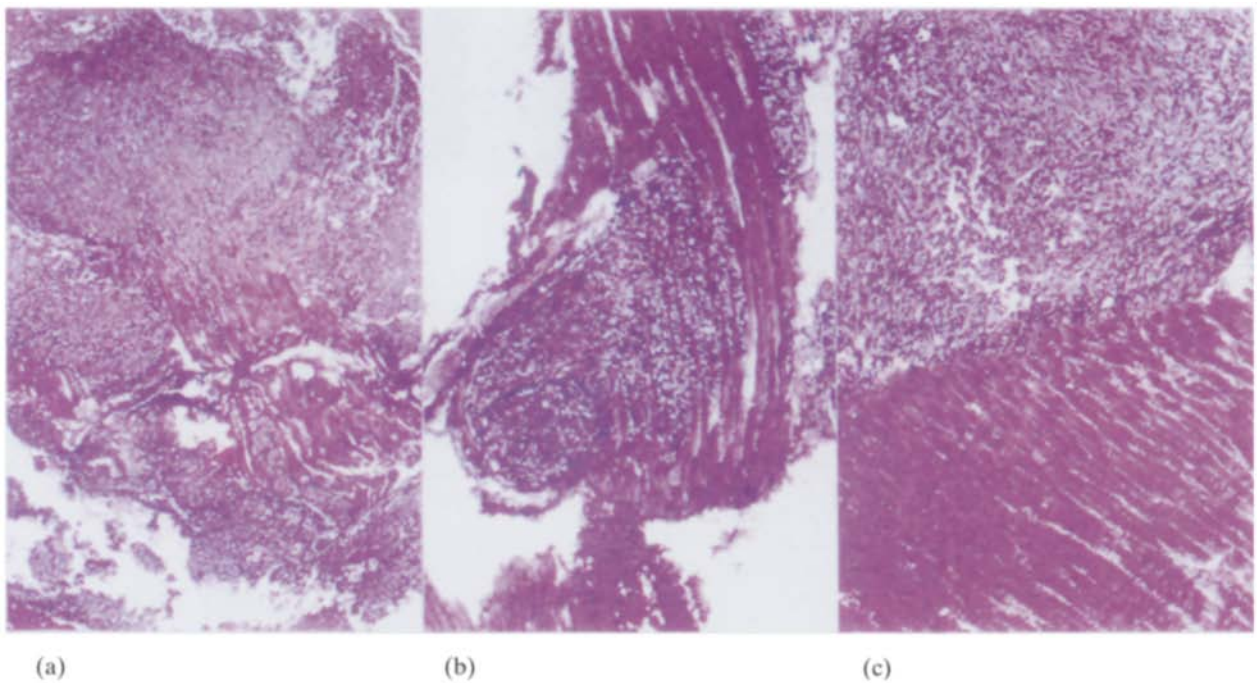

Figure 4. Invasion of control (a, b) and U73122 (c) treated WT DU-145 cells into the diaphragm. Mice were inoculated with 2×10^6 cells in the peritoneal cavity, and q4 day treatment initiated on day 3. Mice were euthanized 45 days later, and tumor growth and invasiveness determined. Shown are representative tumors on the diaphragmatic surface as a measure of invasiveness. Control-treated cells have obliterated the diaphragm (a, 40×; b, 100×). U73122-treated cells form tumors which fail to invade the diaphragm (100×).

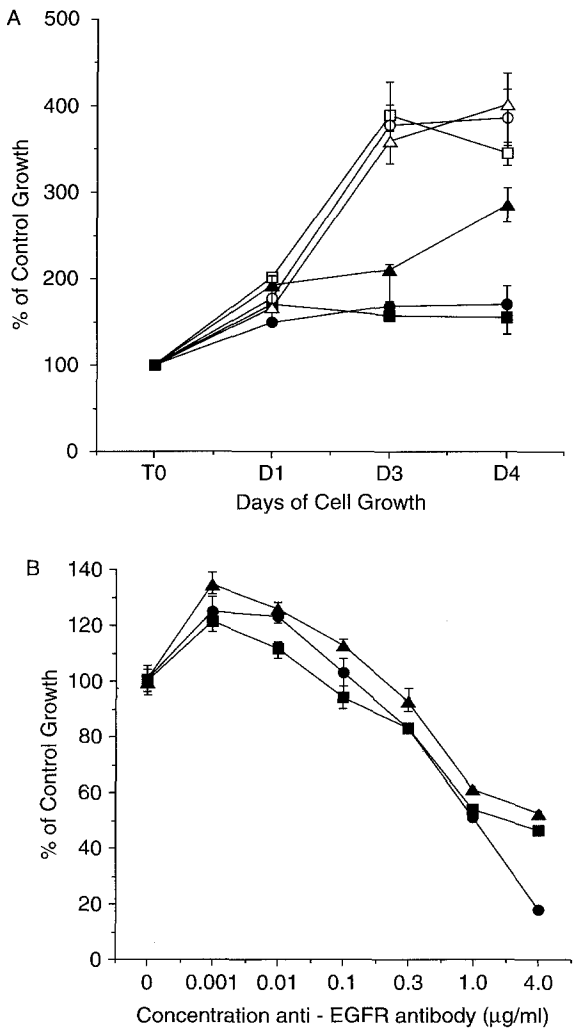

Figure 2. Inhibition of DU-145 cell growth by interruption of EGFR-TGFα autocrine pathway by an anti-EGFR antibody. (A) Parental (□), WT (○) and c'973 (△) infectant lines were exposed to anti-EGFR antibody (4 μg/ml) for up to 4 days in the presence of 7.5% FBS. Time zero (T0) was after 2 days in 0.5% dFBS quiescing medium; this medium was then changed to 7.5% FBS growth medium ± 4 μg/ml of anti-EGFR antibody for the duration of the experiment. Open symbols indicate growth medium only; closed symbols indicate the presence of antibody. (B) The infectant lines were exposed to various concentrations of anti-EGFR for 4 days in the presence of 7.5% FBS. Cell growth is expressed as a percentage (mean ± S.E.M., $n = 3$) of cells (A) at time 0 (T0) or (B) at day 4 (D4). Medium ± antibody was only added at the beginning of the experiments.

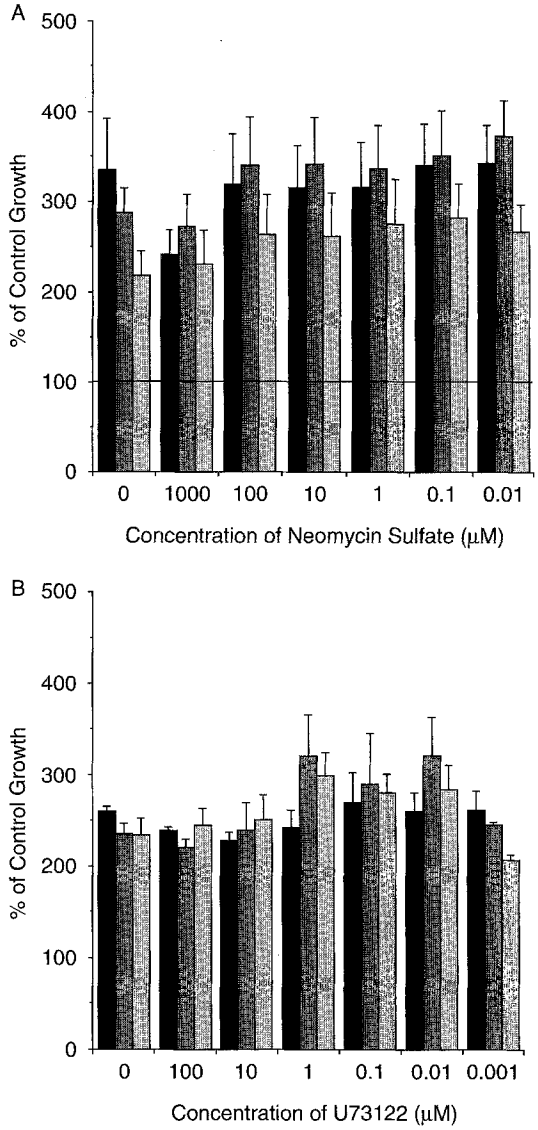

Figure 3. The effects of neomycin sulfate and U73122 on DU-145 cell growth. Parental (filled), WT (slashed) and c'973 (stippled) infectant lines were incubated with various concentrations of (A) neomycin sulfate (0.01–1000 μM) and (B) U73122 (0.001–100 μM) for 3 days in the presence of 7.5% FBS. Cell growth is expressed as a percentage (mean ± S.E.M., $n = 3$) of cells receiving 0.5% dFBS quiescing medium. Medium ± pharmacologic agents were only added at the beginning of the experiments.

injection (WT > PA >> c'973); c'973 tumors formed in the prostate were restricted to site of injection and were not locally invasive. Local (adjoining seminal vesicle; scored as 3+) and distal (through the capsule and into surrounding tissue; scored as 4+) tumor invasion and metastases (preaortic lymph nodes, pancreas, liver) were evident only in the parental and WT sublines, though the WT cells invaded to a greater extent. The incidence of macroscopic lung metastases for parental and WT sublines was similar (50% and 44%, respectively).

Intraperitoneal inoculations were utilized to assess tumor spreading outside of the prostate environment

(Table 1B). Again tumors formed in all groups, with the WT producing the most tumors in various areas of the peritoneal cavity and on the diaphragm serosal surface. Using the extent of tumor invasion into the diaphragm as an indicator of cell invasion [47, 48], DU-145 sublines expressing WT EGFR were aggressively invasive compared to parental and c'973 sublines (WT >> PA >> c'973) (Figure 1). Macroscopic lung metastases were seen only in parental and WT sublines (30% and 36%, respectively). The greater extent of invasion seen in sublines expressing WT EGFR in these *in vivo* models for prostate tumor progression emphasizes the importance of EGFR in tumor invasion.

All three DU-145 sublines require an EGFR-mediated autocrine loop for cell proliferation

One explanation for the differences found in tumor formation and invasiveness would be differential growth rates of the DU-145 sublines. We determined the cell growth rates *in vitro* using the MTT dye reduction method. All three sublines grew at comparable rates (Figure 2A). In an attempt to more closely approximate the *in vivo* situation, we performed soft-agar growth assays on the three DU-145 cell lines. All three lines presented similarly high cloning efficiencies. However, the volumes of the colonies formed after 15–19 days incubation mirrored the differences in *in vivo* tumor size with WT DU-145 cells forming the largest colonies ($144 \pm 7\%$ of parental DU-145 cells) and c'973 DU-145 cells the smallest ($42 \pm 2\%$) ($n = 4$, with 10 colonies calculated for each experiment).

These cells both express EGFR and produce TGFα [36]. This autocrine stimulatory loop has been shown to be important for promoting *in vitro* invasiveness as determined by transmigration of human extracellular matrix [36]. The importance of this EGFR-TGFα stimulatory loop for cell proliferation was investigated by determining cell proliferation in the presence of a antagonistic anti-EGFR antibody [49]. Anti-EGFR antibody reduced cell proliferation in all three sublines in a dose-dependent manner (Figure 2B). In parallel studies over a 4-day time-course study using 4 μg/ml of the anti-EGFR antibody, a decrease in cell proliferation was observed in all sublines. Inhibition of proliferation was evident in all groups by day 3 (Figure 2A). Exposure to the anti-EGFR antibody did not result in reduction of cell number below initial plating density, indicating inhibition of the EGFR is not a result of or resulting in cell death. In this study and a previous report from this laboratory [36], analysis of sublines exposed to anti-EGFR antibody for induced apoptotic cell death using the terminal deoxytransferase method (Apoptag Kit, Oncor, MD, USA) showed only marginal levels of apoptotic cell death (<4% of the exposed cells) resulted from the highest concentration of EGFR antibody (4 μg/ml; data not shown).

An inhibitor of phospholipase C activity reduces tumor invasiveness

In our earlier study [36], we found that *in vitro* invasiveness correlated with the capacity of the EGFR construct to induce cell motility; WT EGFR promotes both proliferation and chemokinesis whereas c'973 induces only proliferation [37]. Furthermore, we have shown that EGFR-mediated cell motility requires PLC activity, and can be inhibited by the pharmacologic agent U73122 (1 μM) (BIOMOL) [38]. We postulated that if *in vivo* invasiveness was promoted by EGFR-mediated motility signaling, U73122 should diminish tumor invasiveness.

First, the cytotoxicity of this agent was tested *in vitro* (Figure 3). In addition, neomycin was used as a control as it binds to the PLC target PIP_2. U73122 had no effect on cell proliferation over a 3-day period, even at doses 100-times greater than that which limits cell motility in NR6 fibroblasts [38]. Neomycin sulfate decreased cell proliferation only at the highest concentration tested, but even in this situation there was still significant cell growth over the 3-day period. These results give further credence to the predicted duality of the EGFR-mediated motility and mitogenesis pathways [37, 50].

The effect of neomycin sulfate and U73122 on prostatic tumor progression was determined in athymic mice bearing intraperitoneal injections of DU-145 WT, as this line was the most aggressive. Treatment with neomycin sulfate and U73122 was given to ascertain if the inhibition of PLC *in vivo* played any role in the inhibition of tumor invasion. The extent of tumor cell penetration into the musculature of the diaphragm serosal surface was the criteria measured. Neomycin sulfate, U73122 or control PBS and U73343 (the inactive congener of U73122) injections were initiated 3 days post tumor inoculation and continued every 4 days until the experiment was terminated on day 45 (Table 2). The WT DU-145 subline formed numerous tumors at several sites, with those on the diaphragm being extremely invasive. Tumor formation within the peritoneal cavity or on the diaphragm occurred in more than 60% of all treatment groups. Extensive invasion of the diaphragm was observed in control-treated animals (Figure 4). This identical pattern of

Table 2. Effects of neomycin sulfate and U73122 on WT DU-145 prostate tumor progression

Treatment	Tumor formation[a]	Diaphragm tumors	Diaphragm invasiveness[b]
Control[c]	7/8	7/8	3.6+ (2–4)
Neomycin[d]	3/4	3/4	3.3+ (3–4)
U73122[e]	8/12	8/12	0.6+ (0–2)

This table is a composite of two independent experimental series. In the first series, one control group (diluent alone; four mice) was compared with U73122-treated mice (five mice). In the second series, two control groups (U73343 or neomycin sulfate treatments; four mice each) were compared to U73122 treatments at two different doses.

[a] The number of mice with macroscopic tumors in the peritoneal cavity over the number of mice challenged.

[b] Invasiveness was scored microscopically on a scale of 0 (non-invasive) to 4 (tumor obliterating the diaphragm); the number is the average invasiveness of all diaphragm tumors (not including mice which did not present tumors on the diaphragm); the range of invasiveness is shown in parentheses.

[c] Control consists of two independent experimental series of four mice each. In the first series, mice were injected intraperitoneally with 0.1 ml of 10% DMSO in HBSS (diluent) q4d; in the second experiment the treatment consisted of U73343 (12 μg/mouse) in diluent. These two groups are listed together as there was no difference between them.

[d] Neomycin (150 μg/mouse) was injected intraperitoneally q4d in diluent.

[e] U73122 treatment consisted of three groups. In the first experimental series five mice were injected q4d at 12 μg/mouse; three of these mice formed tumors. In the second experimental series four mice were treated q4d at 12 μg/mouse (two formed tumors) and three mice (a fourth mouse died before initiation of treatment) received 24 μg/mouse (three formed tumors).

tumor progression was seen in the neomycin sulfate-treated animals (not shown). In U73122-treated animals, an aggressive pattern of tumor invasion was observed in only one animal. In the other seven animals presenting tumors on the diaphragm, only initial or negligible tumor cell invasion was observed histologically (Figure 4); the extent of invasiveness was reduced below that of parental cells. The results suggest that the PLC inhibitor, U73122, may have promise in inhibiting EGFR-mediated tumor progression.

Discussion

Up-regulated EGFR signaling has been correlated with tumor invasion and metastasis [23, 30–33]. However, the cell properties responsible for this increased progression are unknown. In this study, we demonstrate that *in vivo* invasiveness of DU-145 cells depends on EGFR signaling via phospholipase C and is independent of EGFR-mediated cell proliferation.

Recent reports have suggested that EGFR-mediated cell motility may, in part, promote tumor cell invasiveness [32, 36]. We utilized a panel of DU-145 cells which have been genetically engineered to overexpress either WT EGFRs, which promote both cell motility and proliferation, or c'973 EGFRs, which are fully mitogenic but non-motogenic [37]. We had shown earlier that the WT DU-145 cells invaded a human extracellular matrix *in vitro* to a greater extent than parental DU-145 cells. Expression of c'973 EGFR negatively regulated DU-145 invasiveness by down-regulating endogenous WT EGFR [36]. When inoculated either into the prostate or the peritoneal cavity of athymic mice, the same pattern was observed; WT DU-145 cells were the most invasive, whereas c'973 DU-145 cells were virtually non-invasive.

The differences in tumor invasiveness may have been secondary to altered cell growth rates, but *in vitro* all three sublines proliferated at indistinguishable rates. While it is difficult to assess cell growth *in vivo*; programmed cell death was not responsible for the lesser invasiveness of parental and, especially, c'973 DU-145 cells as we could detect few (<5%) apoptotic cells in any of the tumors examined by the TUNEL method. It is possible that the smaller size and number of the c'973 DU-145 tumors in the peritoneal cavity is due to decreased growth secondary to a failure of the cells to spread within the cavity or adhere to underlying structures. Alterations in cell aggregation and adhesion are currently being investigated. Failure to induce neovascularization also may have limited the size of c'973 DU-145 tumors, as autocrine binding of ligand by the non-down-regulating c'973 EGFR may prevent angiogenic TGFα from spreading beyond the tumor mass. However, this would not explain the markedly fewer tumors noted.

More plausibly, the increased invasiveness of the WT DU-145 tumors was secondary to other EGFR-mediated effects. We have previously shown that EGFR-mediated proliferation is separable from

motility [37, 50]. WT EGFR signals both mitogenesis and motility, but c'973 EGFR induces only mitogenesis while down-regulating endogenous WT EGFR [36, 37]. Thus, we could test the hypothesis that cell motility contributes to invasiveness by specifically disrupting the motogenic pathway. U73122, a pharmacologic agent which inhibits PLC [39, 40], inhibits EGFR-mediated cell motility but not mitogenesis [38]. U73122 had no effect on DU-145 cell proliferation *in vitro*. When athymic mice were inoculated intraperitoneally with WT DU-145 cells, equal number and size of tumors were formed in the presence or absence of U73122. However, the tumors were significantly less invasive after treatment with U73122, being less invasive than parental DU-145 and similar in invasiveness to cells expressing the non-motogenic c'973 EGFR. Thus, invasiveness is a tumor property which can be modulated or inhibited independently of tumor growth.

These data strongly support a major role for cell motility of the EGFR-mediated behavior linked to tumor progression. This is not to argue that other mechanisms are not also required for tumor invasiveness. Many reports attest to the necessity of proteases in the invasive process. All three DU-145 sublines produce copious amounts of collagenases, UPa, and other proteases [36], even though c'973 DU-145 tumors were essentially non-invasive. This suggests that, while proteolysis is required for invasiveness, other properties, such as motility, may play a major regulatory role in tumor invasiveness. The results herein also highlight the feasibility of targeting cell motility mechanisms as novel targets for control of nascent and metastasized tumors.

Acknowledgements

The authors thank Drs Steve Barnes and Greg Peterson for assistance and advice, and Mark Van Epps-Fung, Heng Xie and Kiran Gupta for discussion and technical assistance. This study was supported in part by a Merit Award from the Veterans Administration and a grant from the American Cancer Society (CB-118).

References

1. Partin AW and Coffey DS, 1994, *Benign and Malignant Prostatic Neoplasms: human studies*. San Diego, California: Academic Press.
2. Sandberg AA, 1992, *Cytogenetic and Molecular Genetic Aspects of Human Prostate Cancer: primary and metastatic*. New York: Plenum Press.
3. Geldof AA and Rao BR, 1990, Factors in prostate cancer metastasis. *Anticancer Res*, **10**, 1303–6.
4. Linehan WM, 1995, Inhibition of prostate cancer metastasis: a critical challenge ahead. *J Natl Cancer Inst*, **87**, 331–2.
5. Gittes RF, 1991, Carcinoma of the prostate. *N Engl J Med*, **324**, 236–45.
6. Surya BV and Provet JA, 1989, Manifestations of advanced prostate cancer: prognosis and treatment. *J Urol*, **142**, 921–8.
7. McKeehan WL, 1986, Growth factors spawn new cell cultures. *Nature*, **321**, 629–30.
8. Chaproniere DM and Webber MM, 1985, Dexamethasone and retinyl acetate similarly inhibit and stimulate EGF or insulin-induced proliferation of prostatic epithelium. *J Cell Physiol*, **122**, 249–53.
9. Eaton CL, Davies P and Phillips MEA, 1988, Growth factor involvement and oncogene expression in prostatic tumors. *J Ster Biochem*, **30**, 341–5.
10. Marti U, Burwen SJ and Jones AL, 1989, Biological effects of epidermal growth factor, with emphasis on the gastrointestinal tract and liver: an update. *Hepatology*, **9**, 126–38.
11. Connolly JM and Rose DP, 1989, Secretion of epidermal growth factor and related polypeptides by the DU 145 human prostate cancer cell line. *Prostate*, **15**, 177–86.
12. Nishi N, Matuo Y and Wada F, 1988, Partial purification of a major type of rat prostatic growth factor: characterization as an epidermal growth factor-related mitogen. *Prostate*, **13**, 209–20.
13. Wilding G, Valverius E, Knabbe C and Gelmann EP, 1989, Role of transforming growth factor-α in human prostate cancer cell growth. *Prostate*, **15**, 1–12.
14. Liu X-H, Wiley HS and Meikle AW, 1993, Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming gowth factor-α (TGF-α) and epidermal growth factor (EGF)/TGF-α receptor. *J Clin Endocr Metab*, **77**, 1472–8.
15. Aaronson SA, 1991, Growth factors and cancer. *Science*, **254**, 1146–53.
16. Schlegel J, Merdes A, Stumm G, *et al.*, 1994, Amplification of the epidermal growth factor receptor gene correlates with different growth behavior in human glioblastoma. *Int J Cancer*, **56**, 72–7.
17. Collins VP, 1993, Amplified genes in human gliomas. *Sem Cancer Biol*, **4**, 27–32.
18. Yasui W, Sumiyoshi H, Hata J, *et al.*, 1988, Expression of epidermal growth factor receptor in human gastric and colon carcinomas. *Cancer Res*, **48**, 137–41.
19. Neal DE, Marsh C, Bennett MK, *et al.*, 1985, Epidermal-growth-factor receptors in human bladder cancer: comparison of invasive and superficial tumours. *Lancet*, **i**, 366–8.
20. Nguyen PL, Swanson PE, Jaszcz W, *et al.*, 1994, Expression of epidermal growth factor receptor in invasive transitional cell carcinoma of the urinary bladder: a multivariate survival analysis. *Am J Clin Pathol*, **101**, 166–76.
21. Klijn JG, Berns PM, Schmitz PI and Foekens JA, 1992, The clinical significance of epidermal growth factor receptor (EGF-R) in human breast cancer: a review on 5232 patients. *Endocr Rev*, **13**, 3–17.
22. Sainsbury JRC, Farndon JR, Needham GK, Malcolm AJ and Harris AL, 1987, Epidermal-growth-factor receptor status as predictor of early recurrence of and death from breast cancer. *Lancet*, **i**, 1398–402.

23. Radinsky R, Risin S, Fan D, *et al.*, 1995, Level and function of epidermal growth factor receptor predict the metastatic potential of human colon carcinoma cells. *Clin Cancer Res*, **1**, 19–31.
24. Lubrano C, Petrangeli E, Catizone A, *et al.*, 1989, Epidermal growth factor binding and steroid receptor content in human benign prostatic hyperplasia. *J Ster Biochem*, **34**, 499–504.
25. Morris GL and Dodd JG, 1990, Epidermal growth factor receptor mRNA levels in human prostatic tumors and cell lines. *J Urol*, **143**, 1272–4.
26. Davies P and Eaton CL, 1989, Binding of epidermal growth factor by human normal, hypertrophic, and carcinomatous prostate. *Prostate*, **14**, 123–32.
27. Myers RB, Kudlow JE and Grizzle WE, 1993, Expression of transforming growth factor alpha, epidermal growth factor and the epidermal growth factor receptor in adenocarcinoma of the prostate and benign prostatic hyperplasia. *Modern Pathol*, **6**, 733–7.
28. Ching KZ, Ramsey E, Pettigrew N, D'Cunha R, Jason M and Dodd JG, 1993, Expression of mRNA for epidermal growth factor, transforming growth factor-alpha and their receptor in human prostate tissue and cell lines. *Molec Cell Biochem*, **126**, 151–8.
29. Tillotson JK and Rose DP, 1991, Endogenous secretion of epidermal growth factor peptides stimulates growth of DU145 prostate cancer cells. *Cancer Lett*, **60**, 109–12.
30. Haugen DRF, Akslen LA, Varhaug JE and Lillehaug JR, 1993, Demonstration of a TGF-α-EGF-receptor autocrine loop and c-myc protein over-expression in papillary thyroid carcinomas. *Int J Cancer*, **55**, 37–43.
31. Hamada J, Nagayasu H, Takayama M, Kawano T, Hosokawa M and Takeichi N, 1995, Enhanced effect of epidermal growth factor on pulmonary metastasis and *in vitro* invasion of rat mammary carcinoma cells. *Cancer Lett*, **89**, 161–7.
32. Chakrabarty S, Rajagopal S and Huang S, 1995, Expression of antisense epidermal growth factor receptor RNA downmodulates the malignant behavior of human colon cancer cells. *Clin Exp Metastasis*, **13**, 191–5.
33. Korc M, Chandrasekar B, Yamanaka Y, *et al.*, 1992, Overexpression of the epidermal growth factor receptor in human pancreatic cancer is associated with concomitant increases in the levels of epidermal growth factor and transforming growth factor alpha. *J Clin Invest*, **90**, 1352–60.
34. Stone K, Mickey DD, Wunderli H, Mickey GH and Paulson DF, 1978, Isolation of a human prostate carcinoma cell line (DU145). *Int J Cancer*, **21**, 274–81.
35. Connolly JM and Rose DP, 1992, Interactions between epidermal growth factor-mediated autocrine regulation and linoleic acid-stimulated growth of a human prostate cancer cell line. *Prostate*, **20**, 151–8.
36. Xie H, Turner T, Wang M-H, Singh RK, Siegal GP and Wells A, 1995, *In vitro* invasiveness of DU-145 human prostate carcinoma cells is modulated by EGF receptor-mediated signals. *Clin Exp Metastasis*, **13**, 407–19.
37. Chen P, Gupta K and Wells A, 1994, Cell movement elicited by epidermal growth factor receptor requires kinase and autophosphorylation but is separable from mitogenesis. *J Cell Biol*, **124**, 547–55.
38. Chen P, Xie H, Sekar MC, Gupta KB and Wells A, 1994, Epidermal growth factor receptor-mediated cell motility: phospholipase C activity is required, but MAP kinase activity is not sufficient for induced cell movement. *J Cell Biol*, **127**, 847–57.
39. Bleasdale JE, Thakur NR, Gremban RS, *et al.*, 1990, Selective inhibition of receptor-coupled phospholipase C-dependent processes in human platelets and polymorphonuclear neutrophils. *J Pharm Exp Ther*, **255**, 756–68.
40. Powis G, Seewald MJ, Gratas C, Melder D, Riebow J and Modest EJ, 1992, Selective inhibition of phosphatidylinositol phospholipase C by cytotoxic ether lipid analogues. *Cancer Res*, **52**, 2835–40.
41. Wells A, Welsh JB, Lazar CS, Wiley HS, Gill GN and Rosenfeld MG, 1990, Ligand-induced transformation by a non-internalizing EGF receptor. *Science*, **247**, 962–4.
42. Ullrich A, Coussens L, Hayflick JS, *et al.*, 1984, Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. *Nature*, **307**, 418–25.
43. Welsh JB, Gill GN, Rosenfeld MG and Wells A, 1991, A negative feedback loop attenuates EGF-induced morphological changes. *J Cell Biol*, **114**, 533–43.
44. Masui H, Wells A, Lazar CS, Rosenfeld MG and Gill GN, 1991, Enhanced tumorigenesis of NR6 cells which express non-downregulating epidermal growth factor receptors. *Cancer Res*, **51**, 6170–5.
45. Service NTI, 1981–82, *Registry of Toxic Effects of Chemical Substance*. Washington DC: US Department of Commerce.
46. Peterson G and Barnes S, 1993, Genistein and biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *Prostate*, **22**, 335–45.
47. Knox JD, Mack CF, Powell WC, Bowden GT and Nagle RB, 1993, Prostate tumor cell invasion: a comparison of orthotopic and ectopic models. *Invasion and Metastasis*, **13**, 325–31.
48. Powell WC, Knox JD, Navre M, *et al.*, 1993, Expression of the metalloproteinase matrilysin in DU-145 cells increases their invasive potential in severe combined immunodeficient mice. *Cancer Res*, **53**, 417–22.
49. Sunada H, Magun BE, Mendelsohn J and MacLeod CL, 1986, Monoclonal antibody against epidermal growth factor receptor is internalized without stimulating receptor phosphorylation. *Proc Natl Acad Sci, USA*, **83**, 3825–9.
50. Chen P, Xie H and Wells A, 1996, Mitogenic signaling from the EGF receptor is attenuated by a motility-associated phospholipase C-γ/protein kinase C feedback mechanism. *Molec Biol Cell*, **7**, in press.