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In vitro invasiveness of DU-145 human prostate carcinoma cells is modulated by EGF receptor-mediated signals

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Prostate carcinomas often present an autocrine stimulatory loop in which the transformed cells both express the EGF receptor (EGFR) and produce activating ligands (TGFα and EGF forms). Up-regulated EGFR signalling has been correlated with tumor progression in other human neoplasia; however, the cell behaviour which is promoted remains undefined. To determine whether an EGFR-induced response contributes to cell invasiveness, we transduced DU-145 human prostate carcinoma cells with either a full-length (WT) or a mitogenically-active but motility-deficient truncated (c'973) EGFR. The DU-145 Parental and two transgene sublines all produced EGFR and TGFα, but the transduced WT and c'973 EGFR underwent autocrine downregulation to a lesser degree, with more receptor remaining intact. DU-145 cells transduced with WT EGFR transmigrated a human amniotic basement membrane matrix (Amgel) to a greater extent than did Parental DU-145 cells (175±22%). Cells expressing the c'973 EGFR invaded through the Amgel only to about two thirds the extent of the Parental cells (62±23%). A monoclonal antibody which prevents ligand-induced activation of EGFR decreased the invasiveness of WT-expressing cells by half and Parental cells by a fifth, but had little effect on the invasiveness of c'973-expressing cells; with the result that in the presence of antibody, all three cell lines transmigrated the Amgel to the same extent. The different levels of invasiveness between the three sublines were independent of cell proliferation. These findings demonstrated that EGFR-mediated signals increase tumor cell invasiveness and suggested that domains in the carboxy–terminus are required to signal invasiveness. As an initial investigation into the mechanisms underlying the EGFR-mediated enhanced invasiveness, we determined whether these cells presented different collagenolytic activity, as the major constituents of Amgel are collagen types I and IV. All three sublines secreted easily detectable levels of gelatin-directed proteases and TIMP-1, with WT cells secreting equivalent or lower levels of proteases. The proteolytic balance in these cells did not correlate with invasiveness. These data suggest that the TGFα-EGFR autocrine loop promotes invasiveness and that this is accomplished by signalling cell properties other than differential secretion of collagenolytic activity.

Keywords: EGF receptor, invasiveness, prostate carcinoma cells, tumor progression

Introduction

Prostate tumor invasion and metastatic spread present major obstacles to successful cancer control. Transmigration of an extracellular matrix (ECM) is a complex process which requires active interactions between the invading cell and the extracellular matrix and other stromal elements [1, 2]. At least three processes are necessary for cell invasiveness: tumor cell recognition of and adhesion to the ECM, proteolytic remodeling or destruction of the ECM, and cell migration through the resultant defect. The relative contributions of these processes to cell invasiveness may vary under different circumstances. In many situations increased proteolytic activity has been shown to promote cell invasiveness. For example,

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invasion and metastasis of PC-3 prostate carcinoma cells correlate with cellular urokinase-type plasminogen activator (UPa) activity [3, 4]. Conversely, levels of tissue inhibitors of metalloproteases (TIMP) inversely affect cell invasiveness and metastasis [5–10]. Alterations in cell recognition of ECM or cell migration also modulate tumor cell invasiveness [1, 11]. However, modulation of tumor cell behavior by stromal elements, including matrix components and asssociated cytokines [1, 2, 12], complicates deciphering which signals promote these invasive properties. Thus, linking specific signals to invasive processes requires isolation *in vitro*.

The receptor for the epidermal growth factor (EGF) is the peptide growth factor receptor most often found upregulated in human carcinomas [13]. EGF receptor (EGFR) gene amplification or elevated levels of EGF binding sites correlate with tumor progression to invasiveness and metastasis. Gene amplification is noted in the majority of glioblastomas but is not seen in encapsulated gliomas [14, 15]; increased levels of EGFR are detected in invasive bladder carcinoma [16, 17] and advanced gastric carcinoma [18]; and elevated levels of EGFR correlate with metastasis and decreased survival in breast cancer patients [19, 20]. In an experimental model system, metastatic potential of human colon carcinoma cells correlated with EGFR level and function [21]; similarly, metastasis of a non-small cell lung carcinoma line was found to be dependent on the level of the EGFR-related *c-erbB-2/neu* [22]. *In vitro*, exogenous EGF has been shown to promote thyroid tumor cell invasiveness through matrigel [23].

In prostate carcinoma, one notes either increased levels of EGFR [24, 25], production of its activating ligands, EGF and TGFα [26, 27], or both [28, 29]. Thus, an autocrine stimulatory loop is often present in prostate carcinoma, although its role remains obscure. Such an autocrine loop has been detected in the DU-145 human prostate carcinoma cell line [30], which produces TGFα and expresses EGFR [28, 29, 31]. As EGFR activation has been shown to signal production of proteolytic enzymes [3, 32, 33] and extracellular matrix proteins [34, 35], and to enhance cell motility [36, 37], we investigated whether this autocrine stimulatory loop promotes DU-145 invasiveness. We analyzed the DU-145 cells *in vitro*, using a human extracellular matrix which does not contain appreciable levels of EGF, TGFα, PDGF or TGFβ [38], thus isolating the effect of autocrine stimulation from stromal cell factors and matrix-associated cytokines.

Transmigration of the matrix was increased by overexpression of the full-length, wild-type (WT) EGFR [39], in the absence of exogenous EGF or TGFα. A kinase-active but motility-deficient, carboxy-terminal-truncated EGFR (c'973) [36, 37] was overexpressed in parallel, to control for the level of mitogenic EGFR signalling; this EGFR construct reduced cell invasiveness. Invasiveness of both parental DU-145 cells and WT EGFR-expressing cells was diminished by a non-activating, EGFR-inhibitory antibody, thus demonstrating the EGFR-dependency of this cell property.

Materials and methods

Generation of retrovirus vectors containing EGFR

The construction of the EGFR was by standard methods, and has been described previously [39]. WT EGFR is a full-length cDNA [40] derived from a placental cDNA library [41]; when expressed in appropriate cells, this construct elicits all of the responses of wild type EGFR. c'973 EGFR represents a carboxy-terminal-truncated EGFR in which a stop codon was introduced just distal to amino acid 973; this construct presents ligand-induced kinase and mitogenic activities but does not possess phosphotyrosine motifs or induce cell motility. The EGFR constructs were cloned into a murine Moloney leukemia virus-based retroviral expression vector in the *gag* position. An SV40 early promoter and neomycin phosphotransferase gene, as the selectable marker, were cloned in the *env* position. Purified plasmid was transfected into PA-137 amphotropic producer cells using the lipofectin reagent (Gibco/BRL) [36]. Polyclonal producer lines were established from >20 G418-resistant (350 μg/ml) colonies.

Cell culture and infection

DU-145 cells, originally derived from a human prostate carcinoma brain metastasis [30], were grown in DMEM/F12 (50/50) media supplemented with fetal bovine serum (FBS; 7.5%), penicillin (100 U/ml), streptomycin (200 μg/ml), non-essential amino acids, sodium pyruvate (1 mM), and glutamine (2 mM) (37°C, 90% humidity, 5% CO_2). Cells were passaged at subconfluence by trypsinization (0.25%, 1 mM EDTA).

Infection of cells by retroviruses containing EGFR constructs was accomplished by established protocols [39]. Briefly, cells were seeded at ~30% confluence. Once cells had adhered overnight, the medium was replaced with cell-free, virus-containing PA-137 conditioned medium containing polybrene (4 μg/ml). Three hours later, an equal volume of growth medium was added and the incubation continued for an additional 3 h. The cells were then washed and

incubated in growth media for 48 h prior to selection in G418 (Gibco/BRL) (1 mg/ml). Polyclonal lines consisting of >20 colonies were established and maintained in G418-containing media. DU-145 cells transduced with either the WT EGFR or c'973 EGFR construct hereafter are referred to as WT cells or c'973 cells, respectively; uninfected DU-145 cells are referred to as Parental cells.

EGFR detection assays

The presence of EGFR on the surface of infectant cells was determined by EGF binding and immunodetection of EGFR (below). Scatchard analyses determined the apparent binding site number and affinity for EGF. Cells were seeded in 12-well culture plates (~300000 cells/well). The cells were stripped of prebound, autocrine ligand by incubation at pH 2.5 for 10 min [42]. After two washes with PBS, the cells were incubated for 30 min in serum-free media, followed by incubation (4°C for 2 h) in binding buffer (DME with 25 mM HEPES, pH 7.4, and 0.2% BSA) containing 0.1 nM ^{125}I labeled-EGF and unlabeled EGF (0 to 100 nM). Unbound ^{125}I-EGF was collected from the supernatant and two subsequent washes with binding buffer. The cells were lysed to liberate the bound ^{125}I-EGF, which was collected. Free and bound ^{125}I-EGF were counted and the B_{max} and K_d values for each cell line were calculated from Scatchard plots after subtracting background counts (radioactivity bound in the presence of 10–100 nM EGF).

Immunohistochemical detection of EGFR and TGFα

Cells were trypsinized and plated onto glass coverslips. The cells were grown for 2–3 days to allow all surface proteins to be resynthesized. Cells were washed twice in PBS, fixed in 10% buffered formalin, processed, and stained by standard procedures. Briefly, tissues were passed through a series of ethanol and acetone washes for dehydration and fixation. To prevent nonspecific staining, 3% hydrogen peroxide incubation was performed for 5 min to quench endogenous peroxidase activity. For detection of EGFR, cells were pretreated with 0.05% saponin for 30 min, washed, blocked with 1% non-immune rabbit serum for 60 min at room temperature and incubated with a monoclonal anti-EGFR antibody (AB-1; 5 μg/ml, Oncogene Science) for 60 min. For detection of TGFα, cells were blocked with normal rabbit serum and then probed with a monoclonal anti-TGFα antibody (10 μg/ml) (courtesy of Jeffrey Kudlow, UAB [27]) overnight at 4°C. Unbound antibody was removed by several washes in PBS, and antigen was visualized using the biotin-streptavidin detection HRP super-sensitive system (Biogenex). Cells were counterstained with hema-toxylin to visualize nuclei.

In vitro *invasion assay*

Amgel, a human extracellular matrix, was prepared from normal full-term human placenta amniotic membranes as previously described [38]. The major constituents of Amgel are collagen types I and IV, laminin, entactin, tenascin, and heparan sulfate proteoglycan. EGF and TGFα are not detected in this matrix, in contrast to EHS-derived Matrigel [38, 43, 44]. Furthermore, by employing a human-derived ECM, we maintain species homogeneity for all parameters of the assay–cells, ECM, and EGFR.

Cell invasiveness was measured in a modified chamber assay. 100 μg Amgel was layered onto a polycarbonate filter (8 mm pore; 8 mm diameter); uniformity of coating was ascertained by Coomassie blue staining of a parallel well. Cells were labeled metabolically with ^{3}H-thymidine (1 μCi/ml) for 20 h. Cells were washed free of unincorporated thymidine and seeded onto the Amgel-coated filters (50000 cells/well in 0.4 ml DMEM/0.2% bovine serum albumin). The lower chamber contained DMEM supplemented with 10% FBS. The chambers were incubated at 37°C (90% humidity, 5% CO_2) for 24 h, after which the medium in the upper chamber was replaced with DMEM (without bovine serum albumin). After a further 48 h incubation cells were harvested from the lower chamber and the underside of the filter. Quantitation of cells was performed by scintillation counting and the percentage transmigration calculated. All experiments were performed in quadruplicate. In all experiments a highly invasive fibrosarcoma cell line, HT1080, served as the positive control. Determining the percentage of incorporated radiolabel rather than numbers of cells in the lower chamber avoided potential over-estimation of transmigration which may occur secondary to cell proliferation.

In the antibody inhibition studies, cells were mixed with antibodies immediately prior to seeding onto the Amgel-coated filters. A non-activating anti-EGFR monoclonal antibody which prevents EGF binding (mAb 528) [45] was utilized at 4 μg/ml to prevent EGFR-mediated signaling. Nonspecific murine IgG served as a control in these assays.

Collection of secreted proteins

Cells were plated at 60–80% confluence directly onto plastic culture dishes or dishes coated with Amgel. Cells were allowed to attach in media containing 7.5% FBS and 12 to 14 h later were switched to media containing 1% dialyzed FBS (dFBS). This level of

dFBS was required to maintain cell viability whilst inducing quiescence of the cells [36]. Cells plated on plastic were treated with or without EGF (10 nM); a saturating level of EGF was maintained for the entire assay period [46]. Conditioned media were collected over one of two 14 h periods; 0–14 or 34–48 h. The conditioned media were clarified by centrifugation at 1000 ***g*** for 5 min. Protein was precipitated from the supernatant using 60% ammonium sulfate. The protein pellet was dissolved in 50 mM Tris (pH 7.4) and dialyzed against TBS (25 mM Tris (pH 7.4), 150 mM NaCl). Protein quantitation was performed using the Bradford method (Pierce, Rockford, IL).

Immunoblotting detection of proteins

Proteins were separated by SDS-PAGE (polyacrylamide gel electrophoresis), using standard procedures. Samples were boiled in SDS-PAGE buffer under reducing conditions (5% β-mercaptoethanol). After electrophoretic separation, the proteins were transferred to a PVDF membrane (Millipore). EGFR was detected using a monoclonal antibody directed against an extracellular epitope (LA22, Oncogene Sciences); TIMP-1 using rabbit polyclonal antisera; TIMP-2 using a monoclonal antibody; MMP-9 (92 kDa gelatinase) using rabbit polyclonal antisera; MMP-2 (72 kDa gelatinase) using a monoclonal antibody (all gifts from Dr Kirby Bodden, UAB). Visualization was accomplished with a second antibody conjugated to alkaline phosphatase followed by color development (ProtoBlot system; Promega).

Zymography for proteolytic activity

Collagenolytic and plasminogen-dependent collagenolytic activities were detected by the SDS-PAGE-zymography method [47, 48]. 7.5% polyacrylamide gels were copolymerized with 0.15% gelatin; a subset of these gels also included plasminogen (1 μg/ml). Protein samples were mixed with PAGE sample buffer (without reducing agents, and without heating/boiling) before loading onto the 4% stacking gel. SDS was removed from the gels by washing twice for 30 min in 50 mM Tris (pH 7.4) containing 2.5% Triton X-100 and then twice for 5 min in 50 mM Tris (pH 7.4). The gels were incubated either for 3 h at 37°C or overnight at 22°C in digestion buffer (50 mM Tris (pH 7.4), 200 mM NaCl, 10 mM CaCl2, 1% Triton X-100). Lysis zones were visualized after staining with amido black [8].

RNA isolation and message analysis

Cells were grown to ~80% confluence. Total RNA was isolated using the RNAzol B reagent (TEL-TEST Inc). A standard Northern hybridization protocol was used for total RNA analysis. In brief, 30 μg total RNA or 10 μg oligo-dT-binding RNA from each cell line was electrophoresed through a 1% agarose/7% formaldehyde gel. RNA was transferred to a Nitropur nitrocellulose membrane (MSI Micron Separation Inc) by capillary transfer and fixed by baking (2 h at 80°C). Probes were radiolabeled using hexanucleotide random primers in the presence of [α-^{32}P]-dCTP to a specific activity of $>10^8$ cpm/μg DNA. cDNA clones for human TIMP-1 and TIMP-2 were a gift from Dr Unnar Thorgeirsson (NIH).

Results

EGF receptors were expressed on parental and infectant DU-145 cells which also produce TGFα

The presence of both TGFα and its receptor, EGFR, was determined in Parental and infectant DU-145 cells (Figure 1). Cells were plated on glass coverslips under conditions which minimized receptor-ligand internalization and degradation [49, 50]. Immunohistochemical staining detected TGFα in all three sublines (Figure 1), confirming previous reports of DU-145 cells producing TGFα [28, 29]. A monoclonal antibody directed against an epitope in the extracellular ligand-binding domain of EGFR revealed homogenous EGFR expression in all three cell lines (Figure 1), as expected after retroviral transduction [39, 41].

The co-expression of TGFα and EGFR would be expected to downregulate EGFR in these cells. EGFR were detected by immunoprecipitation followed by immunoblotting, while cultured under conditions of normal cell growth, during which EGFR downregulation would proceed (Figure 2A). The carboxy-terminal truncated EGFR is 213 amino acids shorter, and migrates as a ~25 kDa smaller protein in SDS-PAGE [51]. EGFR were barely detectable in the Parental cells. The WT cells presented significantly more full-length EGFR mass than the Parental line (Figure 2A). Thus, full-length EGFR are not down-regulated and degraded to the same extent in the WT cells, presumably due to the combined production from the endogenous and exogenous EGFR genes exceeding the endocytic capacity [49]; though limited EGFR degradation was noted by the presence of the calpain hinge-cleaved product at ~150 kDa [52, 53]. As the transduced WT EGFR migrates identically to endogenous receptor, the source of the EGF binding sites presented on the WT cells could not be ascertained with certainty; however, RNA analysis demonstrated that there was EGFR message from the transduced gene. The majority of the EGFR on the c'973 cells was the transduced EGFR migrating at

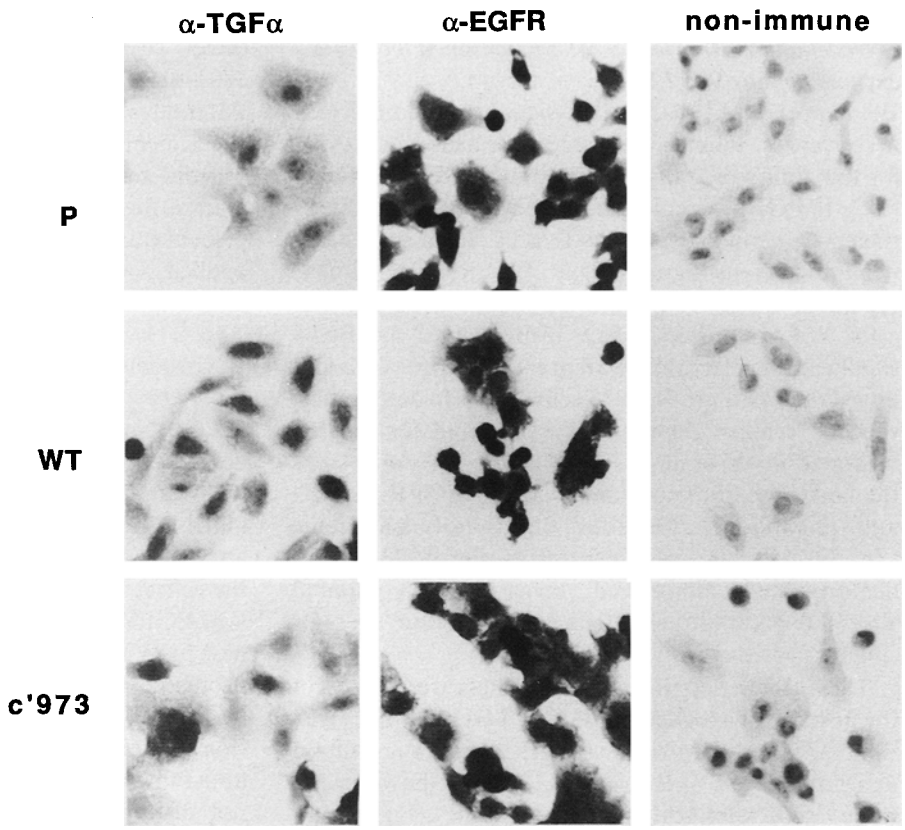

Figure 1. Production of TGFα and expression of EGF receptor on Parental (P) and infectant DU-145 sublines (WT and c'973). Cells were analyzed by immunohistochemistry as described in Materials and methods. TGFα protein was found in a predominantly perinuclear cytoplasmic distribution as expected for a secreted protein. EGFR was detected in a pattern consistent with surface expression.

~150 kDa (Figure 2A). This is most likely the consequence of c'973 being resistant to ligand-induced down-regulation [39] due to removal of internalization domains [51]. Importantly, the level of full-length (endogenous) EGFR, at ~175 kDa, was decreased in this line.

Minimization of EGFR downregulation by cell plating in the absence of trypsin, extensive washing, and brief (~12 h) recovery period, coupled with inhibition of degradation by methylamine (30 nM) [49, 54], demonstrated that all three sublines produced EGFR (Figure 2B). In the c'973 cells, both the endogenous full-length receptor and transduced truncated receptor were noted. Under these conditions, which promote accumulation of EGFR on the cell surface, differences in EGF binding capacity were seen by Scatchard analyses of these cells after removal of bound endogenous ligand. WT and c'973 cells presented significantly more binding sites (136% and 196%, respectively) than the Parental cells; EGFR on all three sublines had similar K_d values (ranging from 0.6 to 0.9 nM). These results are in accordance with the relative levels seen by immunoblotting (Figure 2B). In summary, all three lines produced both ligand and receptor, allowing for autocrine signaling.

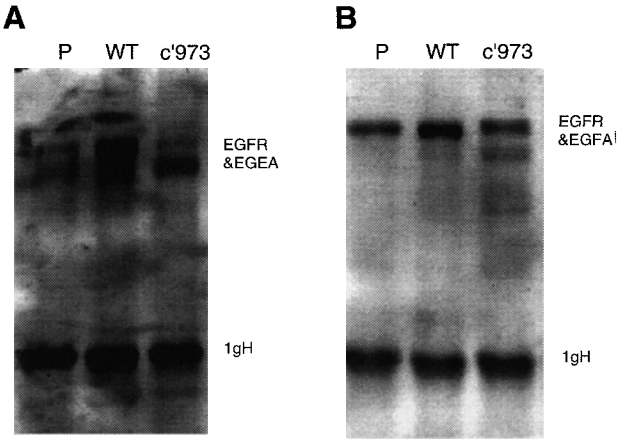

Figure 2. EGF receptor expression in the DU-145 sublines. Cells were analyzed under conditions in which autocrine down-regulation was allowed to occur (**A**) or minimized and endosomal degradation blocked by methylamine (**B**). EGFR were immunoprecipitated from Parental (P) cells and WT and c'973 cells using Mab 528. The immunoprecipitates were separated by 7.5% SDS-PAGE, and detected by immunoblotting with a second antibody. Endogenous and WT EGFR are denoted at ~175 kDa, with a minor amount being the calpain-hinge cleaved version which migrates slightly faster (~150 kDa) [52, 53]. The truncated c'973 EGFR, being 213 amino acids shorter, migrates at ~150 kDa. IgH refers to the heavy chain of the immunoprecipitating antibody.

Cells expressing WT EGFR transmigrated a human extracellular matrix to a greater extent than those expressing the c'973 EGFR

We hypothesized that EGFR signaling promotes cell invasiveness. Parental and infectant lines were tested for the ability to transmigrate a human ECM, Amgel [38]. If EGFR signaling contributed to invasiveness then we would expect WT cells to demonstrate enhanced transmigration, as EGFR were down-regulated to a lesser extent than those on the Parental cells. WT cells did, in fact, transmigrate the ECM significantly better than Parental cells (1.75 ± 0.22-fold on average) (Figure 3). c'973 cells served to determine if specific receptor domains were required for EGFR-mediated invasive-ness. This truncated receptor lacks the unique carboxy-terminus of the EGFR and all auto-phosphorylation sites, but is fully mitogenic [36, 39, 55]. The cells expressing the c'973 EGFR demonstrated diminished invasiveness compared with the parental cells (0.62 ± 0.23-fold on average) (Figure 3).

These differences in cell invasiveness were noted in the absence of exogenous EGF or TGFα. Unlike EHS tumor-derived Matrigel, Amgel does not contain detectable levels of EGF or TGFα [38]; however, all three cell sublines produce TGFα (Figure 1). Furthermore, the differences in invasiveness were not due to differences in cell proliferation for two reasons. Firstly, in a parallel assay, the three sublines proliferated at equal rates when grown on plastic; at three days the cell numbers were $386 \pm 16\%$, $380 \pm 48\%$, and $360 \pm 26\%$ of initial cell number for Parental, WT, and c'973 cells, respectively ($n = 2$, each experiment contained 8 points). Secondly, even if there was differential cell proliferation whilst on the Amgel matrix, this would not affect the determination of the percent transmigration, as we measured the percent of applied, acid-precipitable (i.e. incorporated) radiolabel which appeared in the lower chamber.

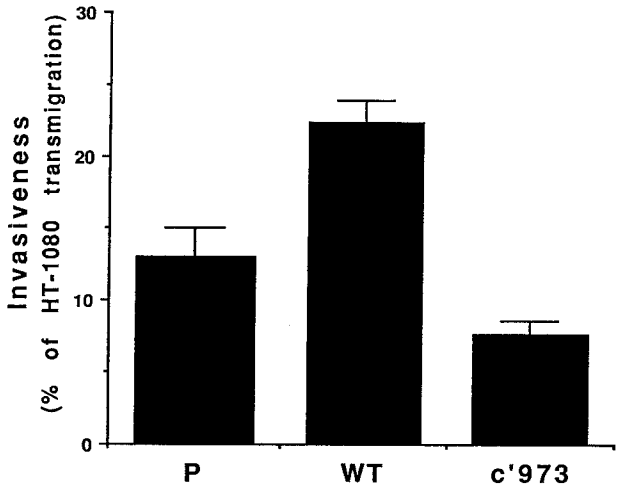

Figure 3 Invasion through an extracellular matrix. The Parental (P) and WT and c'973 cells were tested for the ability to transmigrate a human extracellular matrix, Amgel. The invasiveness of each line was determined as a percentage of that achieved by a highly invasive human fibrosarcoma-derived line, HT1080. The mean ± SEM of three independent experiments are shown, each performed in quadruplicate; $P < 0.01$ comparing each pair of cell lines.

In vitro *invasiveness was inhibited by anti-EGFR antibodies*

If EGFR-mediated signaling contributed to invasive-ness, then blocking EGFR activation should reduce the EGFR dependent transmigration of the Amgel matrix. Addition of a non-activating monoclonal antibody which blocks ligand binding [45] diminished the effect of transduced EGFR (Figure 4). The anti-EGFR monoclonal antibody inhibited invasive-ness by WT cells to the greatest extent (transmigration decreased by $54 \pm 4\%$). Invasiveness by Parental cells was also significantly reduced ($18 \pm 8\%$). Transmigration of the Amgel by c'973 cells was unaffected by the anti-EGFR antibody; the slight increase ($7 \pm 12\%$) was not distinguishable from control IgG alone. In the presence of the anti-EGFR antibody, the levels of

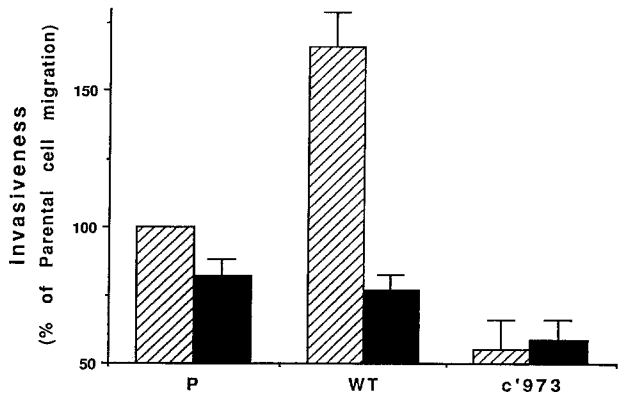

Figure 4. Inhibition of invasiveness by an anti-EGFR antibody. Parental (P) and WT and c'973 cells were tested for Amgel invasiveness in the presence of control murine IgG (hatched bars) or the non-activating Mab 528 (filled bars) which is a competitive inhibitor of ligand binding. Invasiveness is presented as percent of Parental cells. The change in cell invasiveness (mean ± SEM) in the presence of the anti-EGFR antibody is shown for four independent experiments each performed in quadruplicate. $P < 0.01$ for anti-EGFR treated P and WT cells compared with their controls; $P > 0.10$ comparing c'973 with its control and comparing anti-EGFR treated P with anti-EGFR treated WT cells.

invasiveness of the three sublines were not statistically different. These data further support the EGFR-dependency of the increased invasiveness.

The anti-EGFR antibody reduced cell proliferation in all three sublines to a similar extent. In parallel assays of cells cultured on plastic for three days, cell numbers in the presence of the anti-EGFR antibody were $38 \pm 4\%$, $33 \pm 12\%$ and $42 \pm 16\%$ of untreated, control cells for Parental, WT, and c'973 cells, respectively. Furthermore, as we measured incorporated label in the lower chamber, the percent transmigration is independent of cell proliferation. However, as cell death could result in spuriously lower levels of acid-precipitable label appearing in the lower chamber, we determined if antibody treatment increased cell death. Using the terminal deoxytransferase (Apoptag Kit, Oncor) method, the presence of 4 μg/ml of the anti-EGFR antibody only marginally increased the very low levels of apoptotic cell death at 3 days; Parental cells: $1.1 \pm 0.3\%$ in controls to $3.4 \pm 1.6\%$ in treated, WT cells: $1.6 \pm 1.1\%$ to $2.0 \pm 1.1\%$, and c'973 cells: $3.4 \pm 1.6\%$ to $5.0 \pm 1.0\%$ ($n = 2$, each in triplicate; there was no statistically significant different between the cell lines either with or without EGF treatment). Interestingly, the WT cells (invasiveness of which was inhibited to the greatest extent, Figure 4), presented the lowest level of cell death.

Collagenolytic activities of the cell lines did not correlate with invasiveness

Transmigration of ECM is considered to be dependent on three cell properties: matrix recognition, proteolytic ECM remodeling and degradation, and active movement through the resultant defect. Previously, we demonstrated that EGFR signaling enhances NR6 cell motility via activation of PLCγ [37]. Autophosphorylated tyrosines in the carboxy terminus of the EGFR are required for enhanced cell motility [36]. Thus, WT EGFR but not c'973 EGFR enhanced cell motility. This effect on cell motility parallels the results of transmigration of the DU-145 sublines. However, proteolytic activity is a necessary precursor for invasion. We asked whether the DU-145 sublines secreted different levels of ECM-degrading metalloproteases; we focused on collagenolytic activity as collagen types I and IV are the major constituents of Amgel [38].

The secreted levels of the MMP-9 (92 kDa gelatinase) and MMP-2 (72/64 kDa gelatinase) were assessed by gelatin zymography (Figure 5). These two collagenases preferentially degrade collagen type IV, and are more frequently associated with tumor invasion and metastasis than other metalloproteases [56, 57]. All three DU-145 sublines secreted near equivalent amounts of these enzymes (Figure 5A). Addition of exogenous EGF had no discernable effect on the levels of enzyme activities. Control experiments in which the amount of sample applied was varied demonstrated that a 10% difference could be distinguished (data not shown). Metalloproteases are secreted as pro–enzymes and are complexed with inhibitors. It was possible that the cell lines initially secreted different levels of pro–enzymes. Therefore, we first activated the metalloproteases with APMA but did not note significant differences in protease activities. Immunoblots using monoclonal antibodies to detect mass levels of MMP-9 and MMP-2 also demonstrated that WT cells expressed equal or slightly lower levels of these collagenases when compared with the less invasive Parental and c'973 cells (data not shown).

The addition of plasminogen to the zymogram (Figure 5B) revealed the presence of plasminogen-dependent protease activity in all the lines. The major collagenolytic activity in the presence of plasminogen appears at ~ 50 kDa, consistent with identification as UPa [58]. Interestingly, the Parental and c'973 cells secreted more plasminogen-dependent activity than the more invasive WT cells. The Amgel invasion assay was performed over a 72 h period. Therefore, we determined the proteolytic activity secreted between 0–14 h and 34–48 h, corresponding to early and late responses, respectively. No differences in proteolytic activities were noted between these two time periods (Figures 5C & 5D). In no situation did we note a significant EGF effect on secreted activity. This was not unexpected as these cells produce TGFα and possess a functional autocrine loop.

Cells cultured on ECM secreted proteases in a pattern which did not correlate with invasiveness

The initial series of zymograms (Figure 5) investigated secretion of proteases while cells were seeded on plastic. In such a situation, the components of the cell matrix are produced by the cells and derived from the FBS. Signaling from matrix components is a well-documented, widespread phenomenon. Matrix constituents also affect tumor cell growth [59] and metastasis [60, 61]. Because we had measured invasiveness of a biologic ECM, Amgel, it was necessary to determine whether components in the matrix signaled protease production. Cells were seeded onto Amgel-coated dishes, similarly to the invasion assay. Conditioned media was collected from either the 0–14 h (Figures 6A & 6B) or 34–48 h time periods (Figures 6C & 6D), and analyzed as with the cells

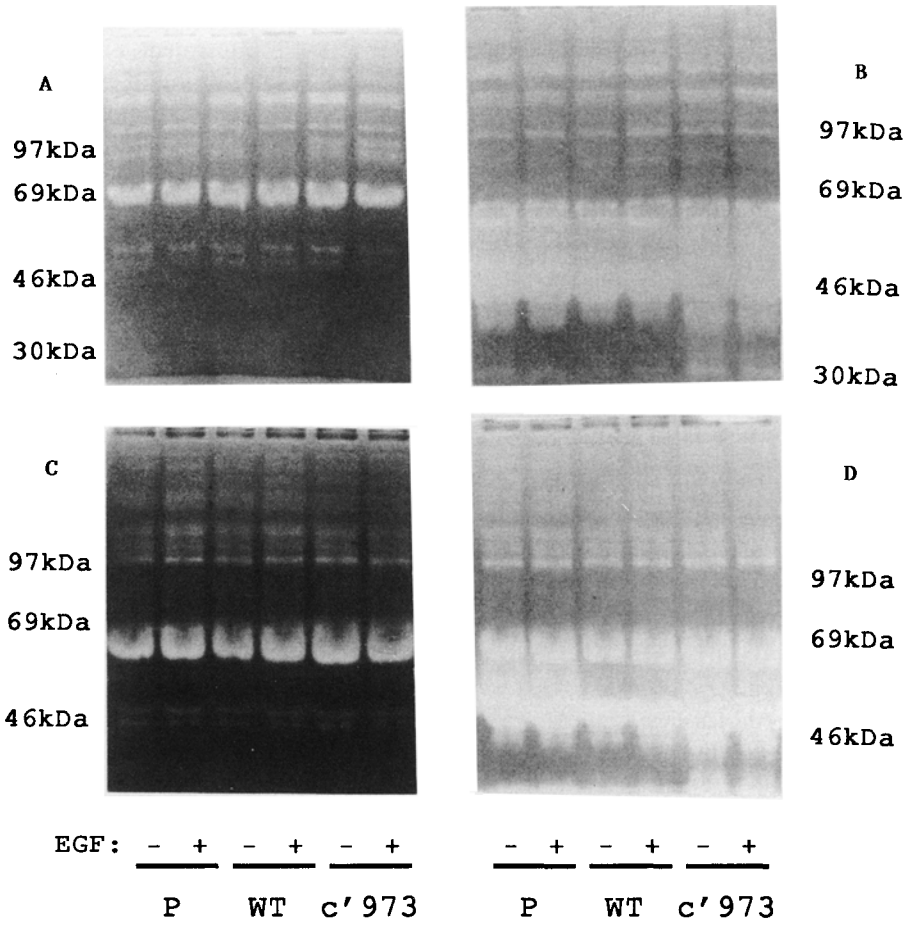

Figure 5. Gelatinase activity produced by DU-145 sublines. Cells were plated on plastic and induced to quiescence in 1% dFBS for 36–48 h. Secreted collagenolytic activity was collected from 0 to 14 h of incubation (**A** & **B**) or from 34 to 48 h (**C** & **D**). The cells were incubated in the absence (−) or presence (+) of EGF (10 nM) during the entire incubation period. Equal amounts of protein from each cell subline and condition were analyzed by zymography using acrylamide gels containing gelatin alone (0.15%) (**A** & **C**) or in conjunction with plasminogen (1 μg/ml) (**B** & **D**). Representative zymograms are shown; repeat zymograms with greater or lesser amounts of protein have been utilized to evaluate individual bands within the linear range of the assay. Each series of experiments was performed independently at least three times.

seeded onto plastic. The pattern of collagenases was similar between the cells on Amgel and those on plastic. Neither activation by APMA (data not shown), nor presence of plasminogen (Figures 6B & 6D) caused differences from the enzyme patterns seen with cells on plastic. Comparisons between the cell lines revealed excess protease activity in the Parental and c'973 cells, not in the more invasive WT cells.

Levels of TIMP-1 did not correlate inversely with invasiveness

Total proteolytic activity is a balance between proteases and their inhibitors. TIMP-1 and -2 are produced by many transformed cells. TIMP-1 binds stoichiometrically to all MMPs but preferentially inhibits MMP-9; TIMP-2 preferentially inhibits MMP-2 [57, 62]. Reverse zymography is used to detect collagenase inhibiting proteins. We could not detect such inhibitors secreted by the DU-145 sublines using this technique (data not shown); therefore, we assessed production by analyzing protein mass and message levels. Immunoblotting detected TIMP-1, but not TIMP-2 (Figure 7). The level of TIMP-1 present in the media was increased by treatmentof the cells with EGF in the WT and c'973 cells. EGF treatment did not alter levels in the Parental cells, which presented the highest level of TIMP-1; Parental cells still demonstrated invasiveness. TIMP-1 and -2 mRNA levels also were ascertained in cells grown under conditions which minimized autocrine stimulation. Total RNA or oligo-dT chromatography-enriched RNA was hybridized with probes specific for human TIMP-1, -2, and γ-actinin (the latter to control for mRNA abundance); relative quantitations were performed by phosphor-image analyses (Molecular Dynamics). TIMP-1 message levels were slightly higher in c'973 cells than in Parental cells (Parental:WT:c'973 were $1.07 \pm 0.14 : 0.54 \pm 0.10 : 1.34 \pm 0.29$, relative to γ-actinin; $n = 3$). TIMP-2 message levels demonstrated the opposite pattern, though at about 20% the level of TIMP-1 message (Parental:WT:c'973 were 0.21: 0.24:0.17; relative to γ-actinin; $n = 1$). EGF exposure resulted in slightly increased TIMP-1 levels and similar, slightly decreased TIMP-2 message levels.

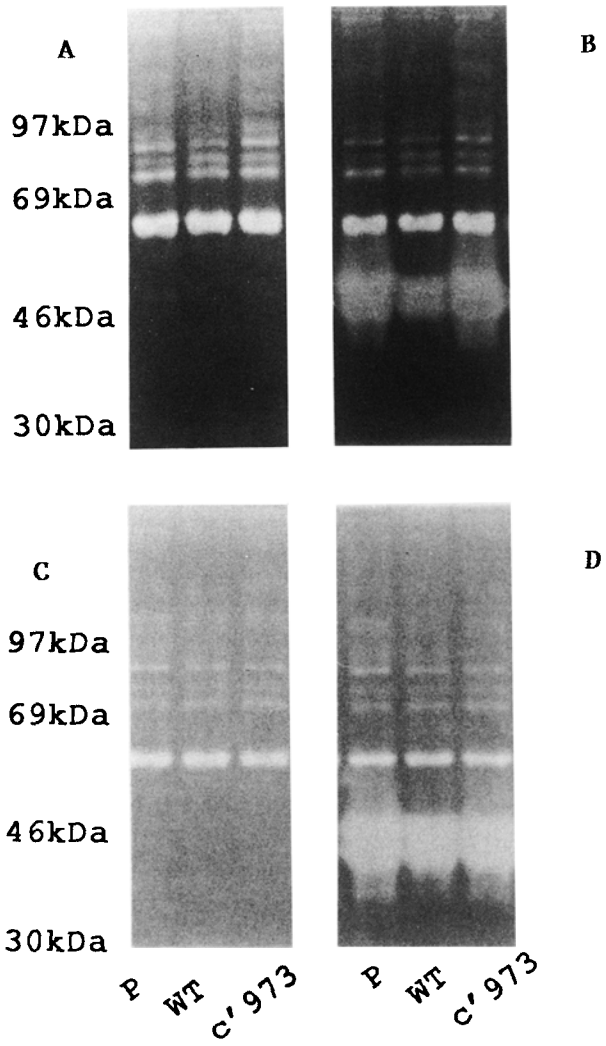

Figure 6. Gelatinase activity produced by DU-145 sublines cultured on Amgel. Cells were plated on Amgel-coated plates, and treated as described in the text and the legend to Figure 5. Collagenolytic activity was collected from 0 to 14 h (**A** & **B** or from 34 to 48 h **C** & **D**). Equal amounts of protein from each cell subline were analyzed in gels copolymerized with gelatin (**A** & **C**) or in conjunction with plasminogen (**B** & **D**). Representative zymograms are shown; repeat zymograms with greater or lesser amounts of protein have been utilized to evaluate individual bands within the linear range of the assay. Each series of experiments was performed independently three times for the 0 to 14 h period and twice for the 34 to 48 h period.

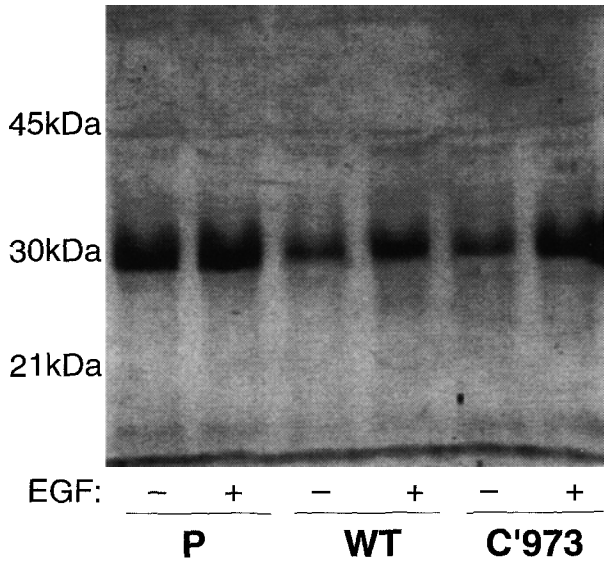

Figure 7. Production of TIMP-1 by DU-145 sublines. Parental (P), WT and c'973 cells were plated on plastic, induced to quiescence in 1% dFBS, and treated for 14 h in the absence (–) or presence (+) of EGF (10 nM). Equal amounts of secreted protein (15 μg) were analyzed by immunoblotting for presence of TIMP-1. A representative immunoblot is shown; a similar pattern was noted in a second independent experiment.

Discussion

Upregulated signaling of the EGF receptor system has been correlated with tumor cell invasion and metastasis [14–21, 23]. In this study, we demonstrate that the extent of invasiveness of DU-145 human prostate carcinoma cells can be modulated by EGFR expression. Cells expressing elevated levels of full-length (WT) EGFR invaded through an extracellular matrix to a greater extent than Parental cells. A monoclonal antibody which prevents ligand binding and subsequent EGFR activation inhibited the transmigration of these cells. This suggests that EGFR signaling, probably secondary to a TGFα-induced autocrine stimulatory loop, promoted invasiveness.

A second question is whether endogenous EGFR signaling contributes to invasiveness of Parental DU-145 cells. EGFR signal blockade by a monoclonal antibody decreased the invasiveness of these cells by 20%. This finding is distinct from blocking invasiveness enhanced by exogenous ligand [23], in that signals intrinsic to the DU-145 cells are promoting invasiveness. These data, coupled with the fact that the monoclonal antibody inhibited invasiveness of both Parental and WT cells down to a similar level, suggests that a component of DU-145 invasiveness is EGFR-mediated secondary to autocrine stimulation.

We expressed a kinase–active, mitogenesis–competent truncated EGFR (c'973) [36, 39] in the DU-145 cells. Cells expressing this truncated receptor

were significantly less invasive than Parental cells (Figure 3), even though they expressed more EGF binding sites and receptor mass. The reduced invasiveness could be secondary either to transmittal of a signal antagonistic to invasiveness such as decreasing ECM recognition, or down-regulation of signaling from the endogenous full-length EGFR. If it were the former, then the blocking antibody would be expected to increase the invasiveness of c'973 cells. In fact, these cells transmigrated the extracellular matrix similarly in the absence or presence of blocking antibody (Figure 4). Thus, c'973 signaling does not contribute to cell invasiveness. The presence of the c'973 EGFR probably results in decreased signaling from the endogenous full-length EGFR in these cells (Figure 2A). We propose that signaling domains in the carboxy-terminus of the EGFR are required for EGFR-mediated invasiveness. The level of invasiveness exhibited by c'973 cells represents the basal invasiveness of DU-145 cells in the absence of EGFR signaling.

The mechanism(s) by which EGFR signaling enhances invasiveness is unknown. EGFR signals have been implicated in modulating cell phenotypes which control all three aspects of invasiveness. We have shown that EGF causes cells to retract lamellipodia and decrease attachment acutely, and that this effect occurs in cells which express both WT and c'973 EGFR [41]. While these signaling responses must be confirmed in DU-145 cells using Amgel as the attachment matrix, the fact that antibody exposure of c'973 cells does not enhance invasiveness, further suggests that differences in matrix recognition are not the underlying mechanism of EGFR-mediated invasiveness.

EGF–induced cell motility is dependent on phosphotyrosine motifs in the carboxy–terminus of the receptor [36, 37]. WT, but not c'973 EGFR, when expressed in NR6 cells signaled enhanced cell motility. This could account for the difference in invasiveness between WT and c'973 cells. The enhanced invasiveness shown by WT cells when compared with Parental cells may be explained through the higher level of EGFR in the WT cells (Figure 2). Partial inhibition of PLCγ signaling in the transduced NR6 cells by pharmacologic and molecular agents [37] resulted in an activity-dependent decrease in cell motility. Thus, differences in EGFR level and overall signaling may result in differential motility.

Proteolytic degradation is required for transmigration of an extracellular matrix. Differential protease production would be expected to result in differing invasiveness. EGF has been shown to increase levels of a number of different proteases [3, 32, 33]. We attempted to determine if the EGFR-mediated invasiveness was accomplished through this mechanism. Initially, we have focused on type IV collagenase production. Zymograms were utilized to determine total collagenolytic activity secreted into the media, and avoid the problems in the interpretation of transcription, translation, and post-translational regulation events. WT cells demonstrated equivalent or lesser levels of collagenases than the less invasive Parental or c'973 cells whether on plastic or on Amgel (Figures 5 & 6). In an additional series of investigations, we did not note greater casein degradation as measured by casein-based zymography to detect non-collagen-directed proteolysis, in the more invasive WT cells (data not shown).

Collagenolytic activity is a balance between collagenases and TIMP species. We could not demonstrate collagenase inhibitory activity by reverse zymography. Therefore, we assessed production of the two major collagenase inhibitory species by protein mass and message levels. TIMP-1 was found to be produced by all three sublines, with WT cells expressing less than the other sublines (Figure 6). However, relative TIMP-1 production (Parental > c'973 > WT) paralleled protease production and was not the inverse of invasiveness (WT > Parental > c'973), as would be expected if this was the underlying mechanism for EGFR-mediated invasiveness. Relative message levels did not parallel the protein mass measurements. We do not know whether this is due to differential translation controls or protein consumption or sequestration. Collagenases and TIMP species bound to the cell surface [5, 7] were not detected in our zymography and immunoblot assays for secreted proteins. Further studies will be required to determine whether cell surface binding contributes significantly to levels of TIMP species and collagenases.

In summary, EGFR-mediated invasiveness of DU-145 does not correlate with increased collagenolysis of specific matrix components. This is not to say that proteolytic degradation of the matrix is not important for invasion and metastasis, as many reports attest to its requirement in this complex process. The role of collagenases and their inhibitors in EGFR-mediated invasiveness awaits further study in which specific effector molecules can be investigated in isolation and the effect of EGFR signaling determined. It is likely that all three sublines produced sufficient proteolytic activity that this was no longer a limiting factor for invasiveness. However, our findings support the contention that tumor cell invasiveness and metastasis involves cell properties in addition to proteolytic degradation of matrix [63]. Animal models of EGFR-mediated tumor progression will be helpful

in defining the relative contributions of various cell responses such as enhanced cell motility and protease production.

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