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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Our overall objective is to understand how dysregulation of cell migration contributes to tumor cell invasiveness in prostate cancer. A combination of correlative epidemiological studies and basic experimental investigations demonstrate a role for upregulated EGF receptor (EGFR) signaling of motility in tumor progression. EGFR-mediated cell motility has been demonstrated to be critical for tumor invasion. *Our central premise is that prostate tumor cell invasiveness can be inhibited by interfering with the specific motility-associated calpain activation that governs the critical underlying biophysical process of de-adhesion.* Prior work has shown that integrin/matrix binding and growth factor stimulation jointly regulate cell locomotion. These studies have identified cell/substratum adhesiveness, especially the ability of a cell to detach at its trailing edge, as a primary governor of cell locomotion. We have recently found that this tail detachment is regulated by calpain activation. We are employing a set of model prostate tumor cell lines including a panel of syngeneic androgen-independent DU-145 cells that vary in invasiveness. Our findings in the first year showed that disruption of calpain activation and de-adhesion can block tumor invasiveness in vitro. This has been extended in the second year to demonstrate that this also applies in animal models of tumor invasiveness.

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Table of Contents

CELL MOTILITY IN TUMOR INVASION

Alan Wells, Douglas Lauffenburger, Timothy Turner

INTRODUCTION

Our overall objective is to understand how dysregulation of cell migration contributes to tumor cell invasiveness in prostate cancer. A combination of correlative epidemiological studies and basic experimental investigations demonstrate a role for upregulated EGF receptor (EGFR) and other receptor signaling of motility in tumor progression. Especially in prostate tumor cells, EGFR-mediated cell motility has been demonstrated to be critical for tumor invasion (2, 5). Since signals from extracellular matrix through integrins and from cell-cell contacts also strongly influence cell motility, the underlying common biophysical processes and biochemical controls of motility offer an attractive target for limiting tumor progression.

Our central premise is that prostate tumor cell invasiveness can be inhibited by interfering with the specific motility-associated calpain activation that governs the critical underlying biophysical process of de-adhesion. Prior work by ourselves and others has shown that integrin/matrix binding and growth factor stimulation jointly regulate cell locomotion (1, 3). These studies have identified cell/substratum adhesiveness, especially the ability of a cell to detach at its trailing edge, as a primary governor of cell locomotion. We have recently found that this tail detachment is regulated by calpain activation. We will employ a set of model prostate tumor cell lines including the moderately invasive androgen-independent PC3 cell and its highly metastatic variant PC3M cell, along with a panel of syngeneic androgen-independent DU-145 cells that vary in invasiveness. We will determine whether targeted disruption of calpain activation and de-adhesion can block tumor invasiveness.

BODY

The original Statement of Work (Table 1) described a series of tasks to accomplish the two Objectives proposed and the additional training Objective. We have tackled these Tasks in the order of greatest yield so that work in areas can progress as systems are being optimized in others. The total three years of effort have led to the accomplishment of these task

Table 1. Original Statement of Work

Work to be performed at University of Pittsburgh (A. Wells Laboratory):

1. determine whether calpain is activated by growth factors and integrins in prostate cancer cells
2. determine whether calpain is limiting for prostate tumor cell motility on complex surfaces
3. determine whether prostate tumor cell transmigration of extracellular matrices is dependent on calpain activity
4. determine whether inhibition of calpain limits tumor invasiveness and metastasis in murine models of progressive prostate cancer

Work to be performed at MIT (D.A. Lauffenburger Laboratory):

1. determine optimal adhesiveness and high and low adhesiveness surfaces for fibroblast motility
2. test prostate tumor cell motility on defined adhesiveness surfaces
3. determine whether calpain activation is required for prostate cell motility

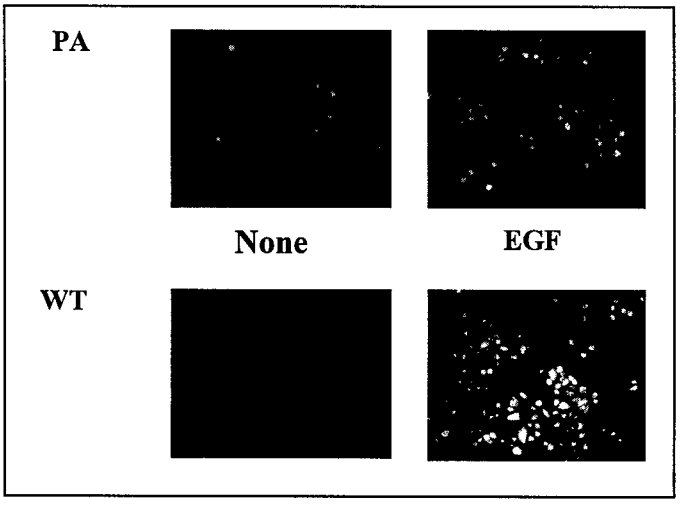
Work to be performed in partnership with Tuskegee (T. Turner Laboratory):

1. trainees will perform prostate cell growth and motility assays at Tuskegee and UPitt
2. trainees will perform in vivo mouse assays at UPitt

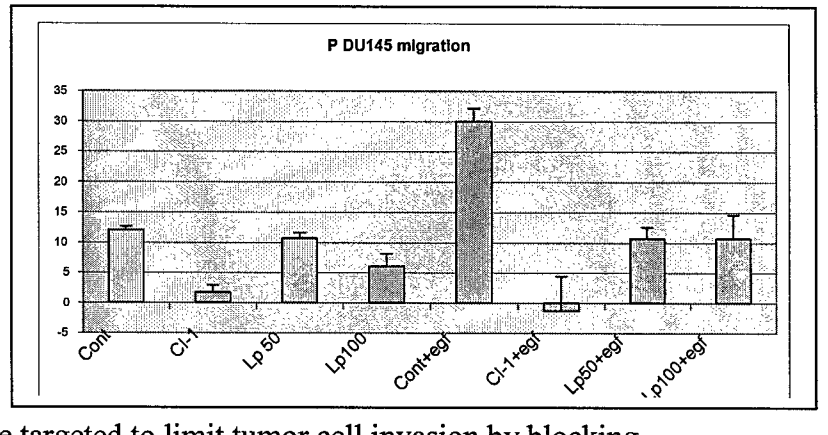
The reader of the Year 2 progress report deemed task completion as the following:

Task 1	No time given	In progress
Task 2	No time given	In progress
Task 3	No time given	Completed
Task 4	No time given	Completed
Task 5	No time given	Completed
Task 6	No time given	In progress
Task 7	No time given	Completed
Task 8	No time given	In progress
Task 9	No time given	In progress

Work to be performed at University of Pittsburgh:

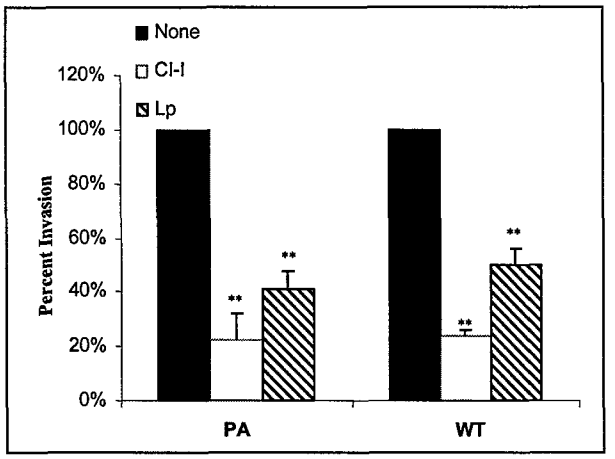


Task 1. determine whether calpain is activated by growth factors and integrins in prostate cancer cells. In Years 1 and 2, we reported that EGF induces calpain activity in DU-145 prostate cancer cells (Fig 1; calpain activity in individual cells is shown by Boc fluorescence). In Year 3, we found that EGF activates ERK MAPkinase, the upstream activator of m-calpain, in a biphasic manner dependent on fibronectin concentration. We now also report that BOC activation occurs across a broad range range of fibronectin concentrations. This task, deemed in progress by the reader of the Year 2 report, is now completed. (Mamoune et al. 2003)

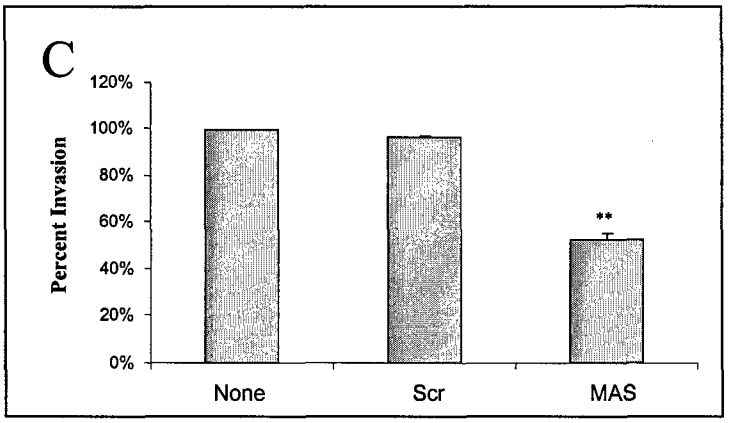


Task 2. determine whether calpain is limiting for prostate tumor cell motility on complex surfaces. Our data, generated during Year 1, demonstrated that calpain inhibitor I and leupeptin can limit DU-145 motility across self-generate matrix. During Year 3 we found that pan-calpain inhibition also reduced EGF-independent motility, though this was not driven by m-calpain (Fig 2; bars showing migration in arbitrary units). These data suggest that calpain could be targeted to limit tumor cell invasion by blocking migration. This task, deemed in progress by the reader of the Year 2 report, is now completed. (Mamoune et al. 2003)

Task 3. determine whether prostate tumor cell transmigration of extracellular matrices is dependent on calpain activity. This task was completed during Year 2. In vitro transmigration of a Matrigel matrix by both Parental and WT EGFR-expressing DU-145 cells is blocked by inhibitors of calpain, CI-I and leupeptin (Fig 3; ** $P < 0.01$ compare to diluent alone/none). Furthermore, antisense downregulation of M-calpain limits this transmigration, providing specificity (Fig 4, below).



These data are presented in Mamoune et al, 2003.



Task 4. determine whether inhibition of calpain limits tumor invasiveness and metastasis in murine models of progressive prostate cancer. This tasked was deemed completed after Year 2. In brief, we challenged mice with DU-145 prostate carcinoma tumor xenografts with inhibitors of calpain. Tumor invasiveness was reduced in the presence of daily injections of the inhibitor leupeptin (Table 1). The differences invasiveness between treated and mock treated were significant ($P < 0.05$) by T-test and ANOVA analyses.

	PA+HBSS	PA+Leupeptin	WT+HBSS	WT+leupeptin
Diaphragm tumors	14/14	13/14	14/14	13/14
Diaphragm invasiver	1.71	0.7**	2.35	1.25*

Antisense constructs to m-calpain were expressed stably in DU-145 cells. These also limited tumor invasion into the diaphragm of mice (Table 2).

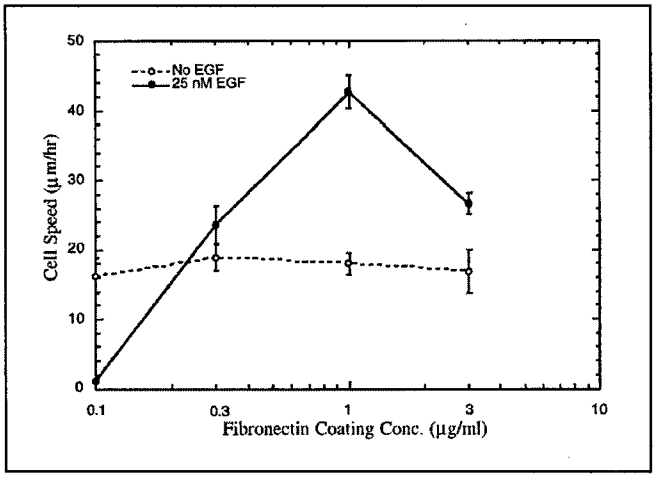
	V PA DU145	C2AS PA DU145	V WT DU145	C2AS WT DU145
Diaphragm tumors	9/10	8/10	4/5	3/5
Diaphragm invasiven	2.33	1.13*	3.50	1.67^

These data are presented in Mamoune et al, 2003.

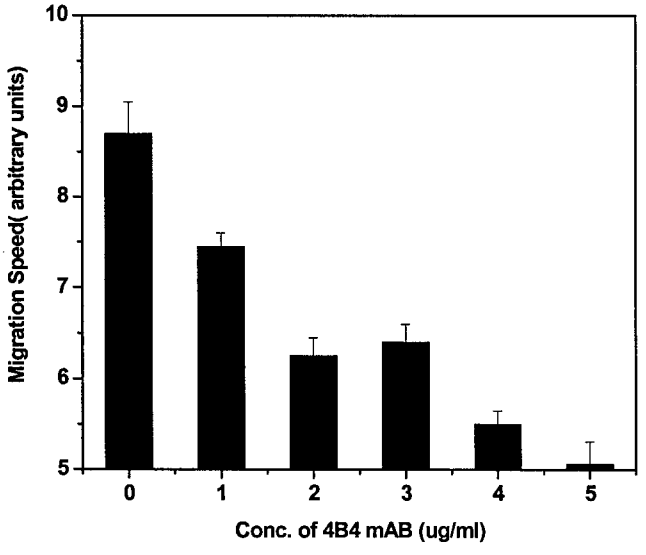
Work to be performed at MIT:

Task 5. determine optimal adhesiveness and high and low adhesiveness surfaces for fibroblast motility. This task was completed in Year 1, and was deemed completed by the Year 2 reviewer.

In brief, for EGF-induced motility, we found that optimal fibronectin coating occurs at around 1ug/ml, with 0.3 ug/ml and 3 ug/ml being low and high adhesiveness, respectively. At these extremes, motility is reduced to levels on par with no EGF stimulation (Fig 5). The optimal adhesive strength of the fibroblasts to the surface is approximately 0.8 nN, with a movement of 0.2 nN either direction wiping out growth factor induced movement.



Task 6. test prostate tumor cell motility on defined adhesiveness surfaces. This task is in progress and should be completed by September 2004. Recent high throughput analyses has examined the ability of the DU-145 cells to migrate in 2 and 3 dimensions on a matrigel-coated surface. Briefly, DU-145 parental cells were plated on glass bottom 96-well Packard plates. The cells were plated in high serum media (final concentration = 7% FBS in DMEM media) and varying concentrations of anti-β1 integrin mAB 4B4 were added to study the effect of anti- β1 integrin on cell speed. The cells were imaged using high throughput Cellomics kinetic scan for 6 hrs, at controlled temperature (37^0 C, controlled CO_2 and humidity conditions) with images taken at ten minute intervals.



Cell Migration speed as a function of varying concentrations of anti-β1 integrin mAB 4B4 on a 2D- glass surface. Addition of 5ug/ml of mAB 4B4 inhibits the migration of DU 145 by as much as 50%. The error bars represent standard deviation in the mean cell speed for each concentration of mAB 4B4. The cell speed was obtained by built-in computerized object tracking algorithm in Imaris 4.06 (Bitplane AG, Inc.).

The results showed that increasing concentrations of anti-β1 integrin mAB 4B4 restrict the migration of DU-145 cells on glass surface (Fig 6.). Adding 5 ug/ml of mAB 4B4 to the media reduced the migration speed of the DU-145 cells by almost 50%.

Currently experiments with various concentrations of matrigel mixed with mAB 4B4 on a glass surface are being performed to study the simultaneous effect of matrix compliance and anti-β1 integrin agent on migratory trends of DU-145 cells.

Task 7. determine whether calpain activation is required for prostate cell motility. This was completed during Year 2, and deemed as such, in conjunction with work at UPitt (Fig 2, above).

Work to be performed in partnership with Tuskegee:
Task 8. trainees will perform prostate cell growth and motility assays at Tuskegee and UPitt. Masters students Clayton Yates, Karlyn Bailey, Roderick Bailey, Charnita Davidson, and Larry Harris have all been trained at Tuskegee University to perform these assays with the DU-145 human prostate tumor lines. Clayton Yates has transitioned to University of Pittsburgh as a PhD degree student in the Cellular and Molecular Pathology graduate program. Mr. Yates is now a thesis candidate and has focused on prostate cancer cell-cell cohesion as a key determinant for metastatic growth. He has been cleared to present his PhD thesis during the coming winter semester. Ms. Bailey has been performing cell growth assays in the presence of EGFR and LHRH inhibitors at Tuskegee. Ms. Bailey has now received her Masters degree for a thesis entitled "Defining the Nature of Protein Kinase C Signaling in the Human Prostate Cancer Cell Line, DU-145 after Exposure to LHRH Analogs".

The post-doctoral fellows, Drs. Ahmed Hassan and Adedayo Ariyibi, have been trained to perform these assays. Since 2002, they have been in contact with the Wells' lab and Dr. Hassan has personally met with Wells at UPitt. In addition, they are in constant contact with Clayton Yates in regards to performing certain techniques pertinent to experiments at Tuskegee University.

This task in now completed. (Yates et al, 2004)

Task 9. trainees will perform in vivo mouse assays at UPitt. Mr. Yates learned and Dr. Hassan the in vivo mouse tumor growth and invasion assays. More importantly, he has developed an ex vivo metastasis model of tumor cell growth in a liver bioreactor. Mr. Yates has learned to generate the liver bioreactor and seed it with prostate tumor cells. This task is now completed.

KEY RESEARCH ACCOMPLISHMENTS

- EGFR signaling enhances prostate tumor motility
- EGFR signaling increases calpain activity in prostate cancer cells
- Calpain inhibitors block prostate tumor invasiveness in vitro
- Calpain inhibitors block prostate tumor invasiveness in vivo
- Downregulation of m-calpain limits prostate tumor invasiveness in vitro
- Downregulation of m-calpain limits prostate tumor invasiveness in vivo
- EGFR signaling enhances fibroblast motility over a narrow range of fibronectin adhesiveness
- EGFR signaling enhances epithelial cell motility over a range of adhesiveness
- One trainnee successfully transitioned from Tuskegee Masters program to be a doctoral thesis candidate at University of Pittsburgh
- The trainee developed a novel prostate tumor metastasis model in an ex vivo liver bioreactor
- One trainee completed a Masters program and two others are in graduate training

lookiing to transition to a doctoral program
- Two trainees are in post-graduate training at Tuskegee in conjunction with the laboratory at University of Pittsburgh

PAID PERSONNEL INVOLVED IN THE GRANT
University of Pittsburgh:
Alan Wells
Kathleen Sullivan
Ashiro Awabu
Latha Satish
Shanmuga Kulasekaran

Tuskegee University
Timothy Turner
Adedayo Ariyibi
Larry Harris
Roderick Bailey
Charnita Davidson
Ahmed Hassan

Massachusetts Institute of Technology
Doug Lauufenburger
Christina Lewis
Kirsty Smith
Bart Hendriks
Clayton Yates

REPORTABLE OUTCOMES
Articles:
A Mamoune, JH Luo, DA Lauffenburger, A Wells (2003). m-Calpain as a target for limiting prostate cancer invasion. Cancer Research 63, 4632-4640.

A Wells, J Grandis (2003). PLCγ-1 in tumor progression. Clinical & Experimental Metastasis 20, 285-290.

C Yates, A Wells, T Turner (2004). Luteinizing hormone releasing hormone (LHRH) antagonist reverses the cell adhesion profile of DU-145 human prostate carcinoma. submitted

Abstracts:
A Mamoune, J Kassis, D Lauffenburger, A Wells (2002) Calpain inhibition reduces prostate tumor invasion. American Association for Cancer Research (AACR) Annual Meeting, San Francisco, CA

C C Yates, K J Bailey, A Wells, T Turner (2001). The Effects of the Luteinizing Hormone Releasing Hormone Antagonist, Cetrorelix on the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-5th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association, Maui, HI

C C Yates, K J Bailey, A Wells, T Turner (2001). Cetrorelix, a Luteinizing Hormone Releasing Hormone Antagonist, Influences the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-Keystone Symposium, Tahoe City, CA

C Yates, D B Stolz, L Griffith, A Wells (2003) An organotypic model for prostate tumor metastasis. 94th Annual Meeting of the American Association for Cancer Research, Washington, DC

K J Bailey, A Hassan, A Wells, T Turner (2002) Protein kinase C signaling in the human prostate cancer cell line DU-145 after exposure to an LHRH analog. Research Centers in Minority Institutions (RCMI) 2002 Spring Symposium, Jackson, MS

A M Hassan, K J Bailey, A Wells and T Turner (2003) Activation of Protein Kinase C/A signaling in the human prostate cancer cell line DU-145 after exposure to a LHRH analog, Cetrorelix. AACR symposium in Phoenix, AZ

C Yates, D B Stolz, L Griffith, A Wells (2004) An organotypic model for prostate tumor metastasis. 95th Annual Meeting of the American Association for Cancer Research, Orlando, FL

Thesis:
K J Bailey (2002) Defining the nature of Protein Kinase C Signaling in the human prostate cancer cell line DU-145 after exposure to a LHRH analog. M.S. Thesis. Tuskegee University.

Training:
C Yates has been accepted as from Tuskegee University with a MA in Biology to a PhD candidate in the program in Cellular and Molecular Pathology at University of Pittsburgh.

K Bailey has received her Masters degree from Tuskegee University and served as program manager of the Tuskegee University/University of Alabama at Birmingham Comprehensive Cancer Center Partnership for three years. She recently took a position at the Environmental Protection Agency as a toxicology specialist.

R Bailey and L Harris are still working towards the completion of their Masters degree at Tuskegee University. Both are keenly interested in following in the steps of Clayton Yates at University of Pittsburgh.

Drs. Hassan and Ariyibi are currently completing experiments in collaboration with Ms. Bailey and Mr. Yates on protein kinase C and A activation in DU-145 human prostate cell line after exposure to the LHRH analogs. Dr. Hassan is currently being strongly considered for a tenure-track faculty position in the Department of Biology at Tuskegee University.

CONCLUSIONS

The third and last year of this multiyear award has seen the attainment of all the original tasks of the award. This has resulted in 3 Articles published or submitted and many abstracts published or presented at national and international meetings. It is has also highlighted new directions for further research.

Importance/Implications: The Key Accomplishments above firmly demonstrate the validity of the model of the tumor biology that calpain-mediated deadhesion is a rate-limiting step in tumor cell motility and invasion. This provide the 'proof a concept' that targeting calpain is a rationale therapeutic option (6, 7). The implications are clear that calpain inhibitors, currently being developed for muscle-wasting conditions, may have a role as adjuvant cancer therapy to limit the spread of prostate carcinoma.

Furthermore, initial data suggest that cell-cell cohesion may be a rate-limiting process in prostate tumor metastasis. Data generated during this award and now being submitted (Yates, et al. 2004) demonstrate this. Work from others suggest that calpain may cleave cadherins in prostate cancer cells (4). Thus, we are now pursuing our postulate that cell-cell cohesion is controlled by calpain cleavage of cadherins (Yates et al. 2004).

Recommended changes: The results have completed the key tasks. The findings on cell-cell cohesion have major implications for the regulation of tumor invasion and thus led us to introduce this project along side the original tasks in Year 3.

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Appendices

Articles:

1. A Mamoune, JH Luo, DA Lauffenburger, A Wells (2003). m-Calpain as a target for limiting prostate cancer invasion. Cancer Research 63, 4632-4640.

2. A Wells, J Grandis (2003). PLCγ-1 in tumor progression. Clinical & Experimental Metastasis 20, 285-290.

3. C Yates, A Wells, T Turner (2004). Luteinizing hormone releasing hormone (LHRH) antagonist reverses the cell adhesion profile of DU-145 human prostate carcinoma. submitted

Abstracts:

4. A Mamoune, J Kassis, D Lauffenburger, A Wells (2002) Calpain inhibition reduces prostate tumor invasion. American Association for Cancer Research (AACR) Annual Meeting, San Francisco, CA

5. C C Yates, K J Bailey, A Wells, T Turner (2001). The Effects of the Luteinizing Hormone Releasing Hormone Antagonist, Cetrorelix on the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-5th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association, Maui, HI

6. C C Yates, K J Bailey, A Wells, T Turner (2001). Cetrorelix, a Luteinizing Hormone Releasing Hormone Antagonist, Influences the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Selected Abstract-Keystone Symposium, Tahoe City, CA

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9. A M Hassan, K J Bailey, A Wells and T Turner (2003) Activation of Protein Kinase C/A signaling in the human prostate cancer cell line DU-145 after exposure to a LHRH analog, Cetrorelix. AACR symposium in Phoenix, AZ

10. C Yates, D B Stolz, L Griffith, A Wells (2004) An organotypic model for prostate tumor metastasis. 95th Annual Meeting of the American Association for Cancer Research, Orlando, FL

[CANCER RESEARCH 63, 4632–4640, August 1, 2003]

Calpain-2 as a Target for Limiting Prostate Cancer Invasion[1]

Appendix 1

Asmaa Mamoune, Jian-Hua Luo, Douglas A. Lauffenburger, and Alan Wells[2]

Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania 15261 [A. M., J-H. L., A. W.]; Pittsburgh VAMC, Pittsburgh, Pennsylvania 15261 [A. W.]; and Biological Engineering Division, Department of Biology and Center for Cancer Research, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139 [D. A. L.]

ABSTRACT

Mortality and morbidity of prostate cancer result from extracapsular invasion and metastasis. This tumor progression depends on active cell motility. Previous studies have shown that calpain-regulated rear detachment enabling forward locomotion is required for cell migration initiated by growth factor and adhesion receptors. Therefore, we asked whether calpain would be a target for limiting tumor progression, using as our model the PA DU-145 human prostate carcinoma cell line and a highly invasive subline, wild-type DU-145, derived from it. *In vitro*, the calpain-specific inhibitor CI-I (ALLN) and the preferential-but-less-specific inhibitor leupeptin decreased transmigration of both cell lines across a Matrigel barrier. These calpain inhibitors limited epidermal growth factor-induced motility but did not alter the growth rate of the tumor cells, as expected. Antisense down-regulation of the growth factor-activated calpain-2 (m-calpain) isoform also reduced transmigration and cell motility. These *in vitro* findings were then buttressed by *in vivo* studies, in which i.p. DU-145 tumor xenografts were treated with leupeptin. Tumor invasion into the diaphragm was reduced by leupeptin treatment for both the PA and wild-type DU-145 cells (from 1.7 to 0.78 for the parental line and 2.3 to 1.2 for the invasive derivative, respectively). Tumor cells of both types engineered to express calpain-2 antisense constructs also demonstrated a similar 50% reduced invasiveness *in vivo*. Finally, we found by gene expression survey of 53 human prostate tumors and 23 normal prostates that calpain was not up-regulated in relationship to invasiveness or metastatic activity, consistent with expectation from the biological role of this effector. Taken together, these results strongly suggest that epigenetic activation of calpain plays an important role in the invasion of human prostate cancer and that it can be targeted to reduce tumor progression.

INTRODUCTION

Prostate cancer is among the most frequent tumors in men (1), with the vast majority of morbidity and mortality resulting from tumor spread beyond the prostate (2, 3). Thus, work has focused on molecular changes that invasive and metastic tumors acquire to enable them to breach the barrier matrices and extend beyond the prostate capsule. Whereas there are a number of cell properties and their controlling signaling pathways, we have focused on cell migration as a critical rate-limiting step in tumor invasion (4–7). Extravasating and metastatic cells have been observed as displaying active motility during these actions (8–10). Therefore, inhibition of tumor cell motility should provide a novel therapeutic approach.

Cell motility is a highly orchestrated process that requires cell protrusion of leading lamellipodia with subsequent new adhesions, contraction through the cell body, and release from the substratum at the trailing edge (11). Each of these biophysical processes is controlled coordinately by biochemical signaling cascades (12). Such cascades can be initiated by adhesion receptors, notably integrins (13), or by growth factor receptors, although the specific elements in signaling chains may vary dependent on the initiating signal (12). The rear detachment step appears to be regulated by convergent signaling from growth factors and integrin (14, 15). Calpains are required for deadhesion of the tail during both haptokinesis (16) and chemokinesis (17, 18), at least on moderately to highly adhesive surfaces (19). However, it appears that integrins activate the calpain-1 (μ-calpain) isoform, whereas growth factor receptors trigger calpain-2 (m-calpain). As these two ubiquitously coexpressed proteins are highly homologous and appear to cleave the same targets, this convergence is likely because of differential regulation of the calpain isoforms (14, 20). Inhibition of calpain does block the motility of fibroblasts and myofibroblasts (16, 17), as well as keratinocytes (21). In the one study to date examining calpain-dependency of motility in carcinoma cells, inhibition of calpain in bladder carcinoma cells limited both motility and transmigration of a Matrigel barrier *in vitro* (22). The effects of inhibiting calpain were similar to when other motility-related signals are blocked, such as peritoneal lymphocyte γ-mediated cytoskeleton reorganization (22–24). Thus, there is promise that calpain may be a target for limited tumor invasiveness. However, this has yet to be determined in animal models.

Calpains are a family of >12 known mammalian intracellular limited proteases that share a similar catalytic structure (25). The two ubiquitous isoforms, calpain-1 and -2, are the best characterized and defined by their calcium requirements for *in vitro* activation. Whereas the biochemistry and structural biology of the ubiquitous calpains is highly advanced (25–28), the cell biology of these enzymes is lagging because of questions of mode of activation *in vivo* (14, 15). Calpains contribute not only to cell motility, as noted above, but also are likely involved in cell proliferation and apoptosis (15, 20, 29). Still less is known about the role of calpains in carcinogenesis and tumor progression. There is a report in a subset of 21 clear cell renal carcinomas of calpain-1, being up-regulated at the mRNA level in metastatic tumors compared with node-negative tumors (30). The gastric-specific calpain-9 is down-regulated in carcinomas from that tissue, although whether it is related to differentiation status or tumorigenesis is still open to question (31, 32). On the other hand, the decrease of muscle-specific calpain-3, and reciprocal increase in calpain-2 and ubiquitin-dependent proteolysis in muscles during cancer cachexia is almost assuredly a secondary organismal effect unrelated to tumor growth and progression (33). However, because calpain is regulated in an epigenetic manner and detection of changes in calpains are not expected, either calpain activity has to be determined directly or challenged in experimental systems to substantiated potential roles in tumor biology.

To investigate the role of calpain in prostate cancer invasion, we used the androgen-independent cell line DU 145 (PA; Ref. 34) and its derivative, WT,[3] which overexpresses the full length of EGFR and which has been shown to be more invasive (35, 36). Because the signature of activated calpain within cells is not known, we could not survey *de novo* tumors for activation status. Rather, we used an interventional strategy to establish proof of concept that calpains contribute to tumor invasion. Both ubiquitous calpains were inhibited

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[3] The abbreviations used are: WT, wild-type; EGFR, epidermal growth factor receptor; EGF, epidermal growth factor; CI, calpain inhibitor; AS, antisense; MAP, microtubule-associated protein; V, vector; ECM, extracellular matrix.

pharmacologically by the calpain-specific inhibitor CI-I (ALLN) or the calpain-preferential but broad-spectrum cysteine-serine protease inhibitor, leupeptin. This latter agent was chosen because it has been used in mice and even, on the basis of compassionate release, in humans with little toxicity evident (37, 38). To confirm calpain targeting and identify the key isoform, AS down-regulation of calpain-2 was performed in these cells. Our findings indicate that calpain may represent a key molecular switch that regulates a rate-limiting step in tumor invasion.

MATERIALS AND METHODS

Cell Lines and Reagents. Human DU 145 prostate carcinoma cell line and its derivative WT DU145 (35, 36) were maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum supplemented with L-glutamine (2 mM), nonessential amino acids (0.1 mM), sodium pyruvate (1 mM), and antibiotics; 350 mg/ml of G418 was added to the medium for the WT cells. Medium was purchased from Life Technologies, Inc. (Gaithersburg, MD). The parental DU145 cells are referred to as PA DU145, whereas those cells overexpressing full length, WT EGFR are referred to as WT DU145. Human recombinant EGF was purchased from BD Biosciences, CI-I (ALLN) from Biomol (Plymouth Meeting, PA), and leupetin and all of the other reagents were purchased from Sigma (St. Louis, MO).

Plasmids and DNA Constructs. To generate a minigene complementary to human calpain-2, we chose a sequence that spanned the translation initiating ATG, as AS to this sequence was productive (17). Human cDNA coding for 80 pb (C2AS) minigene was generated by RT-PCR using the following primers: 5′ oligo sequence 5′ACCGCAGCATGGCGGGCA; and 3′ reverse oligo sequence 5′TGGCCCTCTCGTGGGAGC. The cDNA was cloned into pBluescript II KS vector, digested with *Xho*I and *Bam*HI, and inserted into the *Xho*I and *Bam*HI sites of the mammalian pCEP4 expression vector. cDNA was sequenced to verify correct orientation and sequence. Expression was obtained by electroporation into DU145 cells. Stable transfectant cells were selected by supplementing the medium with 100 μg/ml hygmomycin. These cells are referred to as C2AS WT or PA DU145, whereas the vector only controls are named V WT or PA DU145.

AS Oligonucleotides. Phosphorothioate AS oligodeoxynucleotides were synthesized by DNA synthesis facility (University of Pittsburgh). The sequences of calpain-2 AS have been described previously, 5′CGCGATGCCCGCCCGCCATGCT (39). A scrambled (SCR: 5′TCGTACCGCCCGCCCGTAGCGC) phosphothiorated oligonucleotide was used as a control. These sequences and their complementary sequences presented no similarity with other target mRNA, as best we could determine using the BLASTN program.

Quiescent cells were transfected using the superfectin reagent according to the manufactured protocol. Briefly, cells plated in 12-well plates were incubated with 20 μM of oligonucleotide with 7.5 μl of superfect in a final volume 500 μl for 3 h, then washed twice with PBS and incubated with or without 1 nM EGF for 24 h. For invasion assay, cells were counted and transferred into the transwell chambers. Otherwise, cells were kept in the same plate and used for MAP2 assay or wounded (0 h) for the migration assay.

Migration Assay. An *in vitro* "wound healing" assay was used to assess cell motility in two dimensions (40). Cells (10^5) were plated on a six-well plate and grown to confluence in their regular medium. To minimize the autocrine signaling, confluent cells were kept in 1% dialyzed FBS, then wounded using a rubber policeman (0 h). Cells were washed twice with PBS and treated with or without specific effectors for 24 h. Photographs were taken at 0 and 24 h, and the distance traveled was determined by subtracting the values obtained at 0 from 24 h. Mitomycin C (0.5 μg/ml) was used to limit proliferation (41).

Calpain Activity Assays. Calpain activity was detected in living cells or in the whole cell lysates using BOC or MAP2 assays, respectively, as described previously (17). Briefly, for BOC, cells were plated on glass coverslips at between 50 and 70% confluence in their regular media. Quiescent cells were treated with or without 1 nM EGF, CI-I, or leupetin for 24 h. t-butoxycarbonyl-Leu-Met-chloromethylaminocoumarin (0.5 μM; Molecular Probes, Eugene, OR) is added to the cells for 20 min followed by 1 nM EGF for 10 min. The activity of calpain was detected by the increase of fluorescence noted on the cleavage of the substrate BOC using an Olympus fluorescent microscope (model BX40 with an Olympus M-NUA filter), and representative images were captured using a spot CDD camera. The exposure and time settings were fixed within each experimental series.

To determine calpain activity in cell lysates, MAP2 (Cytoskeleton, Denver, CO) was labeled with DTAF by incubation of MAP2 and dichlorotriazinylaminofluorescein in (pH 8.5) PIPES buffer for 30 min at 4°C. Labeled MAP2 was then isolated by size exclusion column chromatography and dialyzed against (pH 7.5) HEPES buffer overnight. Cells were grown to confluence in six-well plates, quiesced for 24 h, and treated or not with 1 nM EGF. Cells were washed twice with ice-cold PBS and lysed with cell lysis buffer [20 nM HEPES (pH 7.4), 10% glycerol, 0.1% Triton X-100, 500 mM sodium chloride, and 1 mM sodium vanadate]. After removing the cell debris by centrifugation, 0.9 μg of DTAF-labeled MAP2 was added to the samples with 20 μM free Ca^{2+} concentration. Fluorescence was immediately measured by an Aminco-Bowman Series II spectrofluorimeter (Spectronic Instruments Inc., Rochester, NY) at excitation and emission wavelength of 470 and 520 nm, respectively, for 3 min at room temperature

To detect the total potential calpain activity in a cell, we used casein zymography. Twenty μg of cell lysate were resolved under nonreducing conditions by PAGE in HEPES-imidazole buffer with 5 mM EDTA that separates calpain-1 and -2 isoforms. After washing, gels were incubated for 20 h in a calpain activation buffer (20 mM 4-morpholinepropanesulfonic acid 2 plus 5 mM beta-mercaptoethanol) containing 5 mM $CaCl_2$ or in 4-morpholinepropanesulfonic acid buffer without $CaCl_2$ and with EDTA as a control. The gels were stained for protein content with transparent bands identified by comparison to calpain standards. The density of the bands was measured using NIH image.

Immunoblotting. Protein expression was determined as described previously (17). Briefly, cells were washed in PBS and lysed in SDS lysis buffer before analysis by reducing SDS-PAGE. Primary antibodies included anticalpain-2 (clone N-19 and C-19; Santa Cruz Biologics, Santa Cruz, CA), anticalpain-1 (Biomol), and antiactin (Sigma). Bands were visualized using alkaline-phosphatase-coupled secondary antibody (Promega, Madison, WI).

Cell Proliferation Assay. Mitogenic stimulus was determined by direct cell counting. Cells were plated in 24-well plates and cultured for 24 or 48 h in their regular medium, with or without leupeptin or CI-I. The number of cells was determined using a Coulter Counter model Z2 (Miami, FL).

Invasion Assays. Invasive potential was determined *in vitro* by transmigration of an ECM (5). Matrigel invasion chamber plates were obtained from Becton Dickinson/Biocoat (Bedford, MA). The upper surface of the matrix was challenged with 20,000 cells, a number derived from empirical experimentation (22, 23, 35). Cells were kept in serum-free medium containing 1% BSA for the first 24 h and then replaced with only serum-free medium for the remaining 24 h; the lower chamber contained medium containing 10% serum for the entire assay. Enumeration of the cells that invaded through the matrix over a 48-h period was accomplished by visually counting cells on the bottom of the filter, as per routine procedures, after any uninvaded cells were removed from the top of the filter with a cotton swab. In all of the cases, individual experiments were performed in duplicate chambers.

True invasiveness of the cells was determined *in vivo* using the diaphragm invasion model (5, 24, 36). For the first experimental series, 14 male 6-week-old Balb/*c nu/nu* athymic mice (day 0) were inoculated i.p. with 2×10^6 PA or WT DU145 cells and randomly separated into two groups at day 10. After 10 days, the mice received daily i.p. injections of 12 mg/kg of leupeptin or diluent only for 30 days. In the second experimental series, mice were inoculated with either PA or WT DU145 expressing C2AS minigene or V alone to assess AS down-regulation of calpain-2 on tumor invasion after 60 days. In all of the cases, invasion was determined as follows. Mice were sacrificed, and the diaphragm and any tumors were removed, fixed in 10% paraformaldehyde, and stained with H&E. Invasiveness was scored semiquantitatively on a four point scale measuring the greatest extent of invasion into the diaphragm muscle, with 0 being no invasion and 4 being complete transmigration of the diaphragm. Mice without evident diaphragmatic tumors were not included in the invasion scoring. Each experiment was repeated and the data collated for the two experiments. The number of mice challenged was determined *a priori* for a 95% confidence level of determining a difference ($P < 0.05$) using the assumptions of 80% diaphragmatic tumors with a 30% difference in invasiveness between the comparison groups; this yielded a minimum mouse number of 12 mice per test set. These assumptions were based on prior experimentation

of altered EGFR or peritoneal lymphocyte signaling (24, 36). All of the animal experiments were certified by the University of Pittsburgh and Pittsburgh VA Medical Center Institutional Animal Care and Use Committees.

Microarray Gene Expression Analysis. We queried the gene expression profile of 53 prostate cancers and 23 normal donors using the Affymetrix (Santa Clara, CA) system. These human tumor queries were determined as exemption 4 under pre-existing data and excess pathological specimens by the University of Pittsburgh Institutional Review Board; specimens were provided by an "honest broker," and the investigators were blinded as to patient identity. Designation of invasive (aggressive; $n = 29$) and localized (organ-confined; $n = 24$) was per pathology report for clinical use. In addition, 23 normal human prostates from organ donors were run in parallel.

Samples of prostate tissues obtained from prostatectomy were dissected and trimmed to obtain pure tumor (completely free of normal prostate acinar cells) or normal prostate tissues. Sandwich-frozen sections were performed by board-certified genito-urinary pathologists to examine the purity of the tumors. These tissues were then homogenized. Total RNA was extracted and purified with Qiagen RNeasy kit (Qiagen, San Diego, CA). Five μg of total RNA were used in the first strand cDNA synthesis with T7-d$(T)_{24}$ primer [GGCCAGTGAAT-TGTAATACGACTCACTATAGGGAGGCGG-$(dT)_{24}$] by Superscript II (Life Technologies, Inc., Rockville, MD). The second-strand cDNA synthesis was performed at 16°C by adding *Escherichia coli* DNA ligase, *E. coli* DNA polymerase I, and RnaseH in the reaction. This was followed by the addition of T4 DNA polymerase to blunt the ends of newly synthesized cDNA. The cDNA was purified through phenol-chloroform and ethanol precipitation. The purified cDNA was then incubated at 37°C for 4 h in an *in vitro* transcription reaction to produce cRNA labeled with biotin using MEGAscript system (Ambion, Inc., Austin, TX).

Hybridization was as follows. Fifteen to 20 μg of cRNA were fragmented by incubating in a buffer containing 200 mM Tris-acetate (pH 8.1), 500 mM KOAc, and 150 mM MgOAc at 95°C for 35 min. The fragmented cRNA were then hybridized with a pre-equilibrated Affymetrix chip at 45°C for 14–16 h.

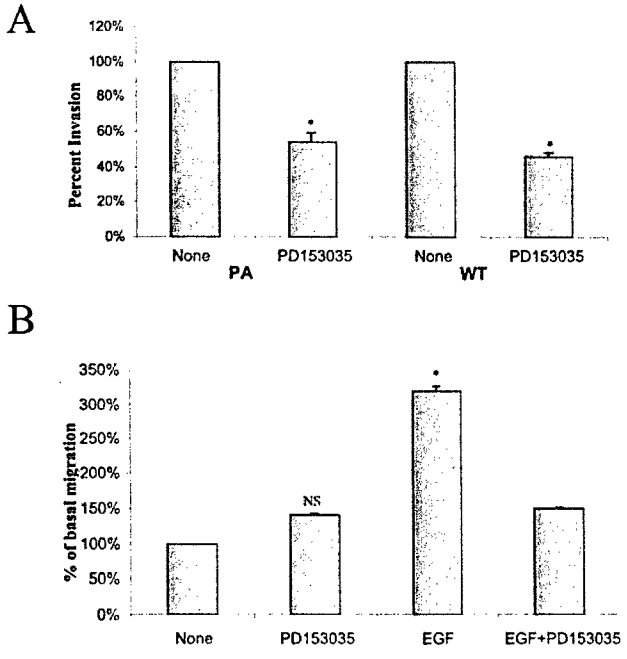

Fig. 1. DU145 invasion and migration are dependent on EGFR signaling. *A*, PA or WT DU145 cells (20,000) were plated in the upper chamber of the transwell plate in medium containing 1% BSA and 500 ng/ml EGFR inhibitor (PD153035). After 24 h, the medium in the upper chamber was replaced with serum-free medium containing PD153035 for another 24 h. The bottom chamber contained complete medium with 10% FCS and PD153035. Cells in the upper compartment were removed by wiping with a cotton swab, and invasive cells were stained according to the manufacture's protocol. *B*, PA DU145 cells were plated in six-well plates and quiesced for 24 h before an *in vitro* wound-healing assay. Cell movement into the denuded space was assessed in the presence of EGF (1 nM) and/or PD153035 (500 ng/ml) or diluent. All of the experiments were performed in triplicate and repeated at least twice. Effects are normalized to diluent alone for the respective cell line; in *A* absolute invasiveness was 2.00 ± 0.24 in WT over PA DU145 cells. $**P < 0.05$ as compared with no treatment, NS, not significant; *bars*, ±SD.

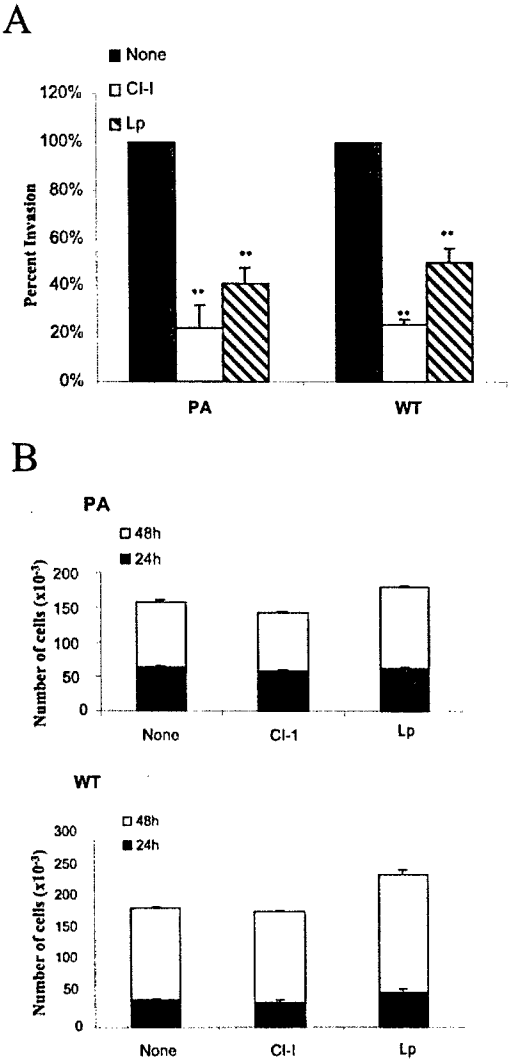

Fig. 2. Calpain inhibition reduces invasiveness of PA and WT DU145 cells *in vitro*. *A*, cells (20,000) were plated in the upper chamber of the transwell plate in medium containing 1% BSA and 2 μg/ml CI-1 or 100 μM of leupeptin (*Lp*). After 24 h, the medium in the upper chamber was replaced with serum-free medium containing CI-1 or Lp for another 24 h. The bottom chamber contained complete medium with 10% FCS and CI-I or Lp. Cells in the upper compartment were removed by wiping with a cotton swab, and invasive cells were stained according to the manufacture's protocol. *B*, cells (20,000 PA or 10,000 WT) were plated in 24-well plates and cultured for 24 or 48 h in CI-I or leupeptin (*Lp*). Cell numbers were enumerated directly. All of the experiments were performed in triplicate and repeated at least twice. $**P < 0.01$; *bars*, ±SD.

After the hybridization mixtures were removed, the chips were then washed in a fluidic station with low-stringency buffer (6× saline-sodium phosphate-EDTA, 0.01% Tween 20, and 0.005% antifoam) for 10 cycles (2 mixes/cycle) and stringent buffer (100 mM 4-morpholinepropanesulfonic acid, 0.1 M NaCl and 0.01% Tween 20) for 4 cycles (15 mixes/cycle), and stained with strepto-avidin phycoerythrin. This was followed by incubation with biotinylated mouse antiavidin antibody, and restained with strepto-avidin phycoerythrin. The chips were scanned in a HP ChipScanner (Affymetrix Inc.) to detect hybridization signals.

Data were analyzed by importing the hybridization data from text files into an Microsoft excel spreadsheet. GeneSpring 4.2 along with Michael Eisen's cluster and tree view software were the primary analysis tools. Principle component analysis and logistic regression were performed using S-Plus (Statistical Sciences, Inc.) statistical software.

RESULTS

Invasiveness of DU-145 Prostate Cell Lines Is EGFR-dependent. EGFR overexpression correlates with tumor progression and invasion (5, 7). We tested the hypothesis that motility induced by

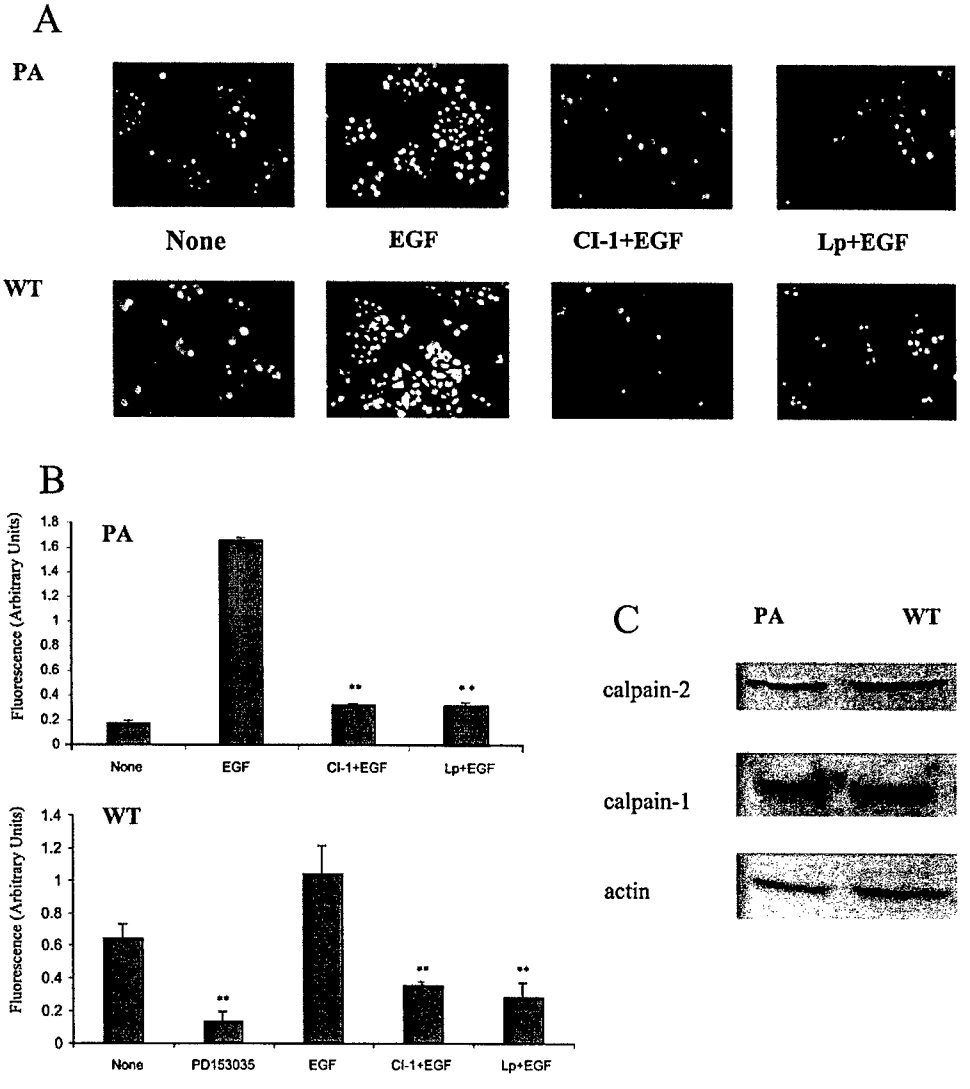

Fig. 3. Leupeptin and CI-I block EGF-induced calpain activation. *A*, quiescent WT and PA DU145 cells were treated with CI-I (ALLN; 2 μg/ml) or leupeptin (*Lp*; 100 μM) for 24 h before loading with BOC-Leu-Met-CMAC for 20 min. Cells were then stimulated with EGF (1 nM) for 10 min before visualizing with a preset CCD camera. The shown exposures are set so only cells with activated calpain are seen; in all of the frames similar numbers of cells were present as determined by phase contrast performed in parallel. *B*, cells were quiesced for 24 h before exposure to EGF (1 nM). Cells were treated with CI-I, leupeptin (as in *A*), or PD153035 (500 nM). Cells were lysed and cleared cytosolic lysates evaluated for their ability to cleave DTAF-labeled MAP2 as described. *C*, cells were grown in complete medium in six-well plates, washed, lysed, and proteins separated by SDS-PAGE. Equal protein loads were immunoblotted for calpain-2, calpain-1, or actin as a loading control. All of the experiments were repeated at least twice with the calpain assays performed in duplicate. $**P < 0.01$, as compared with diluent alone for PD153035 and compared with EGF treatment for CI-I and leupeptin.

autocrine EGFR signaling is a rate-limiting step in the invasion using our model of variously invasive syngeneic DU145 prostate cancer cell lines. Exposure of the moderately invasive PA DU145 or the highly invasive WT DU145 cells to the EGFR kinase inhibitor PD153035 decreased significantly the invasiveness through Matrigel even in the absence of exogenously added EGFR ligand (Fig 1*A*). The data are normalized to the respective mock-treated controls; WT DU145 cells are 1.7 (35) to 2.0 (data herein; $P < 0.05$) times more invasive than PA DU145 cells. EGFR-signaled cell motility was examined under conditions that minimize autocrine EGFR signaling (Fig. 1*B*). As shown in PA DU145 cells, EGF increased motility, which was abrogated by PD153035. These data support the previous literature (22, 35, 36) and demonstrate that the invasiveness of these cells is driven by EGFR signaling.

CIs Reduce DU 145 Cell Invasiveness *in Vitro*. The initial question we asked was whether calpain signaling was required for transmigration of an ECM. Transmigration of Matrigel by the moderately invasive PA DU145 and highly invasive WT DU145 lines was determined in the presence of CI-1 (ALLN; 2 μg/ml) or leupeptin (100 μM; Fig. 2*A*). The number of cells that reached the lower chamber within 48 h was significantly decreased by both inhibitors in both cell lines; the absolute invasiveness of WT DU-145 cells was 2.0-fold that of PA DU-145 cells. This agent-related decrement in cells transmigrated was not secondary to decreased proliferation (15, 20, 29, 39), as the concentrations of CI-1 and leupeptin used in this assay did not block cell proliferation (Fig. 2*B*).

Calpain activation was inhibited by both CI-1 and leupeptin (Fig. 3). First, we ascertained calpain activity *in vivo* by visualizing the bright blue fluorescence after the proteolysis of BOC-LM-CMAC, a calpain-selective substrate (42). Induced calpain activity was inhibited by both CI-1 and leupeptin in both PA and WT cells lines (Fig. 3*A*). In addition, we quantitated calpain activation using cleavage of the prefluorescence substrate DTAF from MAP2; again, both inhibitors limited or eliminated EGF-induced activation of calpain (Fig. 3*B*). WT DU145 demonstrated a somewhat higher basal activity as expected because of increased autocrine EGFR signaling (35), as it was inhibited by the pharmacologic agent PD153035 (Fig. 3*B*). Induced calpain activity was inhibited by both CI-1 and leupeptin to a level similar to that seen in the presence of PD153035 (Fig. 3*B*). The higher basal activity observed in WT compared with PA cells is not attributable to a higher amount of calpain-1 or -2 expression (Fig. 2*C*). Thus, we had evidence for calpain inhibition limiting tumor cell invasion.

Down-Regulation of Calpain-2 Limits PA DU145 Invasion. Molecular targeting of calpain was required, as leupeptin, in particular, and possibly even CI-1 inhibit proteases in addition to calpain. We used AS approaches to calpain-2 to abrogate signaling through this molecule (17). Oligonucleotides against calpain-2 in PA DU145 cells

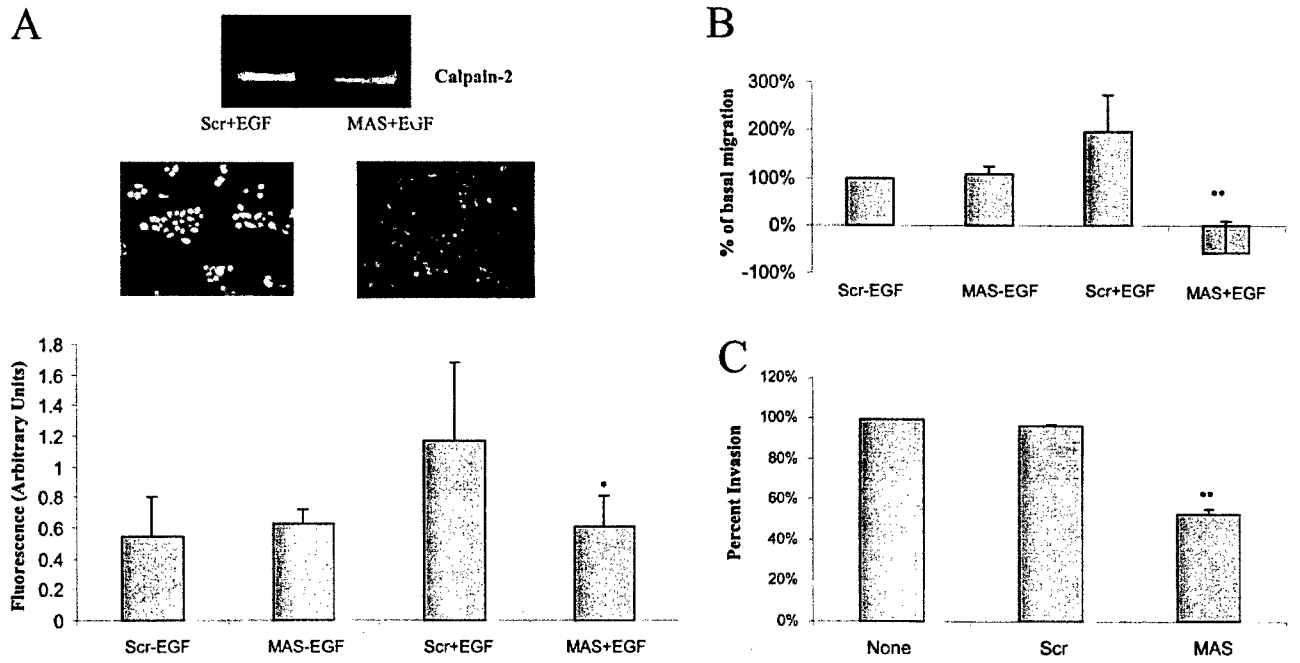

Fig. 4. AS oligonucleotides to calpain-2 decrease PA DU145 cell invasiveness and migration. Phosphothiorated oligonucleotides (20 μM) specific for calpain-2 (*MAS*; Ref. 17) were added to cells; a scrambled oligonucleotide (*Scr*) of similar composition served as a control. *A*, calpain activation was assessed by zymography (*top panel*), BOC-Leu-Met-CMAC (*middle panel*), or MAP2 (*bottom panel*) as described. *B*, the effect of MAS or Scr oligonucleotides on PA DU145 cell migration across a two-dimensional surface were calculated as a percentage of the values obtained with the Scr oligonucleotide alone. *C*, the invasion through Matrigel was evaluated as previously described and the number of cells that transmigrated through the Matrigel were normalized to no treated cells. All of the experiments were repeated at least twice, with the assays performed in duplicate. $^{**}P < 0.01$; NS, not significant; *bars*, ±SD.

limited EGF-induced calpain activation cell migration and transmigration of the Matrigel barrier (Fig. 4). A control scrambled oligonucleotide did not effect these parameters.

We generated a stable PA DU145 derivative in which an 80-bp minigene around the calpain-2 translation initiation site was expressed in the AS direction from the cytomegalovirus promoter. In these cells, calpain-2 levels were reduced by >30% as quantified using an NIH program (Fig. 5); such partial down-regulation was expected because calpain-2 is required for cell viability and growth (15, 20, 29, 39); importantly, a similar level of calpain-2 down-regulation eliminates EGF-induced calpain activity and motility in fibroblasts (17). These cells were significantly less invasive than a PA DU145 derivative expressing the vector alone as a control. This decrement in invasiveness was not because of decreased cell numbers, whether reduced proliferation or survival, because the two derivative polyclonal lines grew at the same rate. In sum, these data strongly suggest that calpain-2 activation is required for increased tumor cell motility and subsequent invasiveness *in vitro*.

Leupeptin and Down-Regulation of Calpain-2 Decreases DU145 Invasiveness *in Vivo*. Our data *in vitro* show that calpain activity is required for cell transmigration throughout a "defined" layer of ECM. To investigate the role of calpain in an *in vivo* environment where complex and various interactions occur, we used the murine tumor xenograft model of diaphragm invasion (24, 36). This assay was used because it is more easily quantitated than invasiveness of orthotopic tumor growth for both technical and biological reasons; however, the semiquantitative scores of diaphragm invasion correlate well with the qualitative assessment of invasiveness of orthotopic tumors (24, 36). The pharmacological agent chosen was leupeptin because this has been used in both mice and humans with minimal toxicity (37, 38). Either PA or WT DU145 cells were inoculated i.p. into athymic mice and allowed to establish for 10 days before treatment with leupeptin or diluent alone. The WT DU145 tumors demonstrated increased invasion *in vivo* ($P < 0.05$ compared with PA DU145 tumors), similarly to the increment *in vitro* transmigration of Matrigel; this finding is consistent with our previous reports (24, 36). For both cell lines, leupeptin treatment significantly reduced the extent of invasion into and through the diaphragm (Table 1). Invasion into other soft organs was not scored because of difficulty in quantitation but qualitatively reflected this difference. The reduction of invasiveness seen with leupeptin was not attributable to decreased tumor growth, because tumors in the diaphragm with the same size from treated or not treated mice showed different level of invasiveness (Fig. 6). This is expected, because leupeptin did not affect cell proliferation (Fig. 2*B*).

Verifying that this invasiveness was because of calpain inhibition required a second approach because leupeptin inhibits other proteases, both intracellularly as well as extracellularly. We repeated the diaphragm invasion assay using the PA and WT DU145 cells expressing the calpain-2 AS minigene or vector alone (Table 2). Mice inoculated with the calpain-2 AS showed 50% less invasiveness compared with the mice carrying the vector alone (Fig. 7). The PA DU145 cells exhibited high significance in themselves, whereas the WT DU145 were marginally inhibited; this affect is likely because of the few mice challenged in this second series, which was curtailed because of the outcome of the P DU145 cells. Again the tumor take rates and size of the diaphragm tumors were indistinguishable between the sublines expressing C2AS or V constructs. This degree of inhibition of invasiveness by slightly more than half was in line with the extent of inhibition shown by leupeptin.

Calpain Levels Are Not Altered in Human Prostate Tumors. The above data strongly suggest an epigenetic role for calpain in enabling tumor cell motility and subsequent invasion. To address whether this is also altered gene expression levels of calpain-2 in prostate tumors, we analyzed 29 aggressive/invasive or metastatic tumors and 24 organ-confined tumors (Table 3). In addition, 23 normal prostates were queried on the same chip set. We also examined the expression of calpain-1 and calpastatin, because these might alter

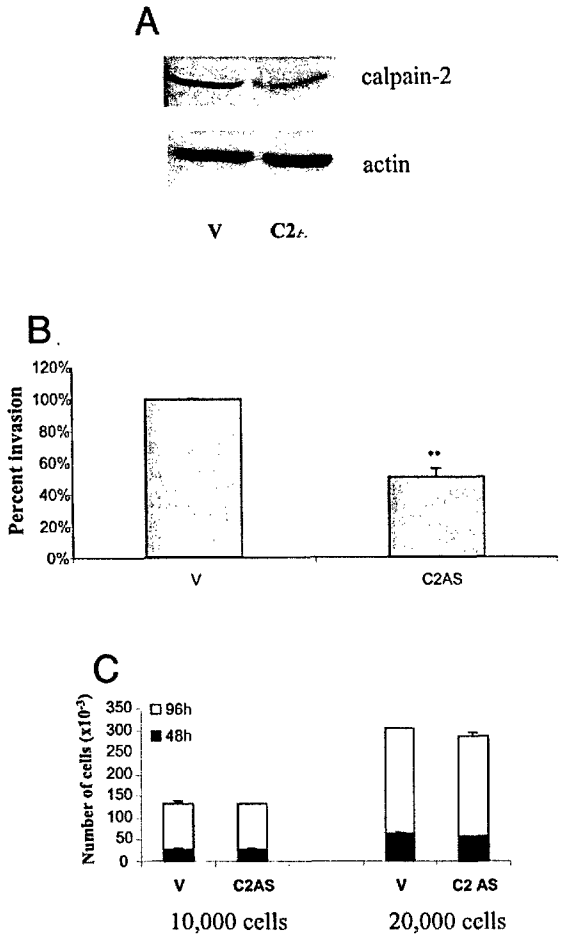

Fig. 5. AS minigene down-regulation of calpain-2 reduces PA DU145 invasiveness. PA DU145 cells were stably transfected with a minigene that expressed AS across the translation initiation site of calpain-2 or an empty vector. *A*, calpain-2 levels were ascertained by immunoblotting. Invasiveness through Matrigel (*B*) and cell proliferation (*C*) were evaluated as described. All of the experiments were repeated at least twice, with the invasiveness assay performed in duplicate and the cell counting in triplicate. $**P < 0.01$; *bars*, ±SD.

the calpain activity balance. Analysis of the expression levels of all five of the calpain-related hybridization spots using the Welch *t* test indicated minimum variation of gene expression across all of the samples. Calpain-2 and calpain-1 expression were characterized as being at moderate levels, whereas expression of calpastatin was minimum. No significant expression change was identified when aggressive prostate cancer was compared with normal donor or organ-confined prostate cancers.

DISCUSSION

Tumor invasion is a complex process that involves cellular migration, interaction with the microenvironment, and survival at the ectopic site. We and others have shown that cell migration is a rate-limiting step in this process (5). Thus, key molecular switches required for functional migration may be successfully targeted to limit tumor spread. Previous studies have shown that the calpain proteases are required for rear deadhesion during productive motility whether initiated by adhesion-related signals or growth factors (14, 16–18). Synthesizing these finding for tumor invasiveness, an initial report demonstrated that blockade of calpain limited both the motility and invasiveness *in vitro* of bladder carcinoma cells (22). As local invasion generates a great part of the morbidity of prostate cancer, we asked whether blockade of calpain signaling would limit this spread. Herein, we report that pharmacological and molecular inhibitors of calpain-2 significantly reduce the motility and invasiveness of DU145 human prostate carcinoma cells both *in vitro* and *in vivo*. These data suggest that calpain may be rationally targeted to limit prostate cancer spread.

Our data strongly implicate calpain-2 control of cell motility as the operative target. However, this assignment is compromised by the lack of selectivity of the pharmacological agents for the calpain-2 isoform; this is especially true for the broad spectrum inhibitor leupeptin. Despite this uncertainty of inhibition, leupeptin was chosen, because it has been used in both animals and humans with minimal reported toxicity (37, 38). Still, a strong case for calpain-2 being the critical element is made by the fact that AS approaches to calpain-2 mimic the findings with leupeptin and CI-I. Whereas leupeptin inhibits both intracellular and extracellular proteases, and ECM remodeling might be hindered (43), the expression of the AS calpain-2 minigene should not alter the myriad of extracellular proteases. Thus, a confluence of data support targeting calpain-2.

A second point of contention may rest on which cell behavior is limited by calpain inhibition. In many settings calpain activity is required for cell proliferation or apoptosis in addition to motility (20). Our *in vitro* data suggest that in this setting our level of calpain inhibition does not affect cell proliferation (Fig. 2*B;* Fig. 5*C*). However, the *in vivo* experiments are not readily amenable to such analyses; although the fact that the tumor take rate (Tables 1 and 2) and size of the tumors were indistinguishable between the calpain-inhibited and control tumors is reassuring that overall cell number is not the main target of calpain inhibition.

It is possible that the increased motility and invasion may be indirectly related to calpain activation because the broad spectrum of calpain targets may also involve regulation of secreted proteases. We feel that this is an unlikely mechanism, because our earlier works failed to demonstrate differences in protease production between PA and WT DU145 (35). Furthermore, because motility over a two-dimensional surface is also affected, the need for extracellular proteases to modify a "barrier" matrix is limited, although others have suggested that matrix metalloprotease 9 is required for dispersion of cohesive keratinocytes even over a matrix surface (44). However, in our earlier survey of cellular proteases produced by DU145 sublines, matrix metalloprotease 9 was secreted at equivalent levels by the three syngeneic lines (35). Lastly, EGF only activates calpain-2 in the immediate subplasma membrane locale (45), and, thus, protease maturation is not likely globally affected by such localized signaling. However, until the identification of the specific target of calpain during induced motility (14), both indirect as well as direct molecular mechanisms must be considered.

Prostate cancer motility and invasion likely uses both ubiquitous calpain isoforms, calpain-2 and calpain-1, for cell movement. This is because prostate carcinoma cells present both integrins capable of promoting haptokinesis and EGFR-mediate autocrine signaling loops that induce chemokinesis (46). Calpain-1 (μ-calpain) has a calcium-dependency that can be attained in living fibroblasts and epithelial

Table 1 *Leupeptin decreases tumor invasiveness in mice*

Control mice inoculated with PA or WT cells were injected with diluent HBSS, and the experimental mice received 12 mg/kg of leupeptin. Mice were sacrified and diaphragm scored for diaphragm invasiveness from 0 to 4. Diaphragm tumors represent the total number of mice with tumors in the diaphragm.

	PA + HBSS	PA + leupeptin	WT + HBSS	WT + leupeptin
Diaphragm tumors	14/14	13/14	14/14	13/14
Diaphragm invasiveness	1.71	0.7[a]	2.35	1.25[b]

[a] $P < 0.01$.
[b] $P < 0.05$.

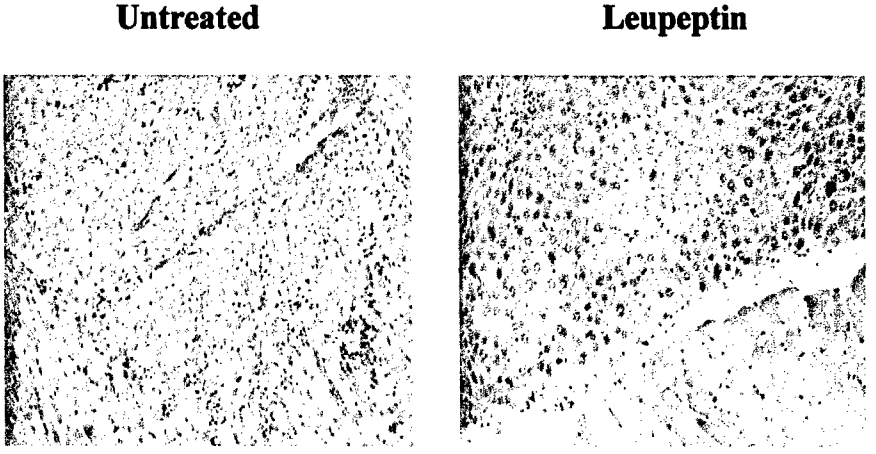

Fig. 6. Leupeptin reduces diaphragmatic invasion *in vivo*. Six-week-old male *BALB/c nu/nu* mice were injected i.p. with 2 million PA DU-145 cells. Ten days later, mice were separated into two groups, one was daily given i.m. injection of 12 mg/kg of leupeptin for 30 days, and the other control group was injected with a similar volume of HBSS. Diaphragms were isolated and evaluated by histopathology. Shown are representative invasion values of 2+ (diluent) and 0 (*Leupeptin*).

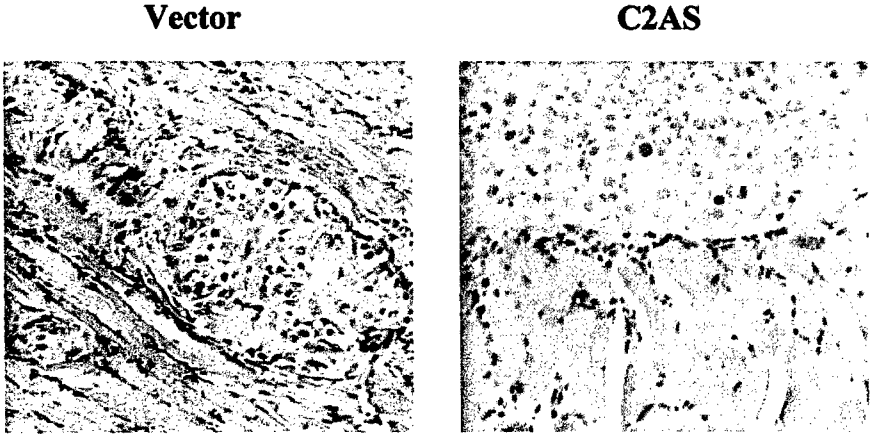

Fig. 7. AS down-regulation of calpain-2 reduces diaphragmatic invasion *in vivo*. Six-week-old male *BALB/c nu/nu* mice were injected i.p. with 2 million PA DU-145 cells expressing the minigene or vector alone. Sixty days later the diaphragms were isolated and evaluated by histopathology. Shown are representative invasion values of 3+ (*vector*) and 1 (*C2AS*).

cells (47). The autocrine EGFR-mediated signaling would activate calpain-2 preferentially via an ERK mitogen-activated protein kinase pathway at the inner face of the plasma membrane (17, 45). Thus, there appears to be a convergent signaling through the two ubiquitous calpain isoforms to regulate cell deadhesion (14). Whereas this might suggest that the best target for intervention is the end target, there are reasons to focus on calpains. First, the presumably common end target(s) might be individually sufficient, but none are actually required, making specific intervention ineffectual. Second, it is likely that the end target of calpain is a structural component and, thus, not readily "inhibitable," although the activation of the rho-GTPase may suggest sensitive points for intervention (48). Third, the ability to inhibit only one isoform may limit toxicity, because homeostatic mechanisms that require low level motility, such as colonic or skin epithelial replacement, would use one of the isoforms and not the other in the absence of injury repair needs (49). Unfortunately, the commonly available inhibitors such as leupeptin and CI-I do not distinguish between the isoforms, making molecular approaches the only viable option at present to determine whether inhibition of a single isoform can accomplish blockade of tumor invasiveness. Obviously, new, isoform-specific small molecule inhibitors would greatly advance our understanding of the physiology of calpain activation.

Table 2 *Antisense down-regulation of calpain-2 decreases prostate tumor invasiveness in mice*

Mice injected with PA or WT DU-145 cells expressing calpain-2 minigene (C2AS) were compared with mice receiving cells transfected with the vector alone (V). Results are the average of diaphragm score of 9 mice *versus* 8 for PA cells and 4 *versus* 3 for WT cells.

	V PA DU145	C2AS PA DU145	V WT DU145	C2AS WT DU145
Diaphragm tumors	9/10	8/10	4/5	3/5
Diaphragm invasiveness	2.33	1.13[a]	3.50	1.67[b]

[a] $P < 0.05$.
[b] $P < 0.10$.

The question remains of whether these findings in model systems translate to the human clinical situation. We surveyed 53 specimens from human prostate tumors and normal prostate tissue. Segregated by tumor stage, invasiveness, and metastases, we found no significant differences in mRNA levels of these tissues. This is in contradistinc-

Table 3 *Calpain expression does not differ in human prostate cancers based on tumor invasiveness*

Probe set	Description	Average of AC[a]	Average of OCC	Average of ND	AC/ND	*P*	AC/OCC	*P*
33384	Calpastatin	64.228	68.457	67.957	0.9451	0.6445	0.9382	0.8002
33385_g	Calpastatin	288.345	298.095	324.826	0.8877	0.1824	0.9673	0.4202
33908_	Calpain-1	2,485.08	2,451.91	2,356.19	1.0547	0.785	1.0135	0.9962
37001_	Calpain-2	1,560.52	1,507.79	1,751.10	0.8912	0.1428	1.035	0.7373
47510_r	CAST Calpastatin	293.507	288.238	256.426	1.1446	0.3642	1.0183	0.9132

[a] AC, aggressive prostate cancer ($n = 29$); OCC, organ-confined prostate cancer ($n = 24$); ND, normal prostate donor ($n = 23$).

tion to a recent report in which calpain-2 mRNA was found to be mildly (1.4 times) up-regulated in prostate carcinomas in conjunction with cadherin cleavage (50). We did not note this increased transcript level in our series of tumors, although the reasons for this discrepancy are not evident at present. However, in a different tissue, calpain-2 levels were not increased over that in normal skin in either squamous or basal cell carcinomas (51). Another calpain isoform reported altered in tumors, calpain-9 (31) is not reliably detectable in our prostate tissues: neither normal donor nor tumor (data not shown). According to accepted models of calpain activation (14, 15, 25), the lack of transcriptional change is not unexpected. Calpains appear to be activated at a post-translational level with calcium or other mechanisms, such as coactivators or phosphorylation (52–54),[4] being the operative event. In fact, in studies that attempt to exogenously express calpains, one usually fails to attain even a doubling of calpain levels, as higher activity leads to apoptosis (55). Thus, to demonstrate increased calpain activation in invasive tumors would require a way to assess *in situ* activation. For live cells, this can be accomplished by fluorescent substrates (Fig. 2). However, in nonliving cells we need to develop reagents to detect either the post-translational modifications that mark activation or colocalization of the activator cofactors.

In summary, we found that targeting calpain can limit prostate cancer cell invasiveness both *in vitro* and *in vivo*. This was likely because of the inhibition of rear deadhesion during growth factor-induced motility. In fact, CI-I limits EGFR-mediated deadhesion of DU145 cells (data not shown) similar to the calpain-dependent detachment of fibroblasts (17) and epithelial keratinocytes (21). Our operative model of calpain function during tumor invasion posits an epigenetic or post-translational activation of calpain-2 rather than significant changes in protein levels. A survey of mRNA profiles of human prostate carcinoma specimens supports this by failing to demonstrate calpain gene expression differences between invasive and noninvasive carcinomas. However, to fully demonstrate the validity of this model will require a knowledge of how calpain-2 is activated and development of tools to detect such changes in activation. Additionally, the targeting of calpain-2 as a rational therapeutic intervention strategy will also necessitate new reagents, isoform-specific inhibitors. Because of the high degree of homology at the amino acid and structure levels (25) molecular agents offer the greatest hope of discriminatory agents. Thus, the full exposition of this potential novel target to limit tumor progression will rely as much on technical developments as on biological insights.

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Review

Phospholipase C-γ1 in tumor progression

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Key words: cytoskeleton reorganization, EGF receptor, growth factor receptors, migration, phosphoinositide hydrolysis, signal transduction, transcriptional regulation, tumor invasion

Abstract

The vast majority of cancer morbidity and mortality arises from tumor progression beyond the primary tumor site. Unfortunately, most therapies are not effective for advanced stage disease with regional extension or distant metastases. Thus, new treatments are needed to target rate limiting steps in tumor progression. The ability of cancers to invade and metastasize requires the acquisition of specific cell behaviors that enable the cell to escape from the localized site, breach the defined boundaries, reach a hospitable ectopic site and grow in this new locale. Recently, dysregulation of cell motility as stimulated by various extracellular factors has gained credence as a rate-limiting alteration in tumor progression in carcinomas and some other solid tumors. This has focused attention on initiators of signaling cascades that regulate tumor migration. In this effort, one molecule, phospholipase C-γ1 (PLCγ), has been shown to function as a key molecular switch.

Phospholipase C-γ

There are 11 known mammalian gene members of phosphoinositide-specific phospholipases, termed phospholipase C (for review of the structure/function of the PLC family see [1]). One family, PLCγ, is activated by receptor and non-receptor tyrosine kinases. PLCγ1 is ubiquitously expressed, whereas the other member, PLCγ2, is largely restricted to cells of hematopoietic lineages [2]; therefore the following discussion will focus on PLCγ1. These two isoforms are distinguished from the other PLCs by presenting a region between the split phospholipase domain (Figure 1) that contains two phosphotyrosine-binding SH2 domains and a polyproline binding SH3 domain. This specific array or Z-domain, residing within a second split PH domain, also contains an autoinhibitory sequence [3]. These domains serve to target PLCγ1 to receptors with intrinsic tyrosine kinase activity (via binding to phosphotyrosines) and to the inner surface of the plasma membrane (via binding to PIP3).

PLCγ is activated by both binding to and being phosphorylated by tyrosine kinases; with the most relevant kinases being members of the growth factor receptor superfamily [1]. PLCγ is strongly activated by all families of growth factor receptors including EGF, PDGF, NGF, FGFs, VEGF, HGF and IGF-1. This phosphorylation and complexing, along with PH-domain binding to plasma-membrane PIP3 allows for juxtapositioning of the split phospholipase domains to hydrolyse phosphatidylinositol (4,5) bisphosphate (PIP2) to its components inositol-trisphosphate (IP3) and diacylglycerol (DAG). Interestingly, while PLCγ is bound and phosphorylated by growth factor receptors both at the inner face of the cell surface membrane and and remains so after internalization of the receptor in endosomes, hydrolysis of PIP2 occurs only from the surface receptors, likely due to lack of available substrate in the endosomes [4–6]. This intracellular restriction limits key PLCγ events to the perimembrane locale.

The role of PLCγ in organismal functioning is incompletely understood. PLCγ1-deficient mice die in early embryogenesis at day 9 [7], whereas mice lacking PLCγ2 survive postnatally and display deficits in cells of the hemopoietic lineage, particularly in B cells. Interestingly, cells from the PLCγ1-deficient animals display normal mitogenic responses to various growth factors suggesting that proliferation is not dependent on this signaling cascade. The lack of a role for PLCγ in mediating cell growth is supported by various *in vitro* and *in vivo* studies in which pharmacologic and molecular inhibitors of PLCγ did not limit cell proliferation [8, 9]. Although others have implicated PLCγ in mitogenic signaling [3, 10, 11], these studies did not directly abrogate only PLC signaling and unintended side events might be responsible for the phenomena observed. While it is possible that PLCγ is involved in mitogenic signaling under certain circumstances, most studies to date have demonstrated that, in carcinoma cells, the studies mitogenesis can occur despite blockade of PLCγ signaling [12, 13].

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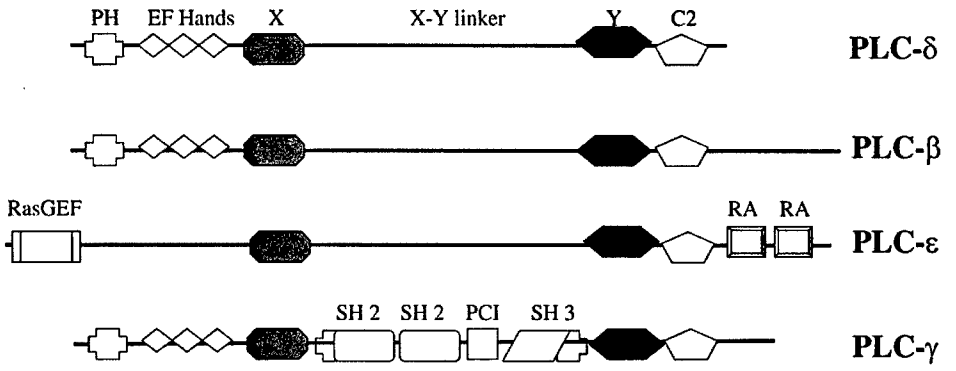


PH : Pleckstrin homology domain
X-Y : Catalytic region
C2 : PKC homology domain
RasGEF : Ras quanine nucleotide exchange factor-like domain
RA : Ras binding domain
SH2 : Src homology 2 domain
PCI : PLC inhibitor region
SH3 : Src homology 3 domain

Figure 1. Structure of phospholipase C isoforms. The schematic shows the general domain organization of the four types of PLC isoforms. The domain sizes are not to scale. The split PH domain that brackets the SH2-SH2-SH3 casette in PLCγ is shown in partial shape. For more details on these structures see [1]

PLCγ is required for cell motility induced by many growth factors, including EGF [8], PDGF [14], IGF-1 [15], and HGF [16], but not FGF [17]. This has been shown in a variety of cell types including fibroblasts, keratinocytes, soft-organ epithelium, glial cells and, most importantly for this discussion, carcinoma cells [12, 13]. Interestingly, cell motility driven by adhesion receptors such as integrins appears not to require active PLC signaling [18], despite complexing together in focal adhesions. This dichotomy of motility signaling may be critical during tumor progression since upregulated autocrine signaling through growth factor receptors is a hallmark of the invasive state [12, 13].

Growth factor receptors and PLCγ in tumor progression

Cumulative data has implicated growth factor receptors in carcinogenesis, starting from their identification as cellular proto-oncogenes [19, 20]. Also, most epithelial tissues produce both growth factors and their cognate ligands, normally separated by cellular polarity into apical and basolateral aspects; autocrine signaling loops are established upon cancer-related dedifferentiation and loss of cell polarity [21]. Interestingly, growth factor receptor activities are associated primarily with tumor progression rather than carcinogenesis, despite the early studies with derivative oncogenes [13, 20]. The best described examples of these involve ErbB2 signaling in breast cancer progression [22] and the EGF receptor in a host of cancers including glioblastoma [23], bladder carcinoma [24], gastric carcinoma [25], breast cancer [26] and head and neck cancer [27]. Other growth factors have been found to be upregulated or dysregulated in various other cancers, usually correlating with progression to invasion and metastasis.

The upregulated activity of these receptors likely indicates subsequent upregulated PLCγ signaling. However, there has not been published evidence linking PLCγ to tumor progression in a large survey of de novo human cancers. An earlier report [28], detected increased levels of PLCγ in the majority of breast cancers compared to normal breast tissue, but they did not link this to disease stage or outcome. Interestingly, they found some of the PLCγ was phosphorylated in those cases in which there appeared to be upregulation of EGF receptor or ErbB2. While intriguing, the lack of further reports could reflect technical challenges due to the fact that upregulation of PLCγ activity is an epigenetic phenomenon that would not be detected by gene expression studies and may be missed by proteomic approaches due to rapid dephosphorylation; such studies may require fresh, living tumor cells in culture for positive verification of PLCγ functioning. This challenging situation would not be unprecedented, as increased EGFR signaling can be noted in the absence of increased receptor mRNA or protein levels in prostate and hepatocellular carcinoma lines [21, 29, 30]. This epigenetic increased signaling is noted

in a large fraction of the archetypal tumor with upregulated EGFR, glioblastoma multiformes [23].

The evidence for PLCγ promoting tumor invasion arises from experimental approaches with human cancer lines *in vivo*, *ex vivo* and *in vitro* [13]. Inhibiting PLCγ signaling blocked invasion into normal brain tissue of human glioblastoma multiformes explants [31]. Similar pharmacologic and molecular inhibition of PLCγ prevented prostate carcinoma cell line invasion in animal models [9, 32]. *In vitro* invasion through matrix barriers of bladder, breast, and head and neck squamous cell carcinomas was similarly diminished by PLCγ inhibitors [33–35]. Thus, experimental data have preceded clinical confirmation of this linkage.

The two putative downstream biological events that derive from PLCγ signaling, cell motility and proliferation, are key to tumor progression. Dysregulated cell migration is central to both tumor invasion through surrounding matrix barriers [12, 13] and during tumor metastasis [36, 37]. Cell proliferation is the sine qua non of tumorigenesis and progression. It is reasonable to explore PLCγ signaling further to unravel these relationships.

PLCγ signaling during induced cell motility

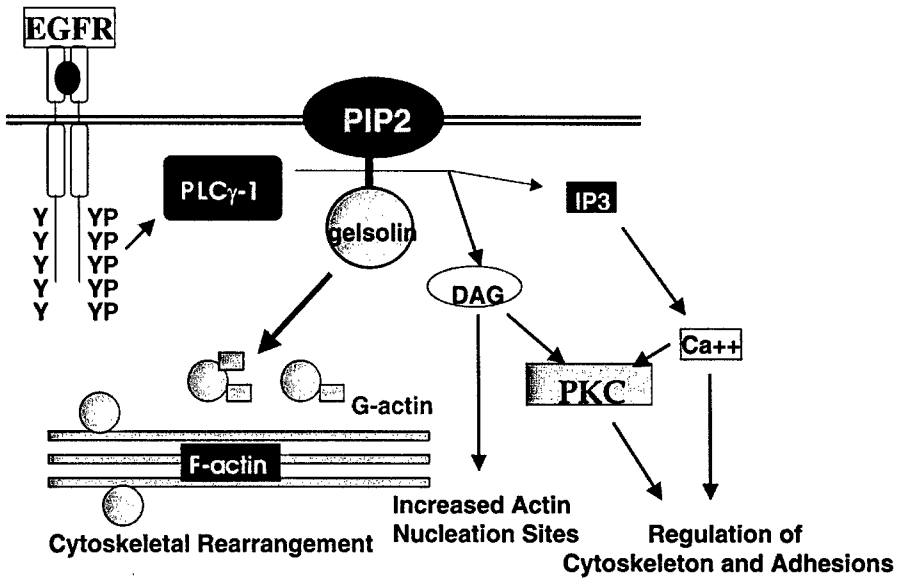
The mechanisms by which PLCγ actuates cell motility in response to growth factors are being defined. In this cellular response, PLC catalytic activity is required in that pharmacologic inhibitors, which do not prevent coupling to targets, abrogate cell motility. PIP2 levels, which are controlled by both kinases and phosphatases in addition to PLCγ, appear critical in two membrane events during motility, stabilization and maintenance of the adhesions [38, 39] and cytoskeletal reorganization behind the leading edge. The cytoskeletal epigenetic events are better understood (Figure 2). Upon growth factor binding to the cognate receptor, PLCγ is phosphorylated on two specific tyrosines, at amino acids 783 and 1253, concomitant with binding to the autophosphorylated receptor [1]. When the receptor-PLCγ complex is resident at the plasma membrane [4], PIP2 is hydrolyzed to DAG and IP3. This action releases actin binding proteins, such as gelsolin [40, 41], that are sequestered by docking to PIP2 [42, 43]. The release of these proteins is regulated by PLCγ phosphorylation in that profilin prevents PIP2 hydrolysis by non-phosphorylated PLCγ but tyrosyl-phosphorylated PLCγ overcomes this steric inhibition [44]. Gelsolin severs established actin filaments [40, 41], while cofilin and profilin serve to nucleate new filament growth [45, 46]. These actin binding proteins appear to regulate Arp2/3 branching at the leading edge [47]. In addition the downstream effectors such as PKC and calcium (activated secondarily to the release of DAG and IP3, respectively), have a multitude of effects on adhesion turnover and acto-myosin contractility, both of which contribute to motility. The result of this decade of investigation is a relatively linear pathway from receptors to actin cytoskeletal alterations at the leading edge to enable protrusion.

What is attractive about this model is the central role played by PLCγ thus supporting it's potential role as a therapeutic target to prevent invasion in a wide spectrum of tumors. PLCγ is required for motility signaling from PDGFR [14], EGFR [8], IGF-1R [15, 48], HGFR/c-met [16], and HER2/erbB2 [33] (although seemingly not FGFRs [17]). These receptors have been implicated in promoting tumor progression in a wide variety of carcinomas and even mesenchymal tumors; often more than one receptor has been shown to be upregulated in invasive/metastatic subsets of the same tumor type. Thus, a point of convergence would be the prefered target. Two tumors are particularly illustrative. In breast cancers, tumors with poor prognosis have been shown to upregulate expression of HER2 in a majority but not all of the cases [49]. However, some of the aggressive tumors display upregulation of EGFR [26], IGF-1R [50] or other growth factor receptors [51]. Despite this, PLCγ inhibition blocks invasiveness of breast carcinoma lines with upregulation of either HER2 or EGFR [33].

In glioblastoma multiformes, EGFR upregulation has classically been related to CNS dissemination [23], though others have implicated PDGFR, NGF or IGF-1 [31, 52]. Again, regardless of whether driven by PDGF, EGF or IGF-1, disruption of PLCγ signaling prevents invasion of glioblastoma cells into normal brain tissue [31]. This suggests that even if there are multiple receptors upregulated in a tumor, targeting a downstream point of convergence could be beneficial. Furthermore, clinical trials of compounds such as tyrosine kinase inhibitors that target a growth factor receptor alone (e.g., ZD1839/Iressa), have shown limited anti-tumor efficacy when administered as monotherapy. Although combining the receptor targeting agent with cytotoxic chemotherapy or radiation therapy appears to be more efficacious, these treatments remain relatively toxic. A potentially attractive strategy may include dual molecular therapy with one compound targeting the upstream growth factor receptor and the second a downstream signaling molecule that regulates tumor cell invasion such as PLCγ.

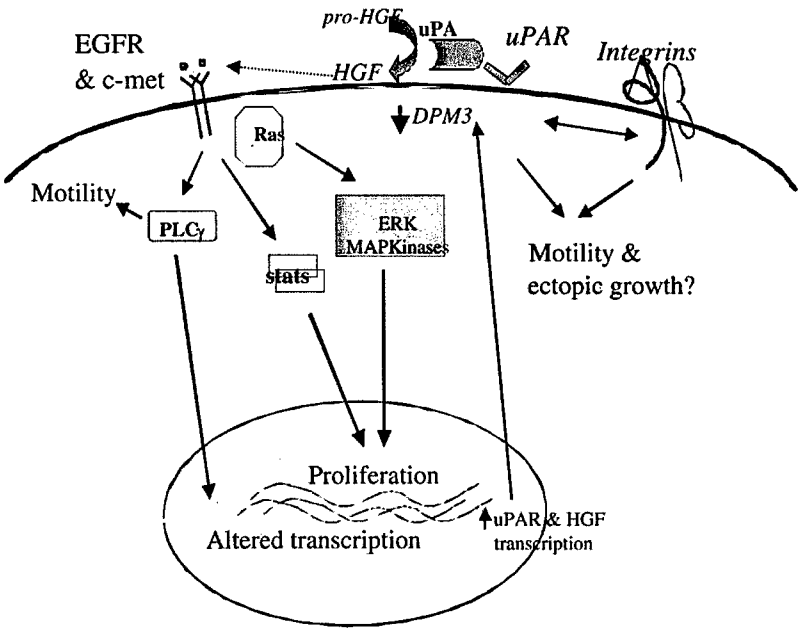
PLCγ in gene expression changes during progression

A new area for exploration is altered gene expression in response to PLCγ signaling. This has come to the fore with the availability of broad profiling of gene expression. We have catalogued gene expression changes over ~5000 gene sequences for DU145 human prostate cancer lines that are isogenic for EGFR variants and vary in their invasiveness [53]. One major signaling effect of the EGFR mutations is to provide for graded PLCγ signaling. We found some 11 genes to change in relationship to PLCγ signaling; seven were increased in parallel with PLCγ signaling, four were decreased (Table 1). For some of the genes, we validated that the changes in expression were due to PLCγ signaling by modulating expression levels by direct inhibition of PLCγ. Interestingly, many of these genes had already been implicated in prostate cancer progression by independent analyses. For instance, increased density of the urokinase receptor (uPAR) [54] and plasma levels of IGF-1 [50, 55]



EGFR	: Epidermal growth factor receptor	IP3	: Inositol trisphosphate
PLCγ	: Phospholipase C-γ	DAG	: Diacyl glycerol
PIP2	: Phosphoinositide (4,5) bisphosphate	PKC	: Protein kinase C

Figure 2. Phospholipase C-γ activation leads to actin cytoskeleton alteration. A simplified schematic of the initial epigenetic events from growth factor activation of PLCγ to alterations in the cytoskeleton that enables lamellipodial protrusion. Not shown are other enzymes that regulate PIP2 levels, such as phospho-inositide specific kinases and phosphatases; also not shown are the putative roles of PIP2 in generating and stabilizing adhesions.



EGFR	: Epidermal growth factor receptor	uPA/R/	: Urokinase-type plasminogen activator/receptor
PLCγ	: Phospholipase C-γ	DPM3	: Dolichol-phosphate-mannose synthase subunit 3/Prostin-1
HGF	: Hepatocyte growth factor/c-met ligand		

Figure 3. Schematic of reinforcing signals from PLCγ-induced genes. This protrays one hypothesized gene expression cycle that reinforces cell motility upon PLCγ activation. The expressed genes, uPAR and HGF, interact to increase signaling through the c-met receptor with intrinsic tyrosine kinase activity, which in turn further activates PLCγ. This cycle is proposed based on the gene array profiles in [53].

Table 1. PLCγ-regulated genes in DU145 human prostate carcinoma sublines.

Invasion promoter	Invasion suppressor
Urokinase receptor (uPAR)	DPM3/prostinv-1
Insulin-like growth factor I (IGF-1)	70kD heat shock protein
Hepatocyte growth factor (HGF)	MHC class I
Platelet-derived growth factor-A (PDGF-A)	EST AA548375
Cyclin I	
EST AA207900	
EST 548375	

Adapted from [53].

has been proposed as markers of prostate cancer invasiveness, and stromally-derived HGF is implicated in invasion in experimental prostate cancer systems [56]. Since IGF-1, PDGF and HGF further activate PLCγ, there is evidence that PLCγ-induced gene expression reinforces the aggressive phenotype (Figure 3).

One gene product that was suppressed by PLCγ signaling provides an interesting conundrum. The regulatory subunit of dolichol-phosphate-mannose synthase complex (DPM3) [57] was found to be downregulated by PLCγ signaling [53]. This machinery is thought to generate the GPI anchors for a variety of cell surface proteins including uPAR [57]. Thus, the downregulation of this gene would seem contrary to upregulation of uPAR. However, when DPM3 was overexpressed in COS-1 cells, apoptosis ensued [53], suggesting either a separate functional role for DPM3 or that increased GPI-anchored proteins may lead to anoikis [58]. Rather, high levels of secreted uPAR may activate HGF over a wide area and thus promote tumor cell dissemination even in cells lacking the initiating upregulation of growth factor receptor/PLCγ signaling.

Future developments

Our present understanding of PLCγ functioning in tumor biology remains incomplete. While the cytoskeletal reorganization is likely to be increasingly detailed in the near future, the gene expression changes downstream of PLCγ are only beginning to be characterized. The secondary effectors from PLCγ that alter gene expression have not been identified – are they the classically described calcium flux and DAG acting via protein kinase C (PKC)? An intriguing report describes the SH3 domain of PLCγ as a guanine nucleotide exchange factor (GEF) for a nuclear phosphatidylinositol kinase, PIKE; with this GEF activity, but not lipase activity, required for PLCγ-mediated mitogenesis [59]. Or is that a non-enzymatic, scaffolding function [60] of PLCγ contribute to such signaling? Such findings need to be deciphered at a finer level and integrated with the other data concerning PLCγ signaling.

Applying the new proteomic tools under development to PLCγ signaling should also yield insightful surprises. This is critical as much of PLCγ regualtion is epigenetic and dependent on specific subcellular localization. Only by placing PLCγ activities in the right time and place in a tumor cell will we be able to determine its suitability as a rationale target to limit tumor progression.

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Luteinizing Hormone Releasing Hormone (LHRH) Analog Reverses the Cell Adhesion Profile of DU-145 Human Prostate Carcinoma

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Abstract

Cetrorelix, a luteinizing hormone releasing hormone (LHRH) analog, has been shown to limit growth of the human androgen-independent prostate cell line DU-145, though other inhibitory actions may also be effected. Both growth and invasion of DU-145 cells are linked to autocrine epidermal growth factor receptor (EGFR) signaling. Invasiveness requires not only cells to migrate to conduits, but also reduced adhesiveness between tumor cells to enable separation from the tumor mass. Thus, we investigated whether Cetrorelix alters the DU-145 cell-cell adhesion and if this occurs via altered EGFR signaling. Pharmacologic levels of Cetrorelix limited the invasiveness of a highly invasive DU-145 subline overexpressing full-length EGFR (DU-145 WT). Extended exposure of the cells to Cetrorelix resulted in increased levels of the cell-cell adhesion complex molecules E-cadherin, α- and β-catenin, and p120. Puromycin blocked the increases in E-cadherin and β–catenin levels, suggesting that *de novo* protein synthesis is required. The Cetrorelix effect appears to occur via transmodulation of EGFR by a protein kinase C (PKC)-dependent mechanism, as there were no changes in DU-145 cells expressing EGFR engineered to negate the PKC trans-attenuation site (DU-145 A654); an inhibitor of EGFR produced a similar upregulation in adhesion complex proteins further suggesting a role for autocrine signaling. Cetrorelix increased the cell-cell adhesiveness of DU-145 WT cells to an extent similar to that seen when autocrine EGFR signaling is blocked; as expected DU-145 A654 cell-cell adhesion also was unaffected by Cetrorelix. The increased adhesiveness is expected as the adhesion complex molecules moved to the cells' periphery. These data offer direct insight into the possible cross-talk pathways between the LHRH and EGFR receptor signaling. The ability of Cetrorelix to downregulate EGFR signaling and subsequently reverse the anti-adhesiveness found in metastatic prostate cancer highlights a novel potential target for therapeutic strategies.

Introduction

Adhesion between normal epithelial cells is usually strong and stable limiting cell movement. In carcinomas, these tight cell associations must first be disrupted or prevented from forming before tumor cells are able to disseminate and metastasize. Cell-cell association is often disorganized in tumors, and has been linked to tumor invasiveness and metastasis (Pignatelli and Vessey 1994; Shino *et al.* 1995; Richmond *et al.* 1997). Acquisition of invasive potential by malignant cancer cells results from an accumulation of characteristics, including increased cell motility, secretion of proteolytic enzymes, and alterations of cell-substrate and cell-cell adhesion (Fidler 2003; Grunert *et al.* 2003). The molecular mechanisms responsible for this latter process, altered cell-cell adhesion in invasive cancer cells are poorly understood (Comoglio and Trusolino 2002). However, the net result is a reduction in cadherin/catenin complexes at the cells' periphery (Morita *et al.* 1999; Davies *et al.* 2000; Chunthapong *et al.* 2004). Thus, to better understand the mechanisms of tumor cell dissociation, the role of cadherins must be taken into account, as they are crucial in cell-cell adhesion (Takeichi 1993; Kim *et al.* 1999; Suyama *et al.* 2002).

Cadherins comprise a family of transmembrane cell surface glycoproteins that mediate calcium (Ca^{++})-dependent, homotypic cell–cell interactions through their extracellular domains, and regulate a variety of biological processes during development, morphogenesis, and tumor metastasis (Gumbiner 1996; Yap *et al.* 1997; Conacci-Sorrell *et al.* 2002). Ca^{++}-dependent cell-cell adhesion usually consists of rapid localization of surface E-cadherin molecules to the regions of contact resulting in homotypic binding that fosters the maintenance of normal cellular structure. However, metastastic cancer cells are able to override or avoid contact inhibition signals employed by normal epithelial cells to control proliferation and cell movement.

The linkage between E-cadherin and the cellular cytoskeleton is a complex interaction involving a number of structural and signaling cytoplasmic proteins such as α- and β-catenin and p120 (Van Aken *et al.* 2001; Mason *et al.* 2002). Early studies identified E-cadherin/catenin interactions as imperative for cell-cell adhesion (Chitaev and Troyanovsky 1998). β-catenin binds with high affinity to the carboxyl-terminal region of the cadherin cytoplasmic tail while α-catenin serves as an anchor, by bridging to α-actinin, to link the complex to the actin cytoskeleton (Aberle *et al.* 1994; Hulsken *et al.* 1994; Funayama *et al.* 1995; Jou *et al.* 1995; Rimm *et al.* 1995). These molecules not only play structural roles but also alter cell responses and phenotypes. β-Catenin is also found to immunoprecipate with the APC tumor suppressor protein (Su *et al.* 1993; Hulsken *et al.* 1994; Shibata *et al.* 1994), and has been recently identified as an oncogene (Kim *et al.* 2002; Minamoto *et al.* 2002; Kielhorn *et al.* 2003; Schneider *et al.* 2003). It is also central to cell signaling, as upon dissociation from E-cadherin, it transits to the nucleus to alter transcriptional profiles (Mason *et al.* 2002; van de Wetering *et al.* 2002) A reduction in β-catenin expression decreases the stability of the adhesion complex and likely results in impairment in E-cadherin function (Willert and Nusse 1998; Lowy *et al.* 2002; Mason *et al.* 2002). Similarly, a reduction in E-cadherin often results in β-catenin degradation (Liu *et al.* 2002). Another protein associated with E-cadherin, p120 (Thoreson *et al.* 2000), is phosphorylated on both tyrosine and serine residues in response to a variety of growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), and colony stimulating factor (CSF)-1, suggesting involvement in active signaling (Downing and Reynolds 1991; Shibamoto *et al.* 1995). Thus, cell-cell adhesion serves not only a structural role but dictates cellular behavior.

As carcinomas progress to the invasive and metastatic stages, select adhesive epithelial cells usually undergo a mesenchymal-like transition that enables their movement from the primary tumor mass (Comoglio and Boccaccio 2001; Conacci-Sorrell *et al.* 2002). During this process in breast, gastric, and pancreatic metastatic carcinomas, E-cadherin expression is frequently downregulated or even undetectable (Birchmeier and Behrens 1994; Lowy *et al.* 2002). This pattern of E-cadherin expression also persists in disseminated prostate carcinomas when compared to non-metastatic prostate cells (Umbas *et al.* 1992; Davies *et al.* 2000; Mason *et al.* 2002). In addition, the loss of E-cadherin expression has been shown as a consequence of autocrine activation of epidermal growth factor receptor (EGFR) signaling (Jawhari *et al.* 1999). This combination of autocrine EGFR signaling and loss of E-cadherin expression leads to cell proliferation, dedifferentiation, and induction of cell motility (Hazan and Norton 1998). Such an association has been suggested in the progression of breast carcinoma cells to a more invasive phenotype, which correlates with downregulation of E-cadherin and overexpression of EGFR (Sorscher *et al.* 1995; Sorscher *et al.* 1995; Hazan and Norton 1998). On a molecular level, EGFR signaling leads to tyrosine phosphorylation of the catenin complex with subsequent breakdown of cell adhesion (El-Bahrawy and Pignatelli 1998; Thoreson *et al.* 2000).

In this study, we examined whether the beneficial anti-cancer effects of Cetrorelix include effects in additon to the established anti-proliferative effects. LHRH receptors are expressed in many cancers (Emons *et al.* 1998; Schally *et al.* 2001; Straub *et al.* 2001) enabling LHRH analogs to directly affect prostate tumor cells (Qayum *et al.* 1990; Halmos *et al.* 2000) in addition to the indirect central androgen suppression. In addition, it has been shown that LHRH agonists directly inhibit cell proliferation of DU-145 and LNCaP prostate cancer cell lines (Dondi *et al.* 1994; Dondi *et al.* 1998; Limonta *et al.* 2001). In line with these observations, the LHRH analog Cetrorelix has been shown to have direct antiproliferative actions on DU-145 cells (Jungwirth *et al.* 1997). As a consequence of this exposure, LHRH analogs have caused decreased levels of EGFR and therefore a decrease in its tyrosine kinase activity (Moretti *et al.* 1996; El-Bahrawy and Pignatelli 1998; Lamharzi *et al.* 1998). Previously we have shown the human prostate carcinoma cell line, DU-145, presents autocrine EGFR signaling that is critical to both cell proliferation and invasion (Xie *et al.* 1995; Turner *et al.* 1996). Recently we demonstrated under both *in vivo* and *in vitro* conditions that a LHRH agonist inhibited EGFR-dependent proliferation through interference with EGFR signaling (Wells *et al.* 2002). Therefore, these data taken together lead us to hypothesize that the LHRH analog, Cetrorelix, would abrogate EGFR signaling. This abrogation would in turn decrease phosphorylation of the associated catenins; thus leading to upregulation of the cell adhesion molecule E-cadherin, which may ultimately result in inhibition of prostatic tumor progression.

MATERIALS AND METHODS

Materials

LHRH analog Cetrorelix ([Ac-D-Nal (2)[1], D-Phe (4Cl)[2], D-Pal (3)[3], D-Cit[6], D-Ala[10]] LH-RH) was obtained from ASTA Medica (Frankfurt/Main, Germany). The primary antibodies used were mouse monoclonal antibodies to E-cadherin, α- and β–catenin, and p120 (Transduction Laboratories, California), phospho- MARCKS (Cell Signaling, Massachusetts), and EGFR (Zymed Laboratories, California). Inhibitors included the EGFR specific tyrosine kinase inhibitor PD153035 (CalBiochem, California) and the transcriptional and translational inhibitor Puromycin (Sigma, Missouri). Other reagents were obtained from Sigma.

DU-145 Cell Lines

The cell line DU-145 was originally derived from a brain metastasis of a human prostate adenocarcinoma (Stone *et al.* 1978); it retains the androgen independence of the original tumor and does not express a functional androgen receptor (Dondi *et al.* 1998). This cell line possesses both LHRH and EGF receptors and produces EGFR ligands, TGF-α and EGF (Xie *et al.* 1995; Jungwirth *et al.* 1997). We have expressed exogenously-encoded EGFR in DU-145 cells (Xie *et al.* 1995). Utilizing established protocols, DU-145 cells were transfected by retroviral-containing EGFR constructs (Wells *et al.* 1990). The Wild Type (WT) EGFR construct is a full-length cDNA derived from a placental cDNA library. Cells expressing WT EGFR at levels which escape down-regulation, demonstrate enhanced invasiveness *in vitro* (Xie *et al.* 1995) and *in vivo* (Turner *et al.* 1996).

The DU-145 WT subline express EGFR that are phosphorylated and negatively modulated by PKC; thus, we have generated an additional DU-145 subline which is not negatively modulated by PKC (Wells *et al.* 2002). This subline is identical to DU-145 WT except it contains a full length EGFR in which the target site for PKC phosphorylation, amino acid threonine 654 (T654), has been replaced with alanine (DU-145 A654) by site directed mutagenesis; this construct is resistance to PKC phosphorylation and negative transmodulation (Welsh *et al.* 1991; Chen *et al.* 1996).

The DU-145 WT and A654 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (4.5g/ml glucose) (Cellgro, Virginia) containing 10 % FBS and supplemented with L-glutamine (2 mM), penicillin/streptomycin (100 units/ml), nonessential amino acids (0.1 mM), and sodium pyruvate (1 mM) (37°C, 90% humidity, 5% CO_2 and 95 % air). For stable selection of WT or A654 EGFR, cells were grown in G418 (1000ug/ml) (Gibco, New York), though all experiments were performed in the absence of G418.

Invasion Assay

Cell invasiveness *in vitro* was determined by the ability of cells to transmigrate a layer of extracellular matrix, Matrigel, in a Boyden Chamber assay. Matrigel invasion chamber plates were obtained from Becton Dickinson Labware (Bedford, Massachusetts). Cells were plated randomly and distributed among the plates. Cells were kept in Cetrorelix containing serum-free media containing 1% BSA for the first 24 hours and then replaced with Cetrorelix serum-free media for the remaining 24 hours. Enumeration of the cells that invaded through the matrix over a 48 hour-period was accomplished by visually counting cells on the bottom of the filter. All experiments were performed in triplicate chambers.

Flow Cytometry

Cells were grown to 80% confluency. LHRH analog Cetrorelix (10^{-5}M) was added for time intervals of 6, 12, and 24 hours. Samples were washed with PBS and fixed with paraformaldhyde, and permeabilized with 1% Triton X 100. Samples were blocked with 5% BSA and incubated with the appropriate FITC conjugated primary antibody or primary antibody (anti-EGFR, anti-E-cadherin, anti-α–catenin, anti-β–catenin, and anti-pp120) at 37°C for 1 hour. For unconjugated samples FITC-conjugated secondary antibody was added. Fluorescence was measured by a flow cytometer (Coulter, Florida).

Immunoblotting

Cells were grown on a six-wells plate for 2 days to 80% confluency. LHRH analog Cetrorelix (10^{-5}M) was added at time intervals of 6, 12, and 24 hours. Protein lysates were prepared from cultured cells in the following buffer: 50 mM Tris, pH 7.5, 120 mM NaCl, 0.5% Nonidet p-40, 40 μM phenylmethylsulfonylfluoride (PMSF), 50 μg/ml leupeptin, and 50 μg/ml aprotinin (all from Sigma). Cells were allowed to lyse 1 h on ice, centrifuged, supernatants extracted, and quantitated using a Bradford assay. 30 μg of protein lysates were separated by 7.5% SDS PAGE, immunoblotted and analyzed using chemiluminescence (Amersham Biosciences). Primary antibodies used included anti-EGFR (Zymed Diagnostics, South San Francisco, California), anti-E-cadherin, anti-β-catenin, and anti-p120 (Transduction Laboratories, Lexington, Kentucky), and anti-α-catenin (Santa Cruz Biotechnology, Santa Cruz, California). The staining was visualized by a secondary anti-mouse IgG or anti-rabbit antibody linked to horseradish peroxidase (Promega, Madison, WI).

Immunofluorescence Microscopy

Cells were grown on glass coverslips and then treated with Cetrorelix. Cells were then fixed in 4% paraformaldehyde, permeabilized with (100 mM Tris-HCl pH 7.4, 150 mM NaCl, 10 mM EGTA, 1% Triton X-100, 1 mM PMSF, and 50 μg/ml aprotinin (all from Sigma)), and subsequently blocked with 5% BSA for 1 h at room temperature. Samples were incubated with indicated primary antibodies diluted in blocking buffer at 4°C overnight. Then FITC-conjugated secondary antibody was added. Cells were then stained with propidium iodine for nuclear staining. Cells were then analyzed with laser confocal microscopy using a Leica TCSNT 3 laser 4 PMT system.

Cell Aggregation Assay

Calcium-dependent aggregation of the DU-145 sublines was measured as described by (Takeichi 1995) with the following modifications. Cell monolayers grown to 80% confluence were incubated for 24 or 48 hours in 10 % FBS in DMEM with or without 10^{-5} M Cetrorelix. Cell monolayers were detached from the culture dishes by incubating in cell stripper (Cell Gro, Virginia), as previously described, for 5-10 minutes at 37°C. Any remaining cells were detached using a rubber policeman, washed once with PBS and collected by centrifugation. Cells were resuspended in 10% FBS in DMEM and single cell suspensions made by trituration with a Pasteur pipette. Cell number was determined in the Coulter Counter Z1 (Coulter, Florida). Cells were plated in triplicate wells of a 24-well plate at 2 x 10^5 cells/well in 10% FBS in DMEM with 1 mM $CaCl_2$ and allowed to aggregate for 60 minutes on a gyratory shaker at 80 rpm at 37°C. Assays were stopped at 0 and 60 minutes by fixing the cells in 0.5% paraformaldehyde. The extent of cell-cell binding was monitored by measuring the disappearance of single cells

using the Coulter Counter. The index of the degree of aggregation was measured utilizing the formula 100 x (N_0/N_{60}), where N_0 is the total cell number per well and N_{60} is the total number of particles after 60 minutes of incubation as determined by counting in a Coulter Counter Z1.

Statistical Analysis

Statistics for all experiments were performed using the Sigma Plot statistical program (Jandel Scientific). Independent Student's T-test was utilized to determine a statistical difference between experimental and the controls for individual experiments.

RESULTS

LHRH Analog Cetrorelix Decreases Invasion in DU-145 Sublines

To confirm and extend the inhibitory effects of Cetrorelix on prostate carcinomas we utilized a genetically engineered human androgen-independent prostate carcinoma cell line that over expresses a full length EGFR, DU-145 WT. This subline is highly invasive in response to upregulation of autocrine EGFR signaling (Xie *et al.* 1995; Turner *et al.* 1996) that exists in practically all prostate carcinomas (Kim *et al.* 1999). In determining the utilized dose of Cetrorelix, we selected the pharmacologic dose of 10^{-5}M based on literature reports (Tang *et al.* 2002); this is equivalent to the dose of a related LHRH analog goserelin (Dondi *et al.* 1994; Jungwirth *et al.* 1997A; Jungwirth *et al.* 1997B; Limonta *et al.* 1998; Wells *et al.* 2002), and one that inhibits DU-145 WT proliferation but does not cause cell death (data not shown). To probe the extent of effectiveness of Cetrorelix against prostate cancer, we determined whether invasion was abrogated. Cetrorelix exposure limited the invasiveness of DU-145 WT through a Matrigel barrier down to 23 ± 14 % ($n = 4$, $P < 0.05$). This level of inhibition is comparable to the decreases noted when EGFR motility signaling via PLCγ or calpain signaling is abrogated (Xie *et al.* 1995; Turner *et al.* 1996; Kassis *et al.* 1999; Mamoune *et al.* 2003).

Cetrorelix Increases Levels of Cell Adhesion Molecules

Invasive phenotypes of many cancers including prostate have extensively been shown to have an increase in EGFR expression and a decreased expression of E-cadherin, α– and β-catenin and p120 catenins as well. These changes are presumed to enable the cells to escape from contact inhibition and/or to detach from the primary tumor mass for dissemination. Therefore we tested whether Cetrorelix would abrogate these phenotypic changes. After six hours of Cetrorelix exposure, EGFR levels measured by flow cytometry were significantly reduced in DU-145 WT cells when compared to non-treated, control levels ($P < 0.05$). This significant reduction in EGFR levels continued throughout the 24 hour experimental time-period ($P < 0.05$) (Figure 1a). As Cetrorelix decreased EGFR surface expression, it induced an increase E-cadherin and β-catenin levels. (Figure 1b and c). Likewise, the E-cadherin associated molecules α– catenin and p120 also showed a continual increase, and all were significant at 24 hours (Figure 1c,d,e). To confirm the flow cytometry experiments, we immunoblotted for whole cell protein content of EGFR and adhesion molecules E-cadherin and _-catenin. A similar pattern was seen with a reduction in EGFR levels and an increase in E-cadherin and _-catenin (data not shown).

To more deeply examine if the increase in E-cadherin and _-catenin protein and expression levels is associated with upregulation in transcription, we used the protein synthesis inhibitor puromycin. Puromycin was able to completely block the enhanced ability of Cetrorelix to restore the E-cadherin and _-catenin expression levels (Figure 2b). This suggests that *de novo* translation is needed for these increases in these cell adhesion molecules by Cetrorelix. However, whether this is compensatory for increased degradation or suppression of transcription awaits further studies.

Reversal in Adhesion Molecule Profile is Related to EGFR Signaling

Growth and invasion of the DU-145 human prostate cell line requires EGFR signaling (Xie *et al.* 1995; Turner *et al.* 1996). Since other LHRH analogs seem to exert their effects through negative attenuation of the EGFR (Wells *et al.* 2002), we probed

further into the mechanism by which LHRH analogs effect EGFR. Cetrorelix stimulates PKC activity as determined by phosphorylation of the MARCKS substrate for classical and novel PKC isoforms (Figure 3) (Fujise *et al.* 1994; Nishikawa *et al.* 1997). This has been confirmed through the use of chelerythrine, pan-PKC inhibitor (Wells *et al.* 2002). As such, we utilized cells expressing an EGFR construct in which the target PKC threonine 654 is replaced by an alanine (DU-145 A654). Since Cetrorelix is decreasing EGFR surface levels (Figure 1a) and increasing surface levels and protein levels of cell adhesion molecules (Figure 1 & 2), then cells expressing this EGFR A654 construct should be at least partly resistant to Cetrorelix. Through the use of immunoblotting we examined the protein levels of the cell adhesion molecules over 24 hours of Cetrorelix exposure. EGFR, E-cadherin, and _-catenin are not extensively changed in the DU-145 A654 cells compared to DU-145 WT cells (Figure 4). Additionally, blocking EGFR specific tyrosine kinase activity with PD153035, demonstrates increased expression of E-cadherin and _-catenin similar that elicited by Cetrorelix (Figure 5). These findings support a functional relationship between EGFR and E-cadherin.

The functional consequences of EGFR transmodulation extend to the invasiveness of the prostate carcinoma cells. While Cetrorelix significantly reduced the invasiveness of the DU-145 WT cells, invasiveness of the DU-145 A654 was limited to much lesser extent ($P < 0.05$ comparing Cetrorelix treatment of DU-145 A654 to WT cells)(Figure 6). These findings suggest that the effect of Cetrorelix on both cell-cell adhesion molecules and cell invasiveness is mediated through its interference with the EGFR signaling cascade.

Cetrorelix Exposure Increases Cell-Cell Aggregation

To assess the functional consequences of the concurrent Cetrorelix-related decrease in EGFR levels and the increase in E-cadherin and its associated proteins observed in the DU-145 WT subline, calcium-dependent aggregation assay was used after 48 hour of Cetrorelix exposure (Figure 7). The aggregation index of DU-145 WT and DU-145 A654 cells treated with Cetrorelix was compared to that of non-treated cells. Cells exposed to Cetrorelix formed a significant number of cell-cell aggregates compared to the non-treated and A654 cells (Figure 7, $P < 0.05$ comparing DU-145 WT to either group, while DU-145 A654 aggregation was indistinguishable from non-treated cells).

If Cetrorelix increases aggregation secondary to negative transmodulation of EGFR signaling, then disruption of this autocrine signaling pathway should also result in increased aggregation. Autocrine EGFR signaling in DU-145 WT cells can be disrupted at least partially by incubating the cells in the presence of specific EGFR kinase inhibtor PD153035. Treatment with PD153035 also showed a significant increase in cell-cell aggregation similar to what was observed with Cetrorelix (Figure 7b). This data suggest that the mechanism by which Cetrorelix induces cell-cell interaction is in part through the disruption of EGFR signaling and increasing E-cadherin and _-catenin (Figure 5).

Cell-cell aggregation requires E-cadherin to be present on the cell surface and its associate molecules at the inner face of the plasma membrane. In DU-145 WT cells, these adhesion complex molecules were distributed throughout the cytosol (Figure 8). Upon Cetrorelix treatment, not only did the levels increase, but the molecules were redistributed to the cells' periphery; this was particularly evident at sites of cell-cell contacts. In aggregate, these data further confirmed with functional application that the increases in E-cadherin, α– and β-catenin and p120 levels observed in Cetrorelix-exposed DU-145 WT cells are reversing the cells invasive phenotype to resemble a more

normal phenotype and that Cetrorelix exerts at least some of its effects via abrogation of autocrine EGFR cell signaling.

DISCUSSION

The LHRH analog Cetrorelix is undergoing evaluation for prostate cancer treatment. While initially considered for treatment due to its central androgen suppression mechanism, direct cancer cell efficacy has been shown. Cetrorelix has been demonstrated to limit proliferation of a variety of human cancer cell lines, including breast, ovarian, endometrial (Yap *et al.* 1997; Schally 1999) and prostate cancer cell lines (Qayum *et al.* 1990; Halmos *et al.* 2000). Herein, we examined whether Cetrorelix altered an important phenotype of tumor cells, decreased cell-cell adhesion. We found that Cetrorelix exposure increased the levels of cell adhesion molecules and enhanced the resultant cell-cell adhesion. Furthermore, Cetrorelix appears to function, at least in part, by cross attenuation of signaling from the EGFR.

Several studies have long established that the loss of the homotypic E-cadherin binding machinery correlates with an invasive phenotype in prostate carcinomas (Behrens *et al.* 1989; Vleminckx *et al.* 1991; Bussemakers *et al.* 1992). Thus, it is logical that this cell-cell zipper (Shibata *et al.* 1994) would disappear concomitant with increased cellular invasion. In fact, re-expression of E-cadherin has been shown to reduce the tumorigenicity of some carcinoma cell lines (Jawhari *et al.* 1999; Lowy *et al.* 2002). This disappearance of E-cadherin and/or any of the major adhesion components affiliated with it, is noted in most advanced carcinoma cells (Takeichi 1977; Hazan and Norton 1998; Takeda *et al.* 1999). Interestingly, Cetrorelix exposure increases the levels of all of the major adhesion molecules probed; this could be secondary to either increased transcription or decreased degradation. This should subsequently lead to the reforming of the zipper. This was corroborated in our invasion and aggregation studies where, after extended Cetrorelix exposure, the highly invasive WT cell line became less invasive and aggregated to a greater extent than non-treated cells.

The ability to exploit the finding that Cetrorelix increases cell-cell adhesion and the levels of the molecules involved is vastly improved by defining the underlying basis for this. Other LHRH analogs have been shown to limit prostate carcinoma cell growth secondary to down-regulation of EGFR (Moretti *et al.* 1996; Jungwirth *et al.* 1997; Jungwirth *et al.* 1997) or through interference with signaling pathways initiated by the EGFR (Wells *et al.* 2002). This occurred via PKC-mediated cross attenuation (Wells *et al.* 2002) secondary to phosphorylation on threonine 654 of EGFR (Lin *et al.* 1986; Welsh *et al.* 1991). In this study, we show direct activation of PKC substrates MARCKS by LHRH receptors in a time-dependent manner. Thus, cells expressing the PKC-resistant A654 EGFR should be impervious to Cetrorelix. This was borne out by finding that EGFR levels remained high and cell adhesion molecule levels low in these cells in the face of Cetrorelix exposure (Figure 2). EGFR's importance was further demonstrated in a time dependent manner with exposure to EGFR specific tyrosine kinase inhibitor, PD153035. Cetrorelix and PD153035 both increased cell-cell adhesion in DU-145 WT, but had little effect on DU-A654 cells (Figure 7). All of this taken together indicates that the ability of Cetrorelix to alter the adhesive profile of these cells is mediated through altered EGFR signaling.

That Cetrorelix restores cell-cell adhesion secondary to disrupting EGFR signaling would be supported if EGFR down-regulates cell-cell adhesion. That this

occurs at least in the DU-145 cells is shown by increased aggregation upon disruption of autocrine signaling (Figure 7). EGFR signaling, upregulated in an autocrine manner in prostate carcinomas (Kim *et al.* 1999), is responsible, at least in part, for the down-regulation of cadherin-mediated adhesion and levels of molecules noted in these tumors as it is in many other carcinomas (Sorscher *et al.* 1995; Sorscher *et al.* 1995; Wilding *et al.* 1996; Jawhari *et al.* 1999; Andl *et al.* 2003). There is a report of EGFR down-regulation decreasing E-cadherin and catenins in OVCAR ovarian carcinoma cells (Alper *et al.* 2000); the reason for this opposite effect in these cells is not obvious, but may be related to the distinct nature of some ovarian cell types. Presumably, such reduced adhesion molecule levels contribute to prostate cancer progression (Wells 2000). How EGFR signaling limits cadherin-mediated adhesions is still being deciphered (Ackland *et al.* 2003; Cozzolino *et al.* 2003). However, this appears to involve both acute phosphorylation and dissociation of components and subsequent degradation. Regardless of the actual mechanism, the result is long-term downregulation of these molecules.

In summary, we found Cetrorelix restored the adhesiveness of the human prostate carcinoma cells (and significantly inhibited cellular proliferation) at high pharmacologic doses, similar to others (Jungwirth *et al.* 1997; Tang *et al.* 2002). Additionally, the LHRH agonist Zoladex was shown to inhibit *in vitro* cell proliferation of androgen-dependent (LNCaP) and androgen-independent (DU-145) cell lines only at similarly high concentrations (Moretti *et al.* 1996; Wells *et al.* 2002). Thus, higher concentrations of LHRH analogs are needed to accomplish direct cell inhibition than to achieve androgen suppression. There are obvious speculative reasons for this, but regardless of the mechanism, these studies serve as proofs of concepts that this signaling axis can be exploited to limit prostate tumor progression. It remains to be determined whether therapeutic interventions will exploit this using higher affinity analogs or indirect augmentation of the described pathway that cross attenuates autocrine EGFR tumor promotion.

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Figure Legends

Figure 1. LHRH analog Cetrorelix (10^{-5}M) reverses cell profile of DU-145 WT cells in time-dependent manner. Expression levels were measured as the mean of percent positive fluorescence at time zero ± SEM at various time intervals. A, DU-145 WT cells labeled with FITC conjugated anti-E-cadherin were analyzed by flow cytometry. B, DU-145 WT cells labeled with FITC conjugated anti-alpha catenin were analyzed by flow cytometry. C, DU-145 WT cells labeled with FITC conjugated anti-beta-catenin were analyzed by flow cytometry. D, DU-145 WT cells labeled with FITC conjugated anti-p120 were analyzed by flow cytometry. E, DU-145 WT cells labeled with FITC conjugated anti-EGFR were analyzed by flow cytometry. Data are the mean ± SEM of 3 experiments each performed in triplicate. * indicates $P < 0.05$ compared to untreated.

Figure 2. LHRH analog Cetrorelix downregulates the EGFR in DU-145 WT cells and up-regulates the Cell adhesion molecules, however it was ineffective on the DU-145 A654 cells. DU-145 WT and A654 cells were expose Cetrorelix (10^{-5}M) for 24 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibodies to E-cadherin, α-catenin, β-catenin, p120 and EGFR respectively. B, DU-145 WT cells were exposed to Cetrorelix (10^{-5}M) for 6,12, and 24 hours. Samples were prepared as above and immunoblotted with antibodies to E-cadherin and β-catenin. Shown are representative examples of 3 experiments.

Figure 3. Adhesion molecules E-cadherin and β-catenin expression levels were decreased in the presence of transcriptional inhibitor puromycin. A, DU-145 WT cells were challenged with ± puromycin (40 μM) in the presence of Cetrorelix (10^{-5}M) for 24 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibody to E-cadherin. B, DU-145 WT cells were challenged as in A, except immunoblotted with antibody to β-catenin. Shown are representative blots of two experiments.

Figure 4. Phosphorylation level of PKC substrates was increased with LHRH analog Cetrorelix. A, DU-145 WT cells were exposed to Cetrorelix (10^{-5}M) from 30 min to 4 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibody recognizing phosphorylated MARCKS. B, DU-145 WT cells were challenged as in A, except immunoblotted with antibody recognizing phosphorylated serine in the context of canonical PKC target sites. Increases observed in figures A and B are comparable to PMA positive control. Shown are representative blots of three experiments.

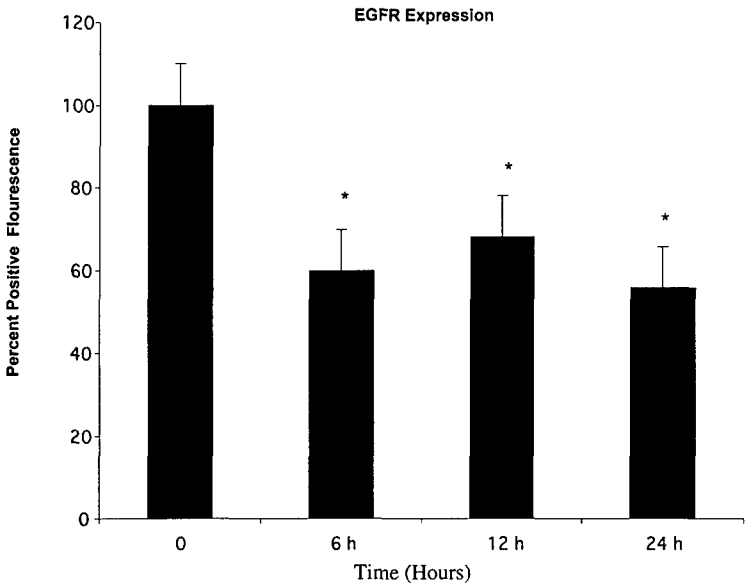
Figure 5. EGFR kinase inhibitor PD153035 (500nM) increases adhesiveness function of DU-145 WT cells. A, DU-145 WT cells were exposed to PD153035 for 1, 6, 12, and 24 hours. Lysates were collected and separated by SDS-PAGE, transferred, and immunoblotted with antibody recognizing E-cadherin. Shown is one of two experiments. B, EGFR inhibitor PD153035 increased the cell-cell aggregation of the DU-145 WT after 48 hours of exposure. Results are expressed as the mean of the index of the degree of aggregation versus time zero ± SEM ($n = 3$, each performed in triplicate) at one hour. *, $P < 0.05$, PD153035-treated (+) groups versus Controls (-), without drug.

Figure 6. Cetrorelix (10^{-5}M) limits invasiveness of DU-145 A654 to a lesser extent than DU-145 WT. Cetrorelix reduced the invasiveness of the DU-145 WT (■) cells while only partly affecting that of DU-145 A654 (▨) cells. Invasiveness was measured by the cells' ability to transmigrate the extracellular matrix, Matrigel, in a Boyden Chamber assay. Data is the mean ± SEM (n=4). *, $P < 0.05$, Cetrorelix-treated (+) groups versus Controls (-), without drug; also $P < 0.05$ between the extent of decreased invasiveness of WT and A654 cells in the presence of Cetrorelix.

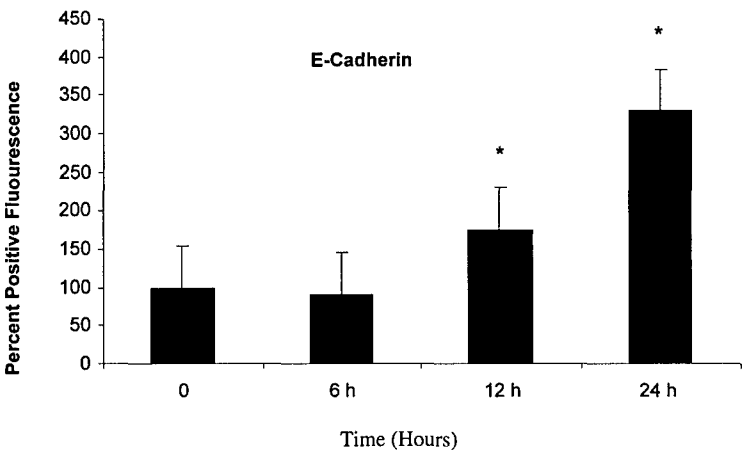
Figure 7. LHRH analog Cetrorelix (10^{-5}M) increases adhesive function of DU-145 WT cells. A, Cetrorelix increased the cell-cell aggregation of the DU-145 WT (■) cells after 48 hours of exposure, while not effecting DU-145 A654 (▨) cells. Results are expressed as the mean of the index of the degree of aggregation versus time zero ± SEM at one hour (n = 3, each in triplicate). *, $P < 0.05$, Cetrorelix-treated (+) groups versus Controls (-), without drug.

Figure 8. Cetrorelix (10^{-5}M) alters the distribution of adhesion molecules in DU-145 WT cells. Cells were exposed to Cetrorelix for up to 48 hours prior to immunofluorescent localization of E-cadherin (top panels), α- and β–catenins (second and third panels, respectively), or p120 (bottom panels). Shown are representative photomicrographs of two independent experiments.

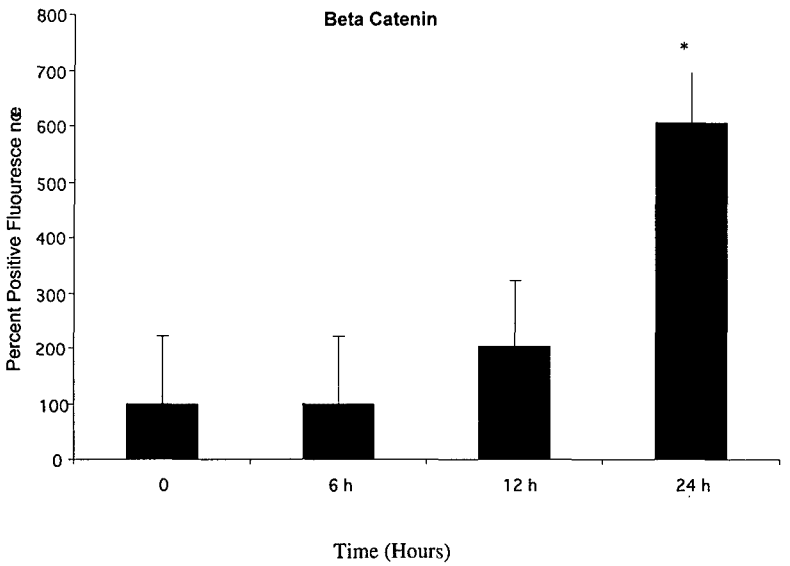
Figure 1
A.


EGFR Expression
Percent Positive Flourescence
120
100
80
60
40
20
0
*
*
*
0
6 h
12 h
24 h
Time (Hours)

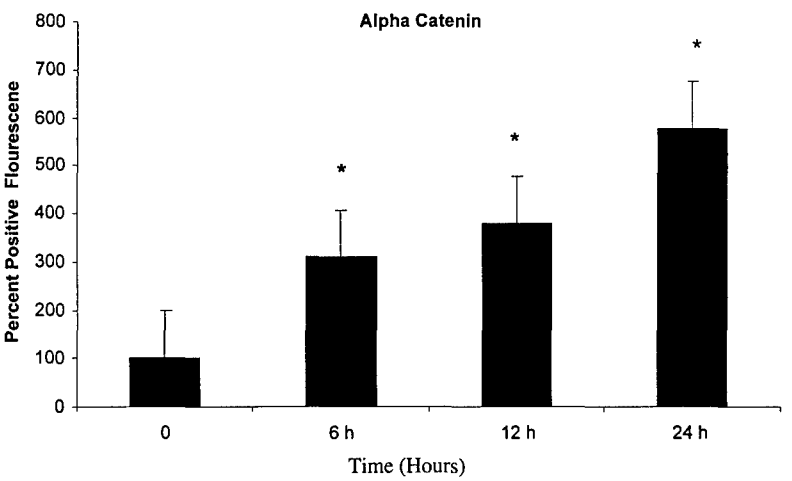
B.


E-Cadherin
Percent Positive Fluourescence
450
400
350
300
250
200
150
100
50
0
*
*
0
6 h
12 h
24 h
Time (Hours)

C.


Beta Catenin
Percent Positive Fluoresce nce
800
700
600
500
400
300
200
100
0
*
0
6 h
12 h
24 h
Time (Hours)

D.


Alpha Catenin
Percent Positive Flourescene
800
700
600
500
400
300
200
100
0
*
*
*
0
6 h
12 h
24 h
Time (Hours)

E.

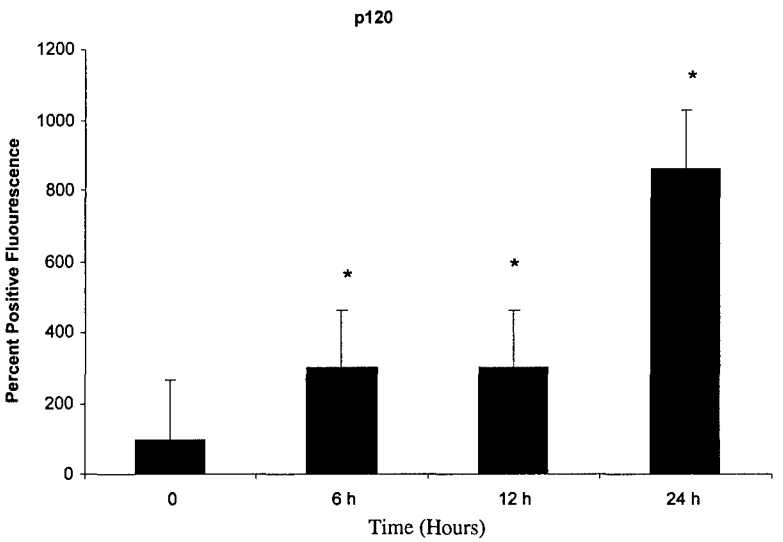

p120
Percent Positive Fluourescence
1200
1000
800
600
400
200
0
*
*
*
0
6 h
12 h
24 h
Time (Hours)

Figure 2

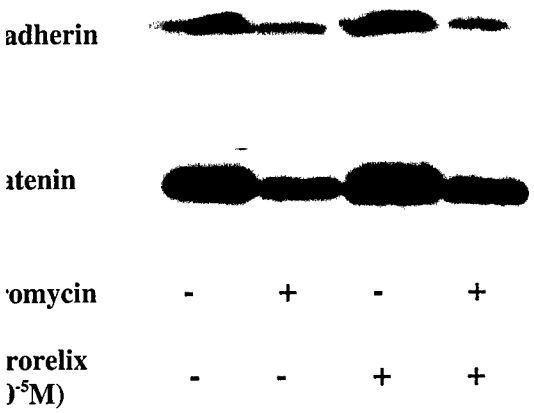


Figure 3

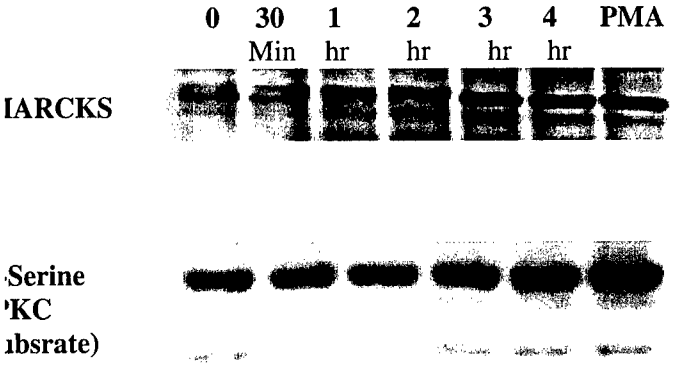


Figure 4

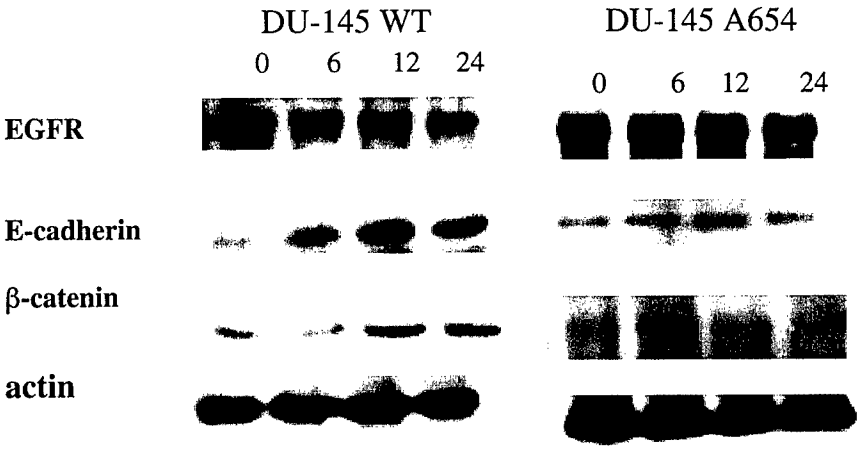


Figure 5

A.

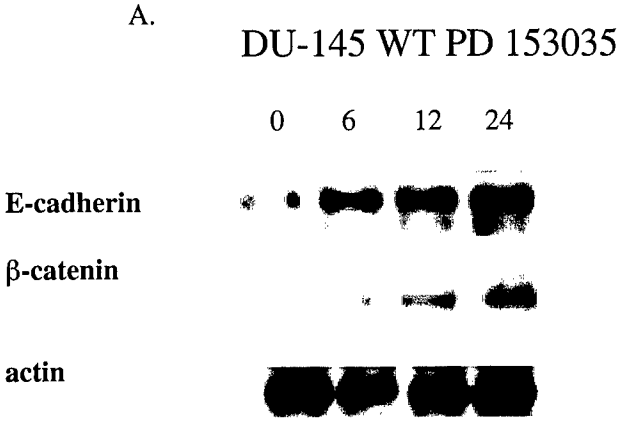


Figure 6

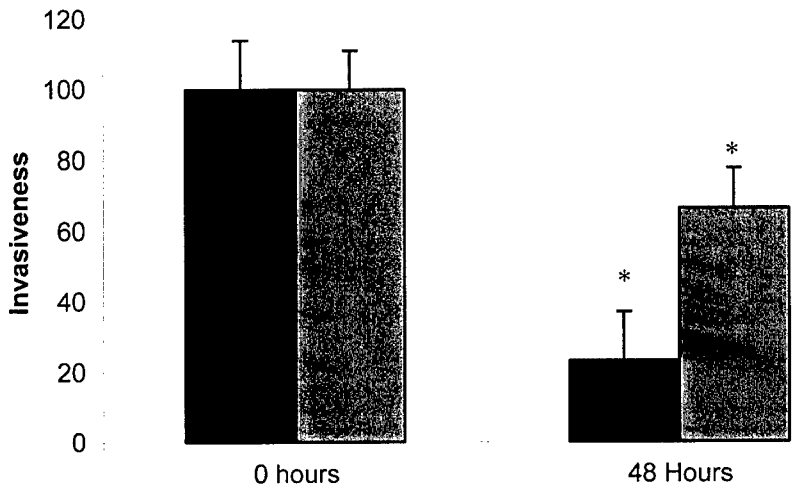
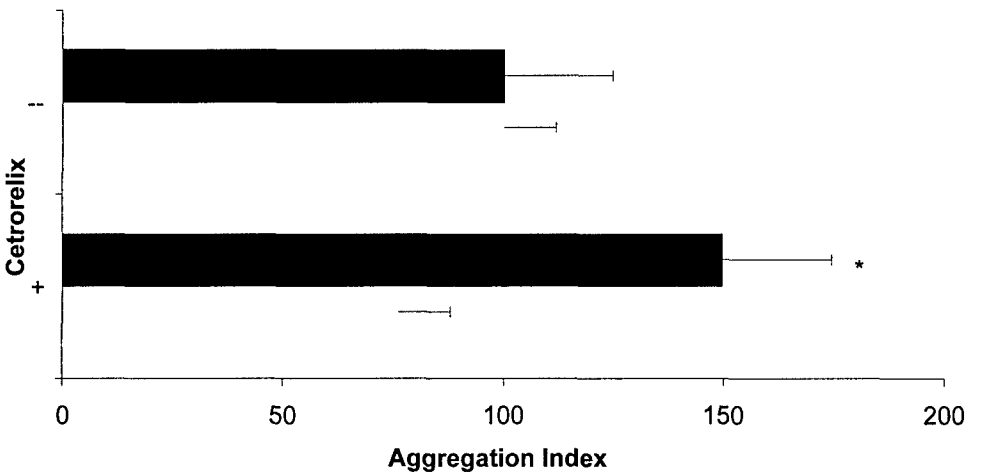


Figure 7

A.


Cetrorelix
-
+
*
0
50
100
150
200
Aggregation Index

B.

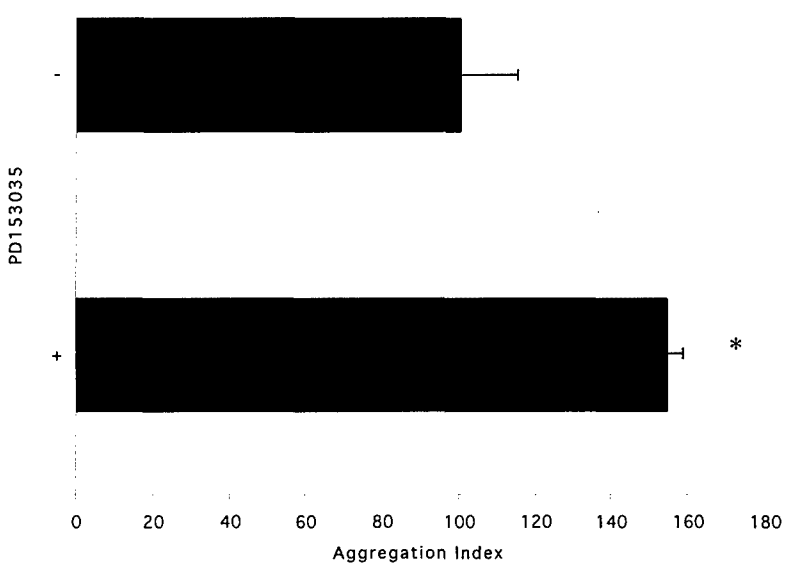
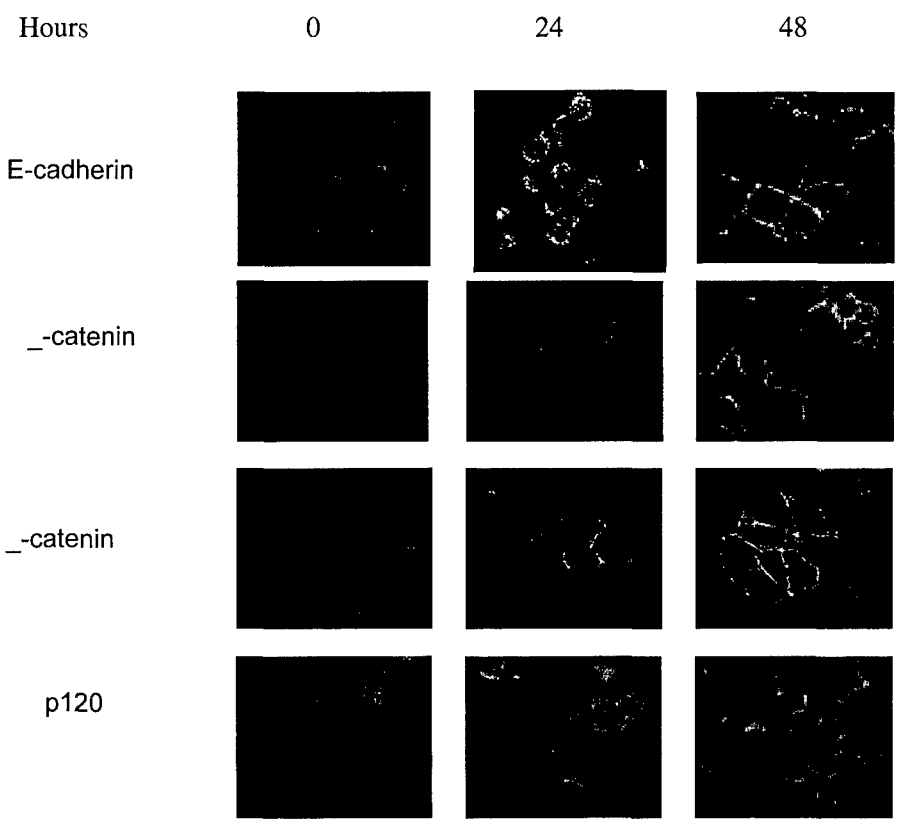

PD153035
-
+
*
0
20
40
60
80
100
120
140
160
180
Aggregation Index

Figure 8



4. American Association for Cancer Research (AACR) Annual Meeting, San Francisco, CA

Calpain inhibition reduces tumor prostate invasion

Asmaa Mamoune, Jareer Kassis, Doug Lauffenburger*, Alan Wells; Department of Pathology, University of Pittsburgh, Pittsburgh, PA 15261 and *Division of Bioengineering and Environmental Health, MIT, Cambridge, MA 02139

The mortality and morbidity of prostate cancer results from extracapsular invasion and metastasis. This tumor progression requires active cell motility. We have found that regulation of calpain activities by growth factor and adhesion receptors is central to cell migration. Calpain regulated rear detachment enabling forward locomotion.

We asked whether this would be a target for limiting tumor progression using moderately (Prntl) and highly (WT) invasive sublines of DU-145 human prostate carcinoma cells. In vitro, the calpain inhibitor CI-I decreased transmigration of a Matrigel barrier by >70% for both cell lines. This correlated with diminished EGF-induced de-adhesion. Intraperitoneal tumor xenografts were treated with the less-specific calpain inhibitor leupeptin as this has limited toxicity in mice. For both the Prntl and WT DU-145 cells, leupeptin treatment reduced tumor invasion into the diaphragm. These results strongly suggest that activation of calpain plays an important role in the invasion of human prostate cancer. Ongoing studies are specifically targeting the two ubiquitous calpain isoforms using molecular techniques to determine the key effector of tumor invasion.

These studies are supported by the DoD Prostate Cancer Program and NIH.

5. Selected Abstract-5th Joint Conference of the American Association for Cancer Research and the Japanese Cancer Association, Maui, HI

The Effects of the Luteinizing Hormone Releasing Hormone Antagonist, Cetrorelix on the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Clayton C. Yates[†], Karlyn J. Bailey[†], Alan Wells* and Timothy Turner[†]. [†]Tuskegee University, Tuskegee, AL, *University of Pittsburgh, Pittsburgh, PA

The therapeutic options for the treatment of androgen-independent prostatic cancers are limited. Among the effective treatments, Cetrorelix, a luteinizing hormone releasing hormone (LHRH) antagonist, has been shown to have antiproliferative actions on the human, androgen-independent prostate cell line, DU-145 (Jungwirth *et al.*, 1997). We have previously documented that DU-145 cell growth and invasion are mediated through epidermal growth factor receptor (EGFR) signaling (Turner *et al.,* 1996; Xie *et al.,* 1995). Consequentially, it has been suggested that the tyrosine kinase activity of the EGFR affects the function of the family of catenins that associate with and appear necessary for the cell adhesion molecule (CAM) E-cadherin's activity (Sorsher *et al.,* 1995). Furthermore, E-cadherin and its associated adhesion complexes have been shown to play an important role in establishing and maintaining intercellular connections associated with tumor progression to the invasive and metastatic stages (Morita *et al.,* 1999). However, studies examining the effect(s) of LHRH analogs on EGFR signaling and CAMs in prostate tumors have not been reported. Therefore, we initiated *in vitro* time-course studies examining the effects of various doses of Cetrorelix (10^{-4}-10^{-8} M) on cell proliferation of an extremely invasive DU-145 cell line, genetically engineered to overexpress the full-length EGFR. In these studies, the LHRH antagonist significantly inhibited DU-145 cell growth at 10^{-4} and 10^{-5} M concentrations ($p<0.05$). We next investigated the effects of Cetrorelix exposure at 10^{-5} M on the cell adhesion profile of the DU-145 WT cell line utilizing flow cytometry at the time intervals of 1, 6, 12, and 24 hours, respectively.) At the 24 hour time-period, our data showed Cetrorelix treatment significantly increased levels of CAMs, E-cadherin, pp120, α, β, and γ-catenins ($p<0.05$) while significantly decreasing EGFR levels ($p<0.05$). Therefore, these data suggest that in addition to Cetrorelix inhibition of prostate tumor proliferation, it also alters its EGFR and CAMs profiles in a manner that could lead to novel therapeutic approaches in the treatment of invasive prostate cancer.

6. Selected Abstract-Keystone Symposium, Tahoe City, CA

Cetrorelix, a Luteinizing Hormone Releasing Hormone Antagonist, Influences the Cell Adhesion Profile of an Invasive DU-145 Human Prostate Cell Line. Clayton C. Yates[†], Karlyn J. Bailey[†], Alan Wells* and Timothy Turner[†]. [†]Tuskegee University, Tuskegee, AL, *University of Pittsburgh, Pittsburgh, PA

The therapeutic options for the treatment of androgen-independent prostate cancers are limited. Among the effective treatments, Cetrorelix, a luteinizing hormone releasing hormone (LHRH) antagonist, has been shown to have antiproliferative actions on the human, androgen-independent prostate cell line, DU-145 (Jungwirth *et al.*, 1997). It has been documented that *in vitro* and *in vivo* DU-145 cell growth and invasion are mediated through epidermal growth factor receptor (EGFR) signaling (Turner *et al.*, 1996; Xie *et al.*, 1995). Furthermore, the cell adhesion molecule (CAM) E-cadherin and its associated adhesion complexes also play an important role in establishing and maintaining intercellular connections associated with invasion and metastasis (Morita *et al.*, 1999). Consequentially, it has been suggested that the tyrosine kinase activity of the EGFR affect the function of the catenins, which associate with E-cadherin, and appear necessary for cadherin activity (Sorsher *et al.*, 1995). However, studies examining the effect(s) of Cetrorelix on EGFR signaling and CAMs in human prostate cancer cell line DU-145 have not been reported. Cell growth time-course studies were initiated utilizing various doses of Cetrorelix (10^{-4}-10^{-8} M) on an extremely invasive DU-145 cell line, genetically engineered to over express the EGFR. The LHRH antagonist significantly inhibited cell growth of this DU-145 subline at 10^{-4} and 10^{-5} M concentrations ($p<0.05$). We next investigated the effects of Cetrorelix exposure at 10^{-5} M on the cell adhesion profile of the invasive DU-145 subline utilizing flow cytometry at the time intervals of 1, 6, 12, and 24 hours, respectively. Our data showed Cetrorelix significantly increased levels of CAMs, E-cadherin, pp120, and α-, β-, and γ-catenins ($p<0.05$) and decreased levels of EGFR ($p<0.05$) at the 24 hour period. In addition, cell aggregation studies indicated DU-145 cells exposed to Cetrorelix (10^{-5} M) for 48 hours aggregated to a significantly greater extent than non-treated ($p<0.05$). These data indicate that Cetrorelix not only inhibits cell growth but also alters the EGFR and cell adhesion profiles in the DU-145 human cell line. Taken together, our data reveal novel avenues for therapeutic approaches in the treatment of invasive prostate cancer.

7. 94th Annual Meeting of the American Association for Cancer Research, Washington, DC

An organotypic model for prostate tumor metastasis.

Clayton Yates, Donna Stolz, Linda Griffith*, Alan Wells, University of Pittsburgh and MIT*

Prostate cancer metastasis is among the leading deaths of American males in the United States. Development of cancer *in situ* has plausible and treatable means. However, the molecular mechanisms contributing to the initiation, progression and ultimate development of androgen independent carcinomas that have the ability to metastases to distal sites such as lung, diaphragm, bone marrow, brain and liver are currently poorly understood (Holleran, 2002). This lack of understanding is due in part to the lack of *ex vivo* metastasis and invasion experimental systems that fully recapitulate the pathophysiological events of this disease. Recently, we have developed a three dimensional liver perfusion culture, which allows for *in situ* observation and ensures a relatively homogeneous distribution of flow and mass transfer throughout the system to meet the metabolic demands of the livers cells augmented by an appropriate scaffold which facilitates morphogenesis of primary cells into tissue-like structures (Powers et al, 2002). This system, formally named a Micro-fabricated Array Bioreactor, affords for the recreation of an *in vivo* environment for *in vitro* observation and provides for an optimal device for the study of physiological events. In order to examine cancer metastasis and invasion we utilized this system. Hepatocytes were obtained from established liver perfusion protocols (Powers et, 2002, Block et al. 1996; Wu et al., 1996). Day 3, 200um spheroids were introduced into the reactor. After allowing 5 days of observed hepatic tissue morphogenesis from spheroids, an endogenously GFP expressing DU-145 WT human prostate cancer cell line genetically engineered to over-express a full length EGFR, which is very invasive, was introduced into the reactor by identical methods as spheroids. *In situ* observation of the co-culture system was observed by light microspopy over a 30 day period and subsequent two-photon scanning fluorescence microcopy and transmission electron microscopy. *In situ* observation of co-cultures revealed cell proliferation of DU-145 WT was observed after only 4 days, with an overgrowth of DU-WT cells from 14 days. The bioreactor medium did not support growth of DU-WT cells in the absence of hepatocytes. The overflowing DU-145 WT migrated across the silica and invaded adjacent hepatocyte filled channels. TEM experiments show that DU-145 WT cells invade the hepatocyte parenchyma within the 30 day incubation. Although only preliminary, these experiments provide the basis for the development of an *ex vivo* model system in which to observe and potentially dissect the dynamic process of tumor invasion and metastasis.

8. Abstract-Research Centers in Minority Institutions (RCMI) 2002 Spring Symposium, Jackson, MS

PROTEIN KINASE C SIGNALING IN THE HUMAN PROSTATE CANCER CELL LINE DU-145 AFTER EXPOSURE TO AN LHRH ANALOG.

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Analogs to luteinizing hormone releasing hormone (LHRH) have been shown to have antiproliferative actions on the human, androgen-independent prostate cell line, DU-145. Although the mechanism by which these analogs exert their antiproliferative effect is unknown, DU-145 cell growth and invasion are known to be mediated through the epidermal growth factor receptor (EGFR). We have hypothesized that the antiproliferative effects of LHRH analogs are mediated through negative attenuation of the EGFR via protein kinase C (PKC) activation. PKC activation in turn limits EGFR tyrosine kinase activity by phosphorylating the EGFR at amino acid threonine 654. The objective of this study was to determine whether Cetrorelix, an LHRH antagonist, attenuates EGFR signaling via PKC-mediated transmodulation. In this study, one subline over-expresses a full length EGFR (Wild Type-WT) the other subline over-expresses a full length EGFR in which amino acid threonine 654 was mutated to an alanine making it resistant to PKC phosphorylation (Wild Type A^{654} -WT A^{654}). Dose response studies conducted on the WT DU-145 subline with PKC activator PMA resulted in significant growth reduction at 10^{-5} M. No significant growth inhibition was witnessed in the WT A^{654} subline exposed to PMA. The PKC inhibitor chelerythrine chloride (10^{-6} M), in the presence of PMA (10^{-5} M) alleviated the inhibition caused by PMA. Treatment of WT A^{654} with Cetrorelix, resulted in a significant inhibition of growth in this subline at 10^{-4} M. Co-treatment of both DU-145 sublines with chelerythrine chloride failed to alleviate this inhibition of growth.

Key words: DU-145 cells, Cetrorelix, PKC, EGFR, Prostate Cancer

9. AACR symposium in Phoenix, AZ

Activation of Protein Kinase C/A Signaling in the Human Prostate Cancer Cell Line Du-145 After Exposure to a LHRH Analog, Cetrorelix.

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Luteinizing hormone releasing hormone (LHRH) analogs have been shown to have antiproliferative actions on DU-145 cell lines. We previously hypothesized the antiproliferative effects are mediated through negative attenuation of the epidermal growth factor receptor (EGFR) via protein kinase C (PKC) activation. Studies done to validate this pathway revealed an alternate route used by Cetrorelix to exert its effect. In addition to PKC, there is an indication of PKA pathway involvement. This study investigates the pathway(s) utilized by Cetrorelix to inhibit DU-145 proliferation. It employs one subline that over-expresses a full length EGFR (Wild Type-WT), and another subline identical to WT, except it is resistant to PKC phosphorylation (Wild Type A^{654}-WT A^{654}). Growth studies were conducted using Cetrorelix (10^{-4}M), PKA activator (Forskolin, 10^{-4}M) and inhibitor (H-89, 10^{-6}M), and a PKC activator (PMA, 10^{-5}M) and inhibitor (chelerythrine chloride, 10^{-6}M). Western blot analyses were done to determine the expression of PKC ε and PKA α after Cetrorelix exposure. PKA activation resulted in significant inhibition of cell proliferation in WT A^{654}, which was alleviated by the PKA inhibitor. PKC activation had no effect on the proliferation of WT A^{654}. In WT, PKC/PKA inhibitors significantly restored cell growth when co-exposed with Cetrorelix. While in WT A^{654}, significant restoration was only observed in PKA inhibitor co-treated cells. Western blot analysis revealed activation of both PKC ε and PKA α in Cetrorelix treated cells. Our data suggests that Cetrorelix may incorporate dual pathways to directly inhibit prostate cancer growth.

10. 95th Annual Meeting of the American Association for Cancer Research, Orlando, FL

An organotypic liver bioreactor model for prostate tumor metastasis

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Currently, most methods available to study the behavior of metastatic tumor cells are indirect or capture only brief periods. Problematic with these assays are an inability to follow the complete metastatic program including extravasation, invasion, establishment and growth of the metastatic mass. As such, we are developing an *ex vivo* model in which tumor cells can be analyzed while in the environment of a major metastatic target, the liver. In this study we assessed whether prostate cancer metastasis, which is among the leading causes of deaths in American males in the United States, could be recreated in a liver bioreactor and observed in real time. In order to accomplish this we utlizied a three dimensional liver perfusion culture (bioreactor), which allows for *in situ* observation and ensures a relatively homogeneous distribution of flow and mass transfer throughout the system to meet the metabolic demands of the livers cells augmented by an appropriate scaffold which facilitates morphogenesis of primary cells into tissue-like structures. This system, formally named a Micro-fabricated Array Bioreactor, affords for the recreation of an *in vivo* environment for *in vitro* observation and provides for an optimal device for the study of physiological events. Hepatocytes and nonparenchymal liver cells were obtained and introduced into the bioreactor utilizing established protocols (Powers et al, 2002, Block et al. 1996; Wu et al., 1996). After allowing 5 days of observed hepatic tissue morphogenesis, DU-145 human prostate carcinoma cells expressing GFP or RFP were introduced into the reactor. *In situ* observation of the co-culture system was observed by fluorescence microspopy over a 30 day period. The rate of growth of prostate cancer cells was assessed with a custom designed spectofluorometer to measure GFP fluorescent intensity throughout the initial growth of the cancer. Tissue architecture of hepatocytes and prostate cancer cells was directly visualized in real time by 2-photon microscropy along with light microscopy. Fluorescent intensity of co-cultures revealed ongoing cell proliferation of the DU-145 WT cells, which correlated with a visually observed overgrowth of DU-WT cells by 14 days. DU-145 WT cells failed to grow without hepatocytes or even in a 2-D mixed culture. This tumor mass growth resulted in a decline in hepatocyte tissue structure and function. Also metastasis of overflowing DU-145 WT to adjacent hepatocyte filled channels not containing DU-WT cells was observed during this process by 14 days. Histological examination of the tissue revealed DU-145 WT cells invading the hepatocyte parenchyma by 15 days. Finally, biochemical analysis revealed expression of biomarkers associated with prostate cancer and decrease in albumin and urea by the hepatocytes. Although only preliminary, these experiments provide the basis for the development of and ex vivo model system in which to observe and potentially dissect the dynamic process of tumor invasion and metastasis.