Prostate cancer-derived angiogenin stimulates the invasion of prostate fibroblasts

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Abstract

Prostate fibroblasts promote prostate cancer progression by secreting factors that enhance tumour growth and induce the migration and invasion of prostate cancer cells. Considering the role of fibroblasts in cancer progression, we hypothesized that prostate cancer cells recruit these cells to their vicinity, where they are most directly available to influence cancer cell behaviour. To test this hypothesis, we performed modified Boyden chamber assays assessing the migration and collagen I invasion of normal primary prostate fibroblasts (PrSCs) and prostate cancer-associated fibroblasts (PCAFs) in response to media conditioned by the metastatic prostate cancer cell lines PC-3, LNCaP and DU145. During 4-hr incubations, PrSCs and PCAFs migrated and invaded in response to the conditioned media. To identify candidate proteins in the conditioned media that produced these effects, we performed cytokine antibody arrays and detected angiogenin in all three media. Angiogenin-blocked PC-3-conditioned medium, obtained using an anti-angiogenin polyclonal antibody or angiogenin siRNA, significantly reduced PC-3-induced PrSC and PCAF collagen I invasion. Furthermore, angiogenin alone at 1, 2 and 5 ng/ml significantly stimulated PCAF collagen I invasion. These results suggest that PC-3-derived angiogenin stimulates the invasion of normal prostate fibroblasts and PCAFs and is sufficient for invasion of the latter. Because prostate fibroblasts play key roles in prostate cancer progression, targeting their invasion using an anti-angiogenin-based therapy may be a strategy for preventing or treating advanced prostate cancer.

Keywords: prostate • cancer • fibroblasts • invasion • angiogenin

Introduction

Metastatic prostate cancer is an incurable disease often treated initially using one of many hormone therapies. Patients inevitably develop resistance to these therapies [1], and only 33% survive more than 5 years after diagnosis of metastatic disease [2]. In efforts to develop more effective therapeutic approaches, researchers have historically studied the prostate cancer cells themselves. However, in recent years, they have begun to study the cancer cells’ interactions with their associated microenvironment, including stromal cells such as fibroblasts [3,4].

Prostate fibroblasts have been shown to promote prostate cancer progression in various ways: for example, by secreting factors that enhance tumour growth [5–9] and by inducing the migration [10] and invasion of prostate cancer cells through Matrigel [11] and collagen [12]. Fibroblasts reside in the collagen-rich connective tissue of the prostate [13,14], a compartment that prostate cancer cells enter after invading through the basement membrane en route to the tumour vasculature [15]. It remains unclear whether prostate fibroblasts follow the cancer cells through this compartment and towards the tumour vasculature. Considering that prostate fibroblasts are naturally motile cells that promote prostate cancer progression, we hypothesized that prostate cancer cells secrete factors that attract prostate fibroblasts towards them.

This study was conducted to test this hypothesis in vitro as well as to identify the specific factors involved. Using a modified Boyden chamber assay, we found that media conditioned by the prostate cancer cell lines PC-3, LNCaP and DU145 stimulated the invasion of prostate fibroblasts through collagen I. Considering that prostate cancer cells express angiogenin (EC 3.1.27) [16–18], a pro-angiogenic polypeptide that stimulates endothelial cell invasion [19], we...
examined the role of this polypeptide in prostate fibroblast invasion. The addition of anti-angiogenin antibody to medium conditioned by PC-3 cells significantly reduced PC-3-induced prostate fibroblast invasion through collagen I. Similarly, medium conditioned by angiogenin siRNA-treated PC-3 cells produced this same effect.

Materials and methods

Cell culture

Normal prostate stromal cells (PrSCs, Lonza, Walkersville, MD, USA) were cultured in Stromal Cell Growth Medium (Lonza). Prostate cancer-associated fibroblasts (PCAFs) were obtained from a Gleason 4+3 tumour and were a kind gift from Dr. John Isaacs (Johns Hopkins University School of Medicine, Baltimore, MD, USA). PCAFs, as well as the prostate cancer cell lines PC-3, LNCaP and DU145 (all from ATCC, Manassas, VA, USA), were cultured under standard conditions in RPMI 1640 containing l-glutamine (Invitrogen, Carlsbad, CA, USA), 10% FBS (Gemini Bio-Products, West Sacramento, CA, USA), and 1% penicillin-streptomycin (Mediatech, Manassas, VA, USA). Cells were passaged using trypsin/EDTA (Mediatech). Passage numbers 9–13 and 4–8 were used for PrSCs and PCAFs, respectively, in all experiments.

Western blotting

PrSC and PCAF whole-cell lysates were prepared using lysis buffer (20-mM Tris-HCl, 135-mM NaCl, 10% glycerol and 1% Triton X-100), according to a protocol from Dr. Beth Pflug’s lab at the University of Pittsburgh Medical Center. Proteins in the lysates were separated by SDS-PAGE and transferred to nitrocellulose membranes, which were then immunostained with monoclonal mouse anti-α-smooth muscle actin (SMA) antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA), monoclonal mouse anti-calponin antibody (Santa Cruz Biotechnology) and polyclonal rabbit anti-collagen I antibody (Rockland, Gilbertsville, PA, USA). The blots were stripped and re-probed with polyclonal rabbit anti-actin antibody (Santa Cruz Biotechnology) as a loading control.

Preparation of conditioned media (CM)

PC-3, LNCaP and DU145 cells were cultured to subconfluence and washed twice with PBS. Phenol red-free RPMI 1640 (15 ml/flask; Invitrogen) was added to each flask. After incubation for 48 hrs at 37°C in 5% CO₂, the medium were collected and centrifuged briefly to remove cell debris. The supernatants were aliquotted into fresh 15-ml conical tubes and stored at –80°C until used.

Cytokine antibody arrays on CM

CM from PC-3, LNCaP and DU145 cells were tested for the presence of various cytokines using RayBio® Cytokine Antibody Array 6 (RayBiotech, Norcross, GA, USA), following the protocol outlined in the kit insert, with the following changes/specifications: array membranes were incubated with the appropriate CM overnight at 4°C with gentle shaking. At the end of the procedure, they were exposed for a series of 10-sec intervals using a Molecular Imager ChemiDoc XRS System (Bio-Rad, Hercules, CA, USA).

Cytokine antibody arrays on prostatic fluids and sera

Prostatic fluids were collected from prostates that had been classified as having minimal or excessive cancer, based on tumour volume, as described previously [20]. As a control, serum samples from prostate cancer patients were collected. Before this study, approval was obtained from our Institutional Review Board, and all patients provided written informed consent. All samples were stored at –80°C until used.

The prostatic fluid and serum samples were tested for the presence of various cytokines using RayBio® Cytokine Antibody Array 6 (RayBiotech), following the protocol outlined in the kit insert, with the following change: before pipetting a sample onto the membrane, the sample was mixed 1:1 with kit extract buffer (2×) containing protease inhibitor cocktail tablets (Roche, Indianapolis, IN, USA). The blots were exposed using Kodak® Biomax™ MR film (Sigma-Aldrich, St. Louis, MO, USA).

Migration and invasion assays

Subconfluent PrSCs or PCAFs were harvested by trypsinization, filtered through 100-µm nylon mesh to remove cell clusters and resuspended in 0.1% bovine serum albumin (BSA, Fisher Scientific, Pittsburgh, PA, USA) in phenol red-free RPMI 1640. The prostate cancer CM were thawed, supplemented with 0.1% BSA and sterile-filtered. Migration assays were performed using a 24-well plate containing a modified 24-well Boyden chamber insert (BD Biosciences), with the upper and lower chambers separated by a filter containing 0.2-µm pores. Medium (1.2 ml of 0.1% BSA/RPMI or 0.1% BSA/CM) was pipetted in triplicate or quadruplicate into the lower chambers, and 50,000 PrSCs or PCAFs were plated in the upper chambers. After a 4-hr incubation at 37°C and 5% CO₂, cells on the upper surfaces of the filters were removed using cotton swabs, and cells that migrated to the lower surfaces were stained with 0.5% crystal violet (Sigma-Aldrich) in 25% methanol (Fisher Scientific) for 20–60 min and washed with water. Plates were stored at 4°C for up to 2.5 weeks, and five images per filter were obtained using a Nikon Eclipse TE2000-E inverted microscope and NIS-Elements AR 3.0 software (Nikon, Melville, NY, USA). The migrated cells in these images were counted manually, and the counts for each filter were summed.

For the invasion assays, a 1 mg/ml collagen I solution was prepared essentially according to the protocol outlined in De Wever et al. (2009) [21], with the following specifications: human collagen I in acidic solution (BD Biosciences) was used, and for standard medium, 10× l-glutamine/10× RPMI 1640 (Sigma-Aldrich) was used. The collagen I solution was plated at 100 µl/filter in 24-well Boyden chamber inserts and incubated overnight in a 37°C, 5% CO₂ incubator before the assays were performed as described earlier.

For the angiogenin-blocking experiments, 0.1% BSA/RPMI or CM from PC-3 cells was incubated with 0.1 µg/ml polyclonal goat anti-angiogenin antibody (Lifespan Biosciences, Seattle, WA, USA) or 0.1 µg/ml polyclonal goat anti-hnRNP A1 (Y-15) antibody (Santa Cruz Biotechnology) for 1.5 hrs at room temperature before use in the assays described earlier. All experiments were performed at least twice.
siRNA knockdown of angiogenin

Angiogenin expression was knocked down in PC-3 cells according to the Thermo Scientific DharmaFECT siRNA Transfection Protocol, with the following specifications: 62,500 cells per well were plated in 24-well plates (BD Biosciences); final concentrations of 25 nM were used for angiogenin siRNA (Abnova, Walnut, CA, USA) and negative control siRNA (pool of four non-targeting siRNAs, Dharmacon, Lafayette, CO, USA); and in Transfection Step 2, 0.25 µl DharmaFECT 2 (Dharmacon) was used for every 49.75 µl serum-free RPMI 1640 medium. At 24 hrs post-transfection, the transfection medium was replaced with 400 µl per well of serum-free, phenol red-free RPMI 1640 medium, which was then collected 48 hr later. The media were stored at −80°C until used. Before the invasion assays, the CM from angiogenin siRNA- and negative control siRNA-treated PC-3 cells were thawed out, and BSA was added to a final concentration of 0.1%. Each medium was plated in triplicate, and invasion assays were performed in duplicate as described in ‘Migration and invasion assays’.

ELISA

The Quantikine® Human ANG Immunoassay Kit (R&D Systems, Minneapolis, MN, USA) was used to determine angiogenin levels in media conditioned by angiogenin siRNA- and negative control siRNA-treated PC-3 cells. The assay was performed three times, using instructions from the kit manual.

Statistics

For all data except those presented in Figures 1 and 3, a two-tailed paired Student’s t-test (one- or two-sample, as appropriate) was performed. Standard errors, indicated by error bars, were determined using Microsoft Excel 2007 (Microsoft, Redmond, WA, USA).

Results

The major goals of this study were (1) to determine the effects of prostate cancer-derived factors on the invasion of prostate fibroblasts, and (2) to identify specific prostate cancer-derived factor(s) that stimulate prostate fibroblast invasion.

Stromal marker expression in normal prostate fibroblasts and PCAFs

To characterize the prostate fibroblasts that we used in later experiments, whole-cell lysates obtained from PrSCs and PCAFs were resolved by SDS-PAGE and transferred to nitrocellulose membranes. These membranes were then immunoblotted for various stromal markers, including SMA, a myofibroblast marker; calponin, a smooth muscle cell differentiation marker; and collagen I, an extracellular matrix molecule secreted by fibroblasts. All three of these stromal markers were expressed by PrSCs and PCAFs [13].

PrSC and PCAF migration and collagen I invasion in response to PC-3-, LNCaP- and DU145-CM

We wanted to determine whether secretions from the prostate cancer cell lines PC-3, LNCaP and DU145 affect the migration and
invasion of prostate fibroblasts. We tested 48-hr CM from these cell lines in modified Boyden chamber assays and found that all three CM stimulated PrSC and PCAF migration and collagen I invasion, albeit to varying extents and levels of significance (Fig. 2). Of the three CM, LNCaP-CM stimulated PrSC migration to the greatest extent, whereas PC-3-CM stimulated PCAF migration to the greatest extent (Fig. 2A). The reverse was true for PrSC and PCAF invasion through collagen I (Fig. 2B). In general, PC-3- and LNCaP-CM stimulated PrSC and PCAF migration and collagen I invasion to greater extents than did DU145-CM.

DU145-CM stimulated PrSC migration and invasion to similar extents: 2.29 ± 0.70- and 2.45 ± 0.55-fold, respectively, relative to unconditioned medium. It did not, however, stimulate these two behaviours to the same level of significance: Its stimulation of migration did not reach statistical significance (P = 0.125), whereas its stimulation of invasion did (P = 0.0225). This difference in the level of significance was due, at least in part, to the fact that the former value (2.29 ± 0.70-fold) was obtained by taking the average of sixfold-change values whereas the latter value (2.45 ± 0.55-fold) was obtained by taking the average of 12-fold-change values. A larger number of fold-change values incorporated into the average leads to a larger degrees of freedom value, which in turn leads to a smaller P value.

Cytokine profiles of CM, prostatic fluids and sera

We next wanted to identify candidate protein(s) in prostate cancer CM that may be involved in the stimulation of PrSC and PCAF invasion. To this end, we performed cytokine antibody array 6 analysis of PC-3-, LNCaP- and DU145-conditioned media. We found that all three CM contained the pro-angiogenic polypeptide angiogenin (Fig. 3A). To determine whether these findings are clinically relevant, we performed cytokine antibody array 6 analysis of prostatic fluid samples obtained at the time of surgery for prostate cancer. Angiogenin was detected in all of the samples tested, including those from 22 patients with minimal prostate cancer and 18 patients with extensive prostate cancer (partial data shown in Fig. 3B). To verify that the source of angiogenin was the prostates rather than the sera within the prostates, we also performed cytokine antibody array 6 analysis of sera from prostate cancer patients. Angiogenin was not detected in these samples (Fig. 3B), suggesting that the source of angiogenin present in the prostatic fluid samples was the prostates themselves.

PrSC and PCAF invasion through collagen I in response to angiogenin-blocked PC-3-CM

Angiogenin has been shown to stimulate the invasion of endothelial cells [19]. Based on this finding and our own data demonstrating that various prostate cancer cell lines secrete angiogenin (Fig. 3A), we wanted to determine whether angiogenin could stimulate the invasion of prostate fibroblasts. We therefore blocked angiogenin in PC-3-CM using a polyclonal goat anti-angiogenin antibody and then tested the CM in collagen I invasion assays. We found that blocking angiogenin completely and significantly abrogated PC-3-induced PrSC and PCAF invasion, while blocking an irrelevant protein, hnRNP A1, did not (Fig. 4). The anti-angiogenin antibody did not affect the viability of PrSCs or PCAFs (data not shown). Together, these results suggest that PC-3-derived angiogenin may be a pro-invasion factor for both normal prostate fibroblasts and PCAFs.
To obtain further evidence for these findings, we inhibited angiogenin production in PC-3 cells using angiogenin siRNA and then collected 48-hr CM from these cells to test in collagen I invasion assays. CM from angiogenin siRNA-treated PC-3 cells significantly reduced PrSC and PCAF invasion relative to CM from negative control siRNA-treated PC-3 cells (Fig. 5A). An angiogenin ELISA on the CM confirmed that the angiogenin siRNA resulted in a decrease in angiogenin protein (Fig. 5B). Together, these data provide additional evidence that PC-3-derived angiogenin stimulates collagen I invasion of both normal prostate fibroblasts and PCAFs.

PrSC and PCAF invasion through collagen I in response to angiogenin

To determine whether angiogenin is sufficient for stimulating PrSC and PCAF invasion through collagen I, we tested angiogenin alone for its ability to produce this effect. Because the angiogenin concentration was determined by ELISA to be 1.55 ± 0.04 ng/ml in CM from negative control siRNA-treated PC-3 (Fig. 5B), which stimulated PrSC and PCAF invasion...
(Fig. 5A), we tested similar angiogenin concentrations (1, 2 and 5 ng/ml) in collagen I invasion assays. Angiogenin stimulated PrSC invasion only minimally but PCAF invasion significantly, especially at higher concentrations (Fig. 6). These results suggest that angiogenin may be sufficient to stimulate the invasion of PCAFs, but not normal prostate fibroblasts, through collagen I.

**Discussion**

In this study, we found that soluble factors derived from the prostate cancer cell lines PC-3, LNCaP and DU145 stimulated the in vitro migration and collagen I invasion of both normal prostate fibroblasts and PCAFs. To our knowledge, this is the first study to...
assess fibroblast migration and invasion in prostate cancer, although these phenomena have been studied for other cancers. For example, some studies have described examples in which bladder cancer stroma invaded the prostate in cancer patients [22,23], and another study showed that fibroblasts invaded mammary and hepatoceular tumours in mice [24].

Both PrSCs and PCAFs responded to PC-3-, LNCaP- and DU145-CM; however, they migrated and invaded to a different extent in response to each CM (Fig. 2). For example, PrSCs invaded the greatest in response to PC-3-CM, whereas PCAFs invaded the greatest in response to LNCaP-CM. We suspect that differential expression of cell-surface receptors for factors in the CM may account for these differences, which are not surprising considering that other studies have shown differences between normal prostate fibroblasts and PCAFs. Among these are differences in their effects on prostate epithelial growth [4,25] as well as their expression of various stromal markers [13] and growth factors [25]. Our work further emphasizes the distinction between normal prostate fibroblasts and PCAFs by demonstrating differences in their migration and invasion.

While trying to identify candidate proteins in PC-3-, LNCaP- and DU145-CM that stimulate prostate fibroblast migration and invasion, we discovered that the pro-angiogenic polypeptide angiogenin is present in all three CM, consistent with other studies showing angiogenin expression in prostate cancer cell lines [17,18] and tissue [16]. Furthermore, we detected angiogenin in prostatic fluids, implying a potential clinical relevance for angiogenin.

When we evaluated the effects of angiogenin on prostate fibroblast invasion, we discovered that blocking angiogenin, using either an anti-angiogenin antibody or angiogenin siRNA, resulted in significantly reduced PC-3-induced invasion of both normal prostate fibroblasts and PCAFs. In addition, angiogenin alone stimulated PCAF invasion, suggesting that angiogenin is sufficient for this effect on PCAFs.

Angiogenin has been shown to stimulate endothelial cell invasion by binding to smooth muscle α-actin on the cell surface [26] to form a complex that then dissociates from the cell membrane [26,27] and activates tissue plasminogen activator (tPA) [19,26]. tPA catalyses the conversion of plasminogen into plasmin, which in turn can activate matrix metalloproteinases (MMPs) [28]. We hypothesized that angiogenin stimulated prostate fibroblast invasion through collagen I in a similar manner: by activating tPA, leading to downstream activation of MMPs. This hypothesis was identified as being false when, in a tPA activity assay, angiogenin did not significantly activate tPA in PrSCs or PCAFs (data not shown).

Based on these and other results (Fig. 6), we now hypothesize that (1) PCAFs, but not PrSCs, secrete and activate MMP1, which breaks down collagen I [29], and then (2) angiogenin acts as a chemoattractant. If this hypothesis is correct, it would explain why angiogenin alone stimulated PCAF but not PrSC invasion. Furthermore, if this hypothesis is correct, then perhaps PrSCs were able to invade in response to PC-3-CM (Fig. 2B) because of the hypothetical presence of MMP1 in PC-3-CM or the hypothetical activation of PrSC-derived MMP1 by factors in PC-3-CM. Further studies need to be undertaken to determine the mechanism by which angiogenin stimulates prostate fibroblast invasion.

Angiogenin has been shown to play a role in prostate cancer growth and metastasis both in vitro and in vivo. For example, prostate cancer growth and/or metastasis were reduced in murine models by antisense targeting of angiogenin [30], a monoclonal anti-angiogenin antibody [31,32], and other angiogenin antagonists [33]. In another study, both in vitro and in vivo prostate cancer growth were inhibited by knocking down angiogenin expression in PC-3 cells [34]. Furthermore, angiogenin expression in tissue [16] and plasma [35] has been shown to positively correlate with prostate cancer progression. Angiogenin has therefore been implicated as a potential target for prostate cancer treatment.

Our study demonstrates a new way in which anti-angiogenin therapies for prostate cancer might be effective. Together, our data indicate that angiogenin stimulates prostate fibroblast invasion,
suggesting that an anti-angiogenin therapy may target not only the prostate cancer cells and endothelial cells but also the prostate fibroblasts. Further studies assessing the in vivo effects of angiogenin on prostate fibroblast invasion need to be undertaken.

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Conflict of interest
The authors confirm that there are no conflicts of interest.

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