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Tumoristatic effects of endostatin in prostate cancer is dependent on androgen receptor status

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Abstract

Background—Although antiangiogenic therapy is a promising new line of therapy for prostate cancer, we recently reported that stable expression of endostatin arrested the progression of prostate cancer to poorly differentiated state and distant metastasis in TRAMP mice. However, the same therapy failed to provide any benefit when given either during or after the onset of metastatic switch. The present study determined the possible mechanisms behind the selective advantage of endostatin therapy in early stage disease.

Methods—Angiogenesis-related gene expression analysis was performed to identify target genes and molecular pathways involved in the therapy effects. Based on the results from *in vivo* studies, and recapitulation of the *in vivo* data *in vitro* using tumorigenic and non-tumorigenic human prostate cancer cells that are either androgen-sensitive or androgen-independent, analyses of possible mechanisms of the selective advantage of early treatment were performed using assays for cell proliferation, apoptosis, migration and cell signaling. The identified mechanisms were further confirmed *in vivo*.

Results—Results indicated that cells with high androgen receptor (AR) expression were more sensitive to endostatin treatment than androgen-independent cells with low or no AR expression. Endostatin was found to significantly downregulate the expression of growth factors, receptor tyrosine kinases, proteases, and AR both *in vitro* and *in vivo* only when the cells express high levels of AR. Cell proliferation was not influenced by endostatin treatment but migration was significantly affected only in androgen-sensitive cells. Targeted downregulation of AR prior to endostatin treatment in androgen sensitive cells and overexpression of AR in androgen independent cells indicated that the effect of endostatin via AR downregulation is mediated by a non-genotropic mechanism on Ras and RhoA pathways, and independently of AR on MAPK/ERK pathway.

Conclusions—These data indicate that systemically stable endostatin expression delays the onset of metastatic switch by acting on multiple pathways involving AR.

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Keywords

Prostate cancer; metastasis; androgen receptor; anti-angiogenesis; TRAMP model

INTRODUCTION

Prostate cancer is the second leading cause of cancer deaths in American men (1). In the last few years, studies have identified several molecular events that occur during the development of more aggressive, androgen-independent tumor, leading to the development of targeted therapies for the management of the disease (2–5). Among promising new therapies for prostate cancer, anti-angiogenic therapy is aimed at arresting the formation of new blood vessels from pre-existing vascular bed, which is crucial for the progression of tumor growth, invasion, and metastasis.

Despite promising preclinical studies, translation of anti-angiogenic therapy in human patients has yielded very limited success (6, 7). Most of the patients in these clinical trials exhibited end-stage disease with poorly-differentiated tumors (8–10). Our recent studies in the transgenic adenocarcinoma mouse prostate (TRAMP) model indicated that sustained systemic levels of endostatin and angiostatin did not eliminate the tumor growth but greatly delayed the progression of the disease into high-grade prostatic intraepithelial neoplasia (PIN) and well-differentiated tumor, only when initiated prior to the onset of metastatic process, suggesting the existence of possible limitations of this therapy for late-stage disease (11). The present study determined the molecular mechanisms that regulate the selective advantage of this therapy prior to metastatic switch. Results of this study indicated the role of androgen receptor (AR) signaling in endostatin effects observed in early-stage disease. Molecular profiling of prostate tissue from TRAMP mice following endostatin therapy and recapitulation of the *in vivo* data using androgen-sensitive and androgen-independent prostate cancer cells indicated a significant downregulation of growth factors known to promote cell invasion and migration. Proteins of Ras, RhoA and MAPK/ERK pathways were down-regulated only in cells with high AR levels. There was a significant decrease in AR levels following endostatin treatment. Further, AR ablation studies indicated a direct effect of endostatin on Src kinase and by an indirect, non-genotropic effect of AR on Ras and RhoA suggesting the involvement of signaling events regulating AR on the effects of endostatin therapy during androgen-dependent and androgen-independent stages of prostate cancer. Overexpression of AR in AR-negative, androgen-independent prostate cancer cells also resulted in inducing the effects of endostatin observed in AR-positive, androgen-sensitive cells. Collectively, these data provide new insight to improve the effects of anti-angiogenic therapy for late-stage prostate cancer.

MATERIALS AND METHODS

Cells and reagents

The human embryonic kidney cell line 293 and human prostate cancer cell lines LNCaP, C4-2, Du145 and PC3 were purchased from ATCC and grown in RPMI 1640 medium (Gibco). LNCaP cells were maintained in the presence of 10 nM dihydrotestosterone (DHT;

Sigma, St. Louis, MO). All antibodies were purchased from Cell Signaling Technology Inc. (Beverly, Massachusetts). The expression vector for human AR was obtained from OriGene Technologies Inc., Rockville, MD. AR-specific and control siRNA were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA.

Transgenic mice

Transgenic males and non-transgenic littermates were obtained as TRAMP C57BL/6 × FVB Breeder F₁. Genotyping for the probasin-Tag transgene was performed by PCR using DNA isolated from tail biopsy (12). Animal care and treatments were conducted in accordance with established guidelines and protocols approved by the University of Alabama at Birmingham Institutional Animal Care Committee.

Treatment and analysis of tissues

Details of the anti-angiogenic therapy in TRAMP mice have been published recently (11). Cell morphology and histology of tumor sections were evaluated in H&E stained formalin-fixed tissue. All prostate lobes, including anterior, ventral, and dorsolateral lobes, were reviewed by an experienced pathologist in a blinded fashion and graded. Prostate lesions were scored using a 1 to 6 scale that has been established for TRAMP mice (13).

Gene expression analysis using angiogenesis-specific microarray

RNA from prostate tissues was isolated using the TRIzol method (Invitrogen). RNA from at least 5 mice were included from each group for each time point for the microarray analysis. Biotin-UTP-labeled cRNA probe was synthesized using 3 µg of purified total RNA and reverse transcriptase (TrueLabeling-AMP 2.0 Kit; S.A. Bioscience Corp., Frederick, MD, USA). Six micrograms of purified cRNA from each sample was then incubated with an Oligo GEArray® Mouse Angiogenesis Microarray (OMM-024, Superarray Inc.). The hybridized arrays were washed and detected with chemiluminescence according to the manufacturer's instructions (G.E. Array, Bethesda, MD). Signals were captured on films and scanned at 300 DPI in TIFF format, and analyzed by the GEArray analysis suite (Superarray Inc.). Standardization of all signals was performed by normalizing the raw data with that of GAPDH.

Effects of endostatin treatment on prostate cancer cells

LNCaP, C4-2, PC3 and Du145 cells were treated with recombinant endostatin or BSA at a concentration of 1 µg/ml for 60 minutes. Then, cells were harvested for cell lysate or RNA preparation, or used for proliferation, apoptosis and migration assays described below.

Real-time PCR

Total RNA was isolated using the TRIzol reagent (Invitrogen). A two-step quantitative real-time, reverse transcription-PCR (RT-PCR) was performed on cDNA generated using the iScript cDNA Synthesis Kit and iQ SYBR Green Supermix (Bio-Rad). The levels of target gene expression were calculated after normalization with GAPDH levels. Normalized expression was determined using the comparative C_t method, and fold changes were derived

from the 2^{-C_t} values for each gene (Livak et al., 2001, Schmittgen et al., 2008). Sequences of the primers used in RT-PCR reactions are given in Table-1.

Immunohistochemistry

Immunohistochemical studies were performed in 5- μ m sections of paraffin-embedded tumor tissues using antibodies for AR, anti-PARP p85, Ras, ERK, RhoA, Src and cytokeratin-8. Antigen retrieval was achieved by incubating the slides in 0.05% trypsin for 20 min at 37°C. Secondary antibodies were respective, isotype-matched, anti-mouse or anti-rabbit antibodies conjugated to HRP or Alexa Fluor, and were used at a dilution of 1:500.

Analysis of cell signaling

Immunoblotting was performed as described (14). Briefly, cell extracts from prostate cancer cells were separated in 10% SDS-PAGE gels and then transferred onto nitrocellulose membranes. Detection of specific proteins was performed using antibodies for pERK1/2, ERK1/2, Ras, pSrc, Src, pAKT, AKT, RhoA and β -actin. Blots were developed with ECL reagent obtained from Amersham Pharmacia Biotech (Piscataway, NJ). Protein bands were detected by chemiluminescence (Pierce).

Cell proliferation, apoptosis and migration assays

For cell proliferation, cells were seeded in 96-well plates (6×10^2 cells/well) and allowed to attach overnight. The cells were incubated in serum-free medium for 24 h, and then treated with 1 μ g/ml of recombinant endostatin for 1 hr. Cell proliferation was measured 24, 48 and 72 hrs later using the Celltiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer's protocol. For apoptosis assay, 100 microliters of the cell suspensions (10^5 cells) were incubated with 5 μ L Annexin V and 5 μ L propidium iodine for 15 min, in the dark, at room temperature and analyzed by flow cytometry. For migration assay, cells were plated as 10^5 cells/well in a 6-well plate coated with fibronectin. The cells were serum starved for 12 hrs following which 10 μ g/ml mitomycin C was added to the media for 2 hrs. Next, the cells were treated with purified endostatin for 1 hour and a "wound" created using a sterile 200 μ l pipette tip. Photographs of the wounded area were taken at the time of wounding and thereafter every 24 hrs for 3 days to determine the rate of wound closure. Percent migration was calculated by measuring the width of the cell-free in five points along the scratch and then averaged.

Modulation of AR in human prostate cancer cells

Abrogation of AR was performed by siRNA transfection using the Transfection reagent (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and constitutive AR overexpression was achieved by transfecting the AR expression vector using Lipofectamin 2000 (Invitrogen). Transfections were performed 24 hrs prior to the treatment of cells with endostatin, either using purified protein or by transfecting subsequently an expression vector for endostatin. The cells were harvested and lysates prepared and analyzed as before.

Data analysis

For the analysis of microarray data, the GEarray software (S.A. Biosciences) was used. The Pathway Architect software (Agilent, CA, USA) was used for pathway analysis and annotation. The Superarray images were uploaded onto the GEarrays software, and minimum signal was used as background. Signal medium was used for signal intensity and student T-test for statistical analysis. Only a differential gene expression of >2-fold was considered significant. The gene lists obtained by filtering both p value and fold changes were used for pathway analysis and annotation. Analysis of gene expression results containing two or more groups was done using the Student's *t* test, assuming equal variance. Student's *t* test was used to determine statistical significance in all other experimental data. Values of $P < 0.05$ were considered statistically significant.

RESULTS

Molecular profiling of angiogenesis-related gene expression in the prostate tissue of TRAMP mice following anti-angiogenic therapy

Towards understanding the possible mechanisms behind the selective advantage of systemically stable anti-angiogenic therapy only in early-stage disease, gene expression analysis was performed to identify differentially-expressed angiogenesis-related genes from prostate tissues of naïve and treated mice with tumor grades 3, 4, 5, and 6. Results of this study is given in Figure 1A.

Following endostatin therapy, there was a significant downregulation in the expression of genes involved in migration, cell growth and apoptosis as shown in Figure 1A. Downregulation of growth factors, FGF, Mdk, PDGF, Ptn, TGF β 1 and TGF β 3, tyrosine kinase receptors Ffgr3 and Nrp1 and proteinases MMP2, MMP9 was observed when the treatment was given during androgen-sensitive stages of the disease. At the stage of prostatic intraepithelial hyperplasia, only Akt1 expression was similar in naïve mice compared to the treated group. The major mechanism of Akt activation is through PI3 kinase pathway (15). Ephrin (Efna2, Efna3) expression was slightly increased in the stage of neoplasia and continued to increase three times in well-differentiated tumors and six times in poorly-differentiated tumors of naïve mice. Ephrin receptors have been implicated in the process of metastasis by activating the Rho signaling pathway and reorganization of cytoskeletal elements (16,17). Mice that received endostatin and angiostatin treatment showed a significant decrease in Ephrins, especially Efna 3, which was restricted to basal levels.

Angiogenic gene expression profile was more pronounced in the transition stage of well-differentiated tumor to moderately-differentiated tumor

Five genes namely *Efna2*, *Eng*, *Mdk*, *MMP2*, *TGF β 3* were highly expressed ($p < 0.003$) in well-differentiated prostate tumors in untreated mice compared to mice treated with endostatin and angiostatin. Endoglin (CD105) is a membrane protein involved in the TGF- β receptor signaling pathway with predominant expression in proliferating endothelial cells (18,19). There was a significant downregulation of midkine family growth factors, Mdk and Ptn following endostatin treatment ($p < 0.003$). During tumor progression, the number of highly expressed genes in naïve group was broadly increased compared to the treated group

($p < 0.05$). These included genes involved in extracellular matrix degradation (MMP2, MMP9, TIMP2), angiogenesis (Adra2b, Ecgf1, Efna2, Efna3, Nrp1, Pofut1, PDGF and FGF), and cell proliferation and metastasis (MAPK14, Ptn, Pecam1, PDGF, FGF). None of these genes were found to be in significant numbers in the prostate tissue of normal, transgene-negative littermates.

Determination of endostatin effects in androgen-sensitive and androgen-independent human prostate cancer cells

Since genes that were downregulated in the prostate tissues of treated mice were associated with cell signaling and kinase activation, which promote cell growth, migration and activation of cancer cell signaling by paracrine and autocrine mechanisms, characteristic of the transitional stage of prostate cancer cells becoming androgen independent, we designed studies to understand the associated molecular events following endostatin treatment in androgen-sensitive and androgen-independent human prostate cancer cells. First, we independently tested the effects of endostatin, angiostatin and the combination of both endostatin and angiostatin and observed maximum effects in endostatin and endostatin plus angiostatin groups. Hence, further efforts have focused on endostatin as the anti-angiogenic factor.

Initially, we performed studies to confirm recapitulation of the effects observed in gene expression analysis of prostate tissues from TRAMP mice, treated with endostatin. Briefly, androgen-sensitive LNCaP cells and androgen-independent C4-2, Du145 and PC3 cells were treated with endostatin for 1 hr. RNA was isolated from the cells and relative expression of key genes, which were downregulated *in vivo* following endostatin therapy, were analyzed by RT-PCR. Results, shown in Figure 1B, indicated a similar pattern of downregulation of gene expression only in the androgen-sensitive (LNCaP) and AR overexpressing (C4-2) human prostate cancer cells. This observation was identical to that observed in gene expression analysis from prostate tissue of TRAMP mice, given therapy at early-stage disease. Based on clustering of genes according to pathways regulating cell function, we further characterized the effects of endostatin treatment on proliferation, migration, apoptosis, cell signaling, and RT-PCR.

Downregulation of MAPK/ERK signaling in AR-positive cells following endostatin treatment

By immunoblotting, we determined that treatment with endostatin significantly inhibited pERK expression in DHT-stimulated LNCaP and C4-2 cells (Figure 2A). However, there was no decrease in pAKT following endostatin treatment in any of the cells. ERK signaling pathways are involved in cell proliferation, differentiation, actin cytoskeleton reorganization, and cell migration. Moreover, ERKs are also involved in stress response and cell death (20–22). However, there was no effect of endostatin on ERK activity in Du145 and PC3 cells, which are androgen independent and express no or low-level AR respectively. One of the important targets for ERK is I κ B kinase- α (23,24). We found a significant decrease in plkB- α level in the endostatin-treated LNCaP and C4-2 cells (not shown). Endostatin has been shown to activate Src in endothelial cells through association with $\alpha_5\beta_1$ integrin and caveolin-1 (25). Upregulation of integrin and caveolin-1 has been implicated in human

prostate cancer progression/metastasis and shown to promote cancer cell survival (26–28). Thus, we determined whether treatment of endostatin exerts differential effects on Src activity in androgen-sensitive and androgen-independent cells. Interestingly, data from our studies indicated a significant decrease in pSrc in androgen dependent LNCaP and AR overexpressing C4-2 cells following endostatin treatment. In contrast, pSrc level was increased in the androgen-independent cell lines Du145 and PC-3 following endostatin treatment (Figure 2A).

Endostatin treatment does not influence proliferation but decreases migration of prostate cancer cells

Results, shown in Figure 2B, indicated that there was no significant effect of endostatin on proliferation in any of the cells. The results of apoptosis indicated no effects following endostatin treatment in androgen-independent prostate cancer cells although there was approximately 25% apoptosis in the androgen-sensitive LNCaP cells at earlier time point (Figure 2C). However, migration showed a significant decrease following endostatin treatment in AR-overexpressing LNCaP and C4-2 cells ($p<0.0003$; Figure 2D). Interestingly, migration was not affected in Du145 or PC3 cells following endostatin treatment (Figure 2D).

Downregulation of Ras and RhoA in endostatin treated prostate cancer cells

Ras and RhoA are two proteins of Ras superfamily of small GTP-binding proteins, which play a central role in actin cytoskeleton organization, oncogenic transformation and adhesion. RhoA also plays an important role in proliferation and migration as well as motility and invasiveness of human prostate cancer (29,30). From the above results on AR overexpressing cells that endostatin did not affect proliferation but significantly decreased migration and also downregulated MAPK/ERK, we determined the status of Ras and RhoA in endostatin treated cells. Data from these studies are given in Figure 3. There was a significant repression of Ras and RhoA in LNCaP and C4-2 cells after treatment with soluble endostatin. In contrast, endostatin treatment increased the level of Ras and RhoA in androgen-independent DU145 and PC3 cells (Figure 3).

Downregulation of activated ERK, Src, Ras and RhoA in prostate tissue of endostatin treated TRAMP mice

From the results obtained *in vitro*, we inferred that a possible arrest in migration of AR overexpressing cells is mediated through suppression of ERK, Ras and RhoA activities. To confirm whether the same mechanism was responsible for delaying tumor progression and arrest of metastasis *in vivo*, ERK, RhoA and Ras were analyzed from prostate tissues of treated and untreated TRAMP mice by RT-PCR and immunohistochemistry. Data, shown in Figure 6, indicated a significant decrease of pErk in the treated group compared to naïve mice. Staining for activated ERK was not permanent but foci of ERK were observed in prostatic intraepithelial hyperplasia lesions and in well-differentiated tumors of naïve mice (Figure 4A). In the prostate lobes of endostatin treated mice, pERK expression was much lower in all stages of tumor progression. Immunoreactivity of RhoA was high in well-differentiated stage of prostate tumor from naïve TRAMP mice and was very weak in endostatin treated group (Figure 4A). Similar to RhoA, Ras expression was found to be

significantly high in the prostate lobes of naïve mice with well-differentiated tumors but the expression was significantly reduced following the therapy (Figure 4B). Analysis of the expression of the three closely related Ras genes *HRas*, *KRas* and *NRas* by RT-PCR indicated significant increase of *HRas* in high-grade PIN in naïve mice. However, following endostatin treatment the *HRas* level was significantly reduced ($p<0.05$). *KRas* activity was highest in the well-differentiated tumors (grade-5) of untreated mice. However, following the therapy, the level of *KRas* was significantly reduced. There was no significant difference in *NRas* between naïve and endostatin treated mice (Figure 4C). Staining of well-differentiated tumor with pSrc antibody indicated significantly higher levels in naïve mice but endostatin treatment during early stages of the disease greatly decreased pSrc in well-differentiated tumors despite the fact that progression to well-differentiated state was greatly delayed following early treatment. However, pSrc level was comparable in mice treated at late stage to the untreated naïve group (Figure 4D).

Expression of AR in prostate cancer cells in vivo and in vitro after endostatin treatment

Since several of the observed changes following endostatin treatment were found more in prostate cancer cells with high AR expression both *in vitro* and in TRAMP mice *in vivo*, we next determined whether the observed effects of endostatin negatively influence AR expression. Prostate cancer cells were stimulated with DHT and then treated with endostatin, and AR expression in LNCaP, C4-2, and PC3 cells was determined. Results, shown in Figure 5A, indicated a significant decrease in AR expression in LNCaP and C4-2 cells after culturing in the presence of endostatin ($p<0.001$). Similarly, RT-PCR using RNA from the prostate tissue of naïve and endostatin treated mice, and immunohistochemistry of prostate tissues showed a significant downregulation of AR in the treated group (Figures 5B and 5C; $p<0.001$).

Targeted abrogation of AR in situ reverses the effects of endostatin function

To determine whether the observed effects on kinase activities are associated with AR function or effected independently of AR, targeted abrogation of AR using siRNA was performed prior to endostatin treatment in LNCaP, C4-2 and Du145 cells. Results, shown in Figure 6A, indicated that only Src activity was found to be decreased whether AR expression was blocked or not in LNCaP and C4-2 cells. However, pSrc level remained unchanged in Du145 and PC3 cells whether AR siRNA was used or not prior to endostatin treatment. Abrogation of AR expression reversed the downregulation of RhoA and Ras to normal levels. Phospho-ERK level was decreased following AR siRNA transfer and further decreased in combination with endostatin treatment in LNCaP and C4-2 cells (Figure 6A). However, endostatin treatment alone, even in the absence of AR blocking, decreased pERK levels greatly in these cells suggesting that the effect of endostatin on pSrc is independent of AR but downregulation of AR influences the phosphorylation of ERK. Interestingly, pERK was also decreased in the androgen independent Du145 cells following AR blocking but as noted before, treatment with endostatin alone increased pERK in these cells (Figure 6A).

Overexpression of AR in androgen-independent prostate cancer cells restores endostatin effects to that seen in androgen-sensitive cells

To confirm the significance of high AR levels on the effects of endostatin, Du145 cells, which do not express detectable levels of AR, were transfected with an AR expression vector. After confirming high levels of AR expression, the cells were either mock-transfected or transfected with a plasmid encoding endostatin. Later, lysates from these cells were tested by Western blot for cell signaling. Results of these studies, shown in Figure 6B, indicated that upon overexpression of AR, there was a significant downregulation of RhoA and pERK but not pSrc.

DISCUSSION

Despite promising preclinical studies that established safety and absence of systemic and organ toxicity, patient response to endostatin therapy for late-stage tumors has encountered little success (31–33). Our recent studies in TRAMP mice, treated with endostatin and angiostatin, indicated that the treatment was highly effective for early stage disease but there was no significant benefit when administered after the onset of metastatic switch (11). Results of the present study indicate that in addition to inhibiting the growth of endothelial cells in the tumor environment, endostatin exerts a profound effect on prostate cancer cells directly. Initial characterization indicated activation of Akt and Erk signaling in early lesions of prostatic neoplasia in untreated TRAMP mice. Foci of activated Erk have been observed in the PIN lesion and well-differentiated tumors. Combined activation of Akt and Erk pathways stimulates epithelial AR function and blocks apoptosis-inducing paracrine signals from the stroma, leading to androgen-independent cancer (34,35). When endostatin treatment was given at early-stage disease, there was a significant decrease in Erk, RhoA, Ras and Src activities. This was accompanied by a decrease in AR levels. Androgen receptor plays a dual role in prostate cancer progression. The AR may also interact with or regulate multiple other proteins that can affect cell growth (36–38). Results of the present study indicate that downregulation of molecular signals involved during the progression of prostate cancer, when the cancer cells become androgen independent, exert profound anti-tumor effects following endostatin treatment. This appears to involve mainly kinases and growth factors that influence AR signaling. Downregulation of these molecules prior to metastatic switch of the disease greatly impacts on AR signaling, which shifts the homeostasis of the primary tumor to an aggressive metastatic disease, when the cells become androgen independent, as well decrease the expression of AR.

Recapitulation of the data from *in vivo* studies using androgen-sensitive and androgen-independent prostate cancer cells indicated that endostatin treatment results in the downregulation of a number of growth factors and receptor tyrosine kinases mainly in androgen-sensitive and AR-overexpressing cells. The effect was more pronounced in Erk, Ras, and RhoA levels. Additionally, there was a concomitant decrease in proteases and growth factors including MMP2, MMP9, PCAM-1, FGF1, EGFR, MDK and Ephrins.

The major pathways involved in the development of androgen escape of prostate cancer cells are MAPK and PI3K pathways. These pathways act to directly modify the AR, altering its sensitivity to both androgens and anti-androgens (39–41). The observation that there was no

decrease in pAKT following endostatin treatment in both androgen-sensitive and androgen-independent cells suggests that endostatin does not function through the PI3K signaling pathway. Thus, signaling through AKT, most commonly encountered in androgen-independent stages, may not be affected by endostatin, which partly accounts for the lack of its therapeutic effects on late-stage tumors. Analysis of Ras expression from the prostate tissues of treated animals and further characterization of the three closely-related *Ras* genes indicated a significant decrease in HRas and KRas when the treatment was given at early stage of the disease. Decreased levels of Rho activity inhibit cell migration through disruption of cell-matrix interactions, reducing membrane ruffling and tail-retraction of migratory cells and delaying the turnover of actin stress fibers and focal adhesions (42–44). Similarly, in prostate cancer, enhanced expression of Ras protein has been shown to correlate with increased tumor grade (45,46).

Targeted abrogation of AR by siRNA in androgen-dependent and AR-positive cells reversed the effect of endostatin on RhoA, and Ras whereas pERK was not restored. This suggests that downregulation of AR by endostatin may cause a non-genotropic effect (36,47,48), indirectly, on MAPK pathway involving Ras and RhoA whereas direct inhibitory action of endostatin on ERK may be independent of AR downregulation. Further support for this mechanism was apparent when the androgen-independent Du145 cells, with no detectable AR expression, was transfected to overexpress AR and treated with endostatin, there was a significant downregulation of RhoA but not other kinases. This observation also suggests a possible influence of endostatin on AR to effect downregulation of RhoA by non-genotropic mechanism, and activation of AR by non-androgen ligands, when the prostate cancer cells acquire androgen-independent stage, may further cause inability of endostatin action in late stage tumors. The observation that PC3 cells, which are androgen independent, and express low level AR did not show the observed effects of endostatin also prompts us to speculate that in addition to the effects of autocrine signaling by non-androgen ligands, the amount of AR expression may be an additional requirement for the treatment response to endostatin.

Results of *in vitro* and *in vivo* studies also indicate that downregulation of AR signaling also impacts on the transactivation of AR target genes. Impairment of AR signaling affects the autocrine production of growth factors that activate key signaling pathways. Decrease in the expression of EGFR, FGFR, VEGFR2, and PDGF following endostatin treatment provides support to this cascade. Once the prostate cancer cells acquire androgen-independent state, activation of AR by cytokines and peptide growth factors, produced by both tumor and non-tumor cells in the microenvironment, is likely to elicit various signaling network for continued proliferation, survival, and metastasis, independent of endostatin effects.

From the data obtained in C4-2 cells, which are androgen-independent, yet have high-level AR expression, it is also possible that intracellular trafficking of endostatin might be different in AR-sensitive and AR-independent cells. C4-2 are derived from LNCaP cells, which are androgen sensitive. Interestingly, signaling effects observed in these cells were identical to that seen in LNCaP cells following endostatin treatment unlike in Du145 and PC3 cells, the other androgen independent cells with relatively undetectable and low AR expression respectively. It will be of further interest to determine whether additional events

such as variation in intracellular trafficking of endostatin also contribute to the difference in treatment effects.

Collectively, these studies suggest that while endostatin therapy may prove to be highly useful in early stages of the disease, combining endostatin with other therapies may be required for anti-tumor effects in late-stage prostate cancer.

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A

Figure 1.

A) Differential expression of angiogenesis-related genes during prostate cancer progression in naïve TRAMP mice and following endostatin and angiostatin therapy.

Following confirmation of tumor grade, RNA was isolated from prostate tissues of untreated (left panel) and treated (right panel) mice and used for microarray analysis with Oligo GEArray® Mouse Angiogenesis array. Relative increase/decrease in the expression of indicated genes was based on comparison with values obtained in the prostate tissue of age-matched, non-transgenic littermates, taken as 100. Different colors in each bar represents tumor grade. Grade-3: high prostatic intraepithelial neoplasia (PIN); Grade-4: well-differentiated tumor; Grade-5: moderately-differentiated tumor; Grade-6: poorly-differentiated tumor. **B) Analysis of gene expression in human prostate cancer cells following endostatin treatment.**

Androgen-sensitive (LNCaP) and androgen-independent (C4-2, PC3 and Du145) human prostate cancer cells were treated with endostatin for 1 hr and RNA was isolated. Relative expression of indicated genes was analyzed by RT-PCR and values represented in comparison to levels in untreated cells indicated as the midpoint.

B

D

Figure 2. Effects of endostatin on kinase activation, proliferation, apoptosis, and migration in androgen-sensitive and androgen-independent prostate cancer cell lines

Androgen-sensitive (LNCaP) and androgen-independent (C4-2, PC3 and Du145) human prostate cancer cells were either mock-treated or treated with BSA or purified recombinant endostatin for 1 hr. Following treatments, the cells were lysed in cold lysis buffer supplemented with proteinase and phosphatase inhibitors. Cell lysates containing equal amount of protein were resolved by 10% SDS/PAGE, transferred to nitrocellulose membrane, and detected with indicated antibodies (A). Cells were incubated with 10 nM DHT for 16 hours followed by treatment with 1 µg/ml endostatin for 1 hr. Cell proliferation

was measured by CellTiter 96 One Solution Cell Proliferation Assay (**B**). Apoptosis was measured using annexin V-FITC Apoptosis Detection Kit. All assays were performed in triplicate (**C**). Cells were analyzed for cell migration using the wound-healing scratch assay. Cells were “wounded” and monitored every 24 h for 3 days to determine the rate of migration into the scratched area (**D**) (* $p < 0.001$).

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Figure 3. Ras and RhoA activities following endostatin treatment of human prostate cancer cells Androgen-sensitive (LNCaP) and androgen-independent (C4-2, PC3 and Du145) human prostate cancer cells were either mock-treated or treated with BSA or recombinant endostatin for 1 hr. Following treatments, the cells were lysed in cold lysis buffer supplemented with proteinase and phosphatase inhibitors. Cell lysates containing equal amount of protein were resolved on 10% SDS/PAGE, transferred to nitrocellulose membrane, and probed with Ras and RhoA antibodies.

A

C

D

Figure 4. Erk, RhoA, Ras and Src levels in the prostate tissue of untreated and endostatin treated TRAMP mice

A) pErk and RhoA were detected in the prostate tissues of untreated TRAMP mice with prostatic intraepithelial neoplasia (PIN), well-differentiated (WD), moderately-differentiated (MD) and poorly-differentiated (PD) tumors, and in PIN and WD tumors of treated mice. **B)** Ras expression in naïve and treated mice was analyzed in WD tumors and cancer cells identified by counterstaining with cytokeratin-8. **C)** RT-PCR analysis of HRas, KRas and NRas in the prostate tissues of naïve and endostatin treated mice. Bars indicate mean values from samples obtained from 5 mice in each group for each tumor grade (* $p < 0.05$). **D)** pSrc expression in naïve mice or mice that were given treatment at 10 weeks or 18 weeks of age was determined when the animals were 24 weeks old. H&E staining was performed for each antibody staining to confirm the location of immunoreactive regions of the prostate.

B

C

Figure 5. Expression of AR in human prostate cancer cells and in prostate tissues of TRAMP mice following endostatin treatment

Total RNA was isolated from indicated prostate cancer cells (**A**) and from prostate tissues of non-transgenic control, untreated TRAMP mice and those treated with endostatin (* $p < 0.001$ compared to mock). (**B**) Quantitation of AR mRNA was performed by RT-PCR. Values from individual samples were normalized with respective GAPDH values (* $p < 0.001$ compared to naïve). (**C**) Prostate tissue sections of naïve and treated TRAMP mice, obtained at 24 weeks of age, were stained immunohistochemically with AR antibody.

A

B

Figure 6. Kinase activity in androgen sensitive and androgen independent human prostate cancer cells following modulation of AR and endostatin treatment

A) AR-positive LNCaP and C4-2, and AR-negative Du145 cells were transfected with endostatin expression vector following AR siRNA transfection. Cell lysates were prepared 24 hrs later and analyzed for indicated signaling proteins by immunoblot. B) Du145 cells were transfected with an expression plasmid for AR. Twenty-four hrs later, the cells were transfected with the endostatin expression vector. Cell lysates were prepared 24 hrs after that and analyzed for indicated signaling proteins by immunoblot.

Table-1

Sequences of primers used in RT-PCR

hAR	F.P.	5'- AAGACGCTTCTACCAGCTCACCAA-3'
	R.P.	5'- TCCCAGAAAGGATCTTGGGCACTT-3'
mAR	F.P.	5'- TCAAGGGAGGTTACGCCAAAGGAT-3'
	R.P.	5'- ACAGAGCCAGCGGAAAGTTGTAGT-3'
hADRA2b	F.P.	5'- TCATCATGGGCACTTTCACCTCT-3'
	R.P.	5'- AGCTCCTGGAAGGCAATCCTGAAA-3'
hEGFR	F.P.	5'- TTTGCCAAGGCACGAGTAACAAGC-3'
	R.P.	5'- ATTCCCAAGGACCACCTCACAGTT-3'
mEGFR	F.P.	5'- CATTGATGGCCCACTGTGTCAA-3'
	R.P.	5'- TACCCAGATGGCCCACTTCACAT-3'
hEphrinA2	F.P.	5'- TCTCGGAGAAGTTCCAGCTCTTCA-3'
	R.P.	5'- TATTGCTGGTGAAGATGGGCTCAG-3'
hEphrinA3	F.P.	5'- AACGTGCTGGAAGACTTTGAGGGA-3'
	R.P.	5'- AGGCCAAGAACGTCATGAGGAAGA-3'
hEphrinA2R	F.P.	5'- CGTGTGGAGCTTTGGCATTGTCAT-3'
	R.P.	5'- AGCACTGCATCATGAGCTGGTAGA-3'
hEphrinA3R	F.P.	5'- TGGAGCAGGTGAATTTGGAGAGGT-3'
	R.P.	5'- ATGCTTGCTTCTCCCAGGAAGTCT-3'
hGAPDH	F.P.	5'- TCGACAGTCAGCCGCATCTTCTT-3'
	R.P.	5'- ACCAAATCCGTTGACTCCGACCTT-3'
mGAPDH	F.P.	5'- TCAACAGCAACTCCCACTCTTCCA-3'
	R.P.	5'- ACCCTGTTGCTGTAGCCGTATTCA-3'
hMDK	F.P.	5'- AGCCAAGAAAGGGAAGGGAAGGA-3'
	R.P.	5'- AAGCTAACGAGCAGACAGAAGGCA-3'
hMMP-9	F.P.	5'- TGGGCTACGTGACCTATGACAT-3'
	R.P.	5'- GCCCAGCCACCTCCACTCCTC-3'
hMMP-2	F.P.	5'- GAAGGTGAAGGTCGGAGT-3'
	R.P.	5'- GAAGATGGTGATGGGATTTC-3'
hNrp-1	F.P.	5'- TAACGACCCACTCTGCTTCTTGCT-3'
	R.P.	5'- TTGCCAAATCTTCAGAGCCCTTGC-3'
hPleiotrophin	F.P.	5'- TGTGGATACTGCTGAAGCAGGGAA-3'
	R.P.	5'- GGGTCTTCATGTTTGCTTGCACT-3'
hPI3K p110	F.P.	5'- ATCCCAGGTGGAATGAATGGCTGA-3'
	R.P.	5'- AATGGAAAGGCAAAGTCGAGCAGC-3'
mN-Ras	F.P.	5'- TCCTAGCTGGCCTCAAACACAA-3'
	R.P.	5'- TCAGTCAGGTGTGTGGTGGGAAT-3'
mH-Ras	F.P.	5'- AGTCCGTGAGATTCGGCAGCATAA-3'
	R.P.	5'- GGACAGCACACATTTGCAGCTCAT-3'
mK-Ras	F.P.	5'- AGACACGAAACAGGCTCAGGAGTT-3'
	R.P.	5'- TAGAAGGCATCGTCAACACCCTGT-3'