Anti-Angiogenic Activity of PSA-Derived Peptides

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BACKGROUND. PSA is a biomarker for diagnosis and management of prostate cancer. PSA is known to have anti-tumorigenic activities, however, the physiological role of PSA in prostate tumor progression is not well understood.
METHODS. Five candidate peptides identified based upon computer modeling of the PSA crystal structure and hydrophobicity were synthesized at >95% purity. The peptides in a linear form, and a constrained form forced by a di-sulfide bond joining the two ends of the peptide, were investigated for anti-angiogenic activity in HUVEC.
RESULTS. None of the five PSA-mimetic peptides exhibited PSA-like serine protease activity. Two of the peptides demonstrated significant anti-angiogenic activity in HUVEC based on (i) inhibition of cell migration and invasion; (ii) inhibition of tube formation in Matrigel; (iii) anti-angiogenic activity in a sprouting assay; and (iv) altered expression of pro- and anti-angiogenic growth factors. Constrained PSA-mimetic peptides had greater anti-angiogenic activity than the corresponding linearized form. Complexing of PSA with ACT eliminated PSA enzymatic activity and reduced anti-angiogenic activity. In contrast, ACT had no effect on the anti-angiogenic effects of the linear or constrained PSA-mimetic peptides. Modeling of the ACT-PSA complex demonstrated ACT sterically blocks the anti-angiogenic activity of the two bioactive peptides.
CONCLUSIONS. The interaction of a hydrophilic domain on the surface of the PSA molecule with a target on the cell membrane of prostate endothelial and epithelial cells was responsible for the anti-angiogenic or anti-tumorigenic activity of PSA: enzymatic activity was not associated with anti-angiogenic effects. Furthermore, since PSA and ACT are both expressed within the human prostate tissue microenvironment, the balance of their expression may represent a mechanism for endogenous regulation of tissue angiogenesis. *Prostate 75:1285–1299, 2015.* © 2015 Wiley Periodicals, Inc.

KEY WORDS: **prostate cancer; HUVEC; PSA-ACT complex; theoretical model; QPCR; in-vitro angiogenesis**

INTRODUCTION

Prostate cancer (CaP) is the leading cause of non-cutaneous cancer diagnoses in men and the second leading cause of cancer-related death [1]. The biology of CaP is not understood fully and the clinical behavior remains unpredictable. Prostate-Specific Antigen (PSA) is a valuable biomarker used

[This article was corrected in June 2015 after original online publication; the affiliation of Alejandro Godoy was incorrect.]
Conflicts of interest: The authors have no conflicts of interest.
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Received 18 February 2014; Accepted 2 April 2015
DOI 10.1002/pros.23010
Published online 11 May 2015 in Wiley Online Library (wileyonlinelibrary.com).

extensively in diagnosis and management of prostate cancer [2,3] despite the fact that the physiological role of PSA within the prostate tissue microenvironment, or in the pathogenesis of prostate cancer, is not known. The sole well-characterized physiological function of PSA is the enzymatic cleavage of semenogelins I & II in the seminal coagulum to facilitate sperm motility [4]. Recent evidences from both in vitro and in vivo models suggest strongly that PSA has both anti-tumorigenic and anti-angiogenic activity [5–8]. However, at present the anti-tumoricidal potential of PSA is controversial [7,9]. Williams et al [10] reported that enzymatically active PSA conferred an enhanced growth rate to human prostate cancer cells, suggesting a causal role in prostate cancer progression. On the contrary, however, transfection of PC-3 CaP cells with PSA before transplantation into nude mice prolonged doubling time, reduced tumorigenic activity and reduced metastasis [11]. Furthermore, in vitro treatment of CaP cells with PSA released angiostatin-like peptides by proteolytic digestion of extracellular matrix components and plasminogen that exhibited anti-angiogenic activity [12]. Consequently, the anti-tumorigenic activity of PSA, as for the degradation of semenogelins, was proposed to be related to the serine protease enzymatic activity of PSA in that denatured PSA did not demonstrate biological activity in these assays [13]. However, our group demonstrated in CaP cells (PC-3M) that both purified, enzymatically active PSA (free-PSA: f-PSA) and enzymatically inactive PSA, where enzymatic activity was inactivated by incubation with $Zinc^{2+}$ [14], equivalently down-regulated expression of pro-angiogenic factors/cancer-related genes/proteins, including VEGF, EphA2, Bcl2, Pim-1 oncogene, CYR61 and uPA, and up-regulated expression of anti-angiogenic genes/proteins, including interferon (IFN) and IFN-related genes [15]. In addition, both enzymatically active and enzymatically inactive f-PSA comparably modulated gene expression in human umbilical vein endothelial cells (HUVEC) of multiple growth factors, including suppression of expression of bFGF and VEGF, and up-regulation of IFN, and of proteins that have a direct role in blood vessel development, including FAK, FLT, KDR, TWIST-1, P-38, and Cathepsin-D. Furthermore, maintenance of PSA enzymatic activity was not required for inhibition of endothelial cell tube formation in the in vitro Matrigel Tube Formation Assay or for inhibition of endothelial cell invasion and migration by HUVEC cells [15]. Therefore, our data suggests strongly that PSA has a significant physiological role in prostate tissue, potentially affecting both prostate epithelial cells/prostate cancer cells and prostate endothelial cells, which is independent of the serine protease enzymatic activity of PSA.

PSA is an androgen-regulated gene produced predominantly in the male prostate, but also produced in multiple other AR-expressing tissues, including breast tissue in human females and in the parotid gland [16,17]. Consistent with our hypothesis that PSA represents an endogenous anti-tumoricidal molecule, others also reported that tissue PSA levels declined with increasing disease stage, and tissue PSA expression levels had an inverse correlation with prognosis in both prostate and breast cancer [18–23]. Furthermore, PSA production in the prostate decreases with reduction of circulating testicular androgen levels due to age or tumor progression, and is lost with androgen-deprivation therapy, suggesting that decreases in endogenous PSA in the prostate could contribute to a more tumor-supportive tissue microenvironment. However, circulating levels of PSA usually are higher as disease progresses and this increase in circulating PSA level is not due to increase in production of PSA in the prostate epithelium, but primarily is due to damage to prostate endothelium [24]. The increase in circulating PSA level generally is small relative to the 1000-fold higher level of PSA in prostate tissue compared to in circulation, and because essentially all of the PSA in circulation is complexed with the chaperone molecule alpha-1-antichymotrypsin (ACT) [25,26].

Our evidence that the anti-angiogenic activity of PSA was maintained in f-PSA protein in which the enzymatic active site was inactivated by incubation with Zn^{2+} [15] is in contrast to reported data where the anti-angiogenic activity was not preserved in enzymatically inactive internally cleaved PSA-isoforms [27]. This study suggests that rather than through enzymatic activity, the anti-tumoricidal activity of PSA may be mediated through the interaction of f-PSA with a cell surface protein(s) on prostate cancer cells and/or prostate endothelial cells. Domains of the PSA protein likely to be present on the external surface of PSA in aqueous solution were identified based upon hydrophilicity mapping of the external surface in 3-D structures of human PSA. Five candidate peptides were identified and evaluated for their effect on gene expression, invasion, migration and anti-angiogenic activity as evaluated by inhibition of vascular tube formation by HUVEC cells. The goal was to demonstrate that candidate peptides derived from human PSA protein would maintain significant anti-tumorigenic and anti-angiogenic activity in the absence of enzymatic function. Peptides derived from PSA may not elicit an immune response, and would not be subjected to complexing with and inactivation by ACT, allowing systemic administration to obviate

the loss of endogenous intact tissue PSA due to age, disease progression or iatrogenic intervention.

MATERIALS AND METHODS

Cells

Human umbilical vein endothelial cells (HUVEC) were obtained from PromoCell (Heidelberg, Germany). HUVEC were grown in Endothelial Cell Basal Medium supplemented with Endothelial Cell Growth Medium Supplemental Pack (PromoCell) and 5.0 nM metribolone. HUCEC were maintained at 37°C in 5% CO_2. Cells were used between passages two and five, with experimental populations >95% viable. We reported that HUVEC express AR and are androgen responsive (28).

f-PSA Isolation

A two-step chromatographic procedure was used to obtain homogeneous preparations of f-PSA from human seminal plasma [29]. Briefly, seminal plasma was dialyzed against 25 mM HEPES buffer that contained 1.0 M sodium sulfate, pH 7.0, and was applied to a column packed with T-gel (Fractogel TA 650(s), Merck Darmstadt, Germany). The column was washed with column equilibration buffer (dialysis buffer) and the bound proteins were displaced with 25 mM HEPES buffer, pH 7.0 containing no salt. The bound fraction contained immunoglobulins, seminal fluid proteins, and both free and complexed PSA. The eluted fractions that contained bound proteins were pooled, concentrated and applied to a Molecular Size Filtration Chromatographic column (Ultrogel AcA-54; fractionation range of 5,000–70,000 kDa) that was pre-equilibrated with 10 mM sodium acetate buffer, pH 5.6, that contained 0.15 mM sodium chloride. The Ultrogel column fractionated the immunoglobulins, f-PSA and PSA complexed with chaperone protein. Column fractions that contained f-PSA were identified using Western blot analysis of the eluate: f-PSA was recovered in the molecular weight range between 25 and 40 kDa. The f-PSA was characterized for purity using 2D-gel electrophoresis/Western blot analysis using anti-PSA- and anti-PSA-chaperone-complex, specific monoclonal antibodies [29]. Several individual preparations of f-PSA were subjected to amino-terminal sequencing, and did not reveal the presence of proteins other than PSA (W.M. Keck Foundation Biotechnology Resources Laboratory, Yale University).

Identification of PSA-Derived Peptides

Computer modeling of the crystal structure of PSA in conjunction with hydrophobicity mapping based on the primary amino acid sequence allowed identification of five candidate peptides exposed on the surface of PSA in aqueous solution. These peptides were hypothesized to represent regions of PSA molecule capable of binding/interacting with a target(s) on a cell membrane surface. The sequence of these peptides and their location within the PSA primary amino acid sequence are given in Figure 1. The peptides were synthesized and purified to >95% purity by a commercial source. The five PSA-derived linear peptides are referred as PSA-P1L; PSA-P2L; PSA-P3L; PSA-P4L and PSA-P5L respectively. Because of the fluidity of peptide structure in aqueous solution, and potential sensitivity to proteolytic degradation in culture, Peptides one & three were redesigned to include cysteine residues at both ends of the peptide sequence to produce peptides that display a constrained (rigid) structure in solution forced by di-sulfide bonding of the cysteines.

Enzymatic Activity of f-PSA, and PSA-Derived Peptides

The enzymatic activity of f-PSA and the candidate PSA-derived peptides was determined using a PSA-specific fluorogenic substrate, Mu-His-Ser-Ser-Lys-Leu-Gln-AFC (Calbiochem, San Diego, CA). The substrate was diluted in 20 μL of enzymatic activity buffer (50 mM Tris, 0.1 M NaCl, pH 7.8) to a final concentration of 38 μM. The f-PSA and PSA-derived peptides were diluted in enzymatic activity buffer to a

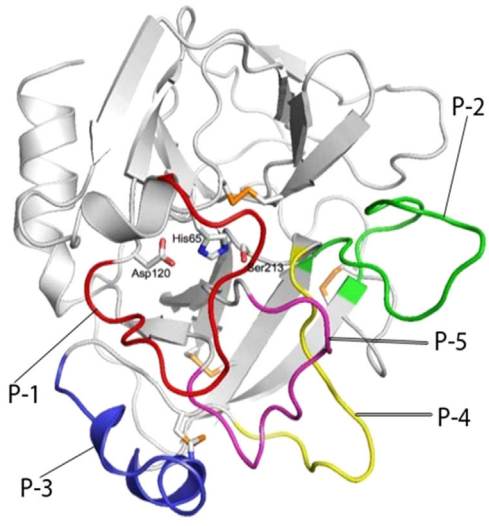

Fig. 1. Computer modeling of PSA protein showing the five candidate PSA-derived peptides (P1-P5) in corresponding color.

concentration of 5.7 nM (0.1 μg/10 μL). The total assay volume (530 μL) contained 10 μL PSA, or PSA-derived peptide, 20 μL of fluorogenic substrate, and 500 μL enzymatic assay buffer. The fluorescent signal due to hydrolysis of the fluorogenic substrate was determined with a LS 45 Luminescence Spectrophotometer: excitation at 400 nm and emission at 505 nm. The fluorescent intensity was determined by recording the fluorescent intensity at 200 sec intervals for a total of 20 min, and quantitated using the FL-WinLab software (PerkinElmer, Waltham, MA). The enzymatic activity was expressed as described previously [30].

In Vitro Angiogenesis Assay

The in vitro endothelial cell tube formation assays using Matrigel basement membrane matrix (BD Biosciences, San Jose, CA) were performed as described elsewhere [31] with some modifications. The day before performing the tube formation assay, appropriate volumes of Matrigel were thawed overnight in an ice bucket placed in a 4°C cold room. Similarly, 24-well tissue culture plates (Corning Life Sciences, Lowell, MA) and 200 μL pipette tips were chilled overnight at 4°C. Matrigel (200 μL) was added to each well of the culture plates and incubated for 30 min at 37°C to allow the liquid Matrigel to solidify. During the 30 min incubation, HUVEC cultures were trypsinized, endothelial cell media added to neutralize the trypsin, cells pelleted and the cell pellet re-suspended in 1.0 ml of fresh media. After the Matrigel had solidified, approximately 75,000 HUVECs in 0.5 ml of media or media containing either f-PSA, chymotrypsin, scrambled peptide or PSA-derived peptide, was added to each well. The plate was incubated for 16–18 hr at 37°C. Live cell images (4x magnification) were taken using a Nikon Eclipse TE300 inverted microscope and images of the endothelial tubes analyzed using the Spot Advance software program (Diagnostic Instruments, Inc., Sterling Heights, MI). Five images were taken per well and the images were processed using Analyze 7.0 (Analyze Direct Inc., Overland Park, KS) and AngioQuant v1.33 [32] to obtain the average tube length (in pixels) for each image. Percent inhibition of tube formation is expressed relative to tube formation in untreated control cells. Statistical analysis was performed using Student's *t*-test. *P*-values < 0.05 were considered statistically significant.

Cell Invasion Assay

The effect of intact PSA or PSA-derived peptides on the invasion property of HUVEC was analyzed using the CytoSelect 24-well Cell Invasion Assay [33,34]. The assay kit contains polycarbonate membrane inserts (8 μm pore size) in 24-well plates. The upper surface of the insert membrane is coated with a uniform layer of dried basement membrane matrix that serves as the barrier to discriminate invasive cells from non-invasive cells. Invasive cells degrade the matrix proteins in the basement membrane matrix layer, and pass through the pores of the polycarbonate membrane. At the conclusion of the assay, cells on the top of the membrane, and cells that have invaded through the membrane, are removed separately, stained, and quantified. Briefly, after overnight serum-starvation, HUVEC cells were seeded in the upper chamber (3×10^4 cells per well) in serum-free medium, in the absence or presence of PSA or PSA-derived peptide (10 μM), and the cells allowed to migrate toward the FBS containing medium in the bottom chamber for 24 hr. Migratory cells were stained and quantified by absorbance at 540 nm.

Cell Migration Assay

The CytoSelect™ 24-well Wound Healing Assay is performed in 24-well plates that contain proprietary plastic inserts. The inserts create a wound field with a defined gap of 0.9 mm for measuring the migration and proliferation rates of cells. Migratory cells extend protrusions, and ultimately invade into the gap to close the wound field. Cell proliferation and migration rates can be determined using microscopic imaging. A fixing solution is provided for stopping cells at specific time points. Briefly, HUVEC cells were plated in 24-well culture plates and grown in endothelial cell conditioned medium with reduced FBS. Cell monolayers were disrupted to produce a linear wound (0.9 mm in width), the wells washed with PBS to remove debris, and the cultures incubated with PSA-derived peptide (10 μM) or f-PSA that was demonstrated previously to inhibit HUVEC cell migration [15] and was used as the positive control. Wound fields were examined at different time points using phase contrast microscopy (Olympus) to measure wound size.

Cell Proliferation Assay

The MTT assay was performed as described previously [35]. Briefly, HUVECs in suspension were seeded at 1×10^4 cells per well in 96-well micro titer plate, and cultured in a humidified atmosphere of 5% CO_2 in air at 37°C. The cells were exposed to free-PSA and PSA-derived peptide three (PSA-P3L) for 48 hr. After that, culture medium was carefully removed and exchanged for fresh medium. . MTT solution (5 mg/ml in PBS) was added and plates were incubated for 2 hr. at 37°C and 5% CO_2; metabolically active cells reduced MTT to blue formazan crystals,

At the conclusion of the incubation, MTT- formazan crystals were dissolved in DMSO and absorbance was measured at 570 nm on a multifunctional BioTek ELISA reader (Molecular Device, Sunnyvale, CA) and absorbance in treated cells compared with control, untreated cells.

Angiogenic Sprout Assay

Angiogenic sprout assays were done using HUVEC cells following a protocol taken from Nakatsu et al. [36]. Dextran-coated Cytodex-3 micro carrier beads (Amersham Biosciences) were mixed with HUVEC (400 cells/bead) in EGM-2 medium and incubated overnight at 37°C and 5% $C0_2$. HUVEC-coated beads were washed three times with 5 ml of EGM-2 medium, and resuspended in clotting medium containing fibrinogen (2.5 mg/ml; Sigma) at a density of 200 beads/ml in the presence of aprotinin (0.15 units/ml; Sigma) at pH 7.4. To induce clotting, 0.5 ml of fibrinogen/bead solution was added into one well of a 24-well tissue culture plate that contained 0.625 units of thrombin (Sigma), and incubated for 5 min at room temperature and for 20 min at 37°C in 5% CO_2. The clot was equilibrated in 1 ml of EMG-2 containing 2% FBS and with 0.15 units/ml of aprotinin for 30 min at 37°C in 5% CO_2 The medium was replaced with 1.0 ml of clotting medium containing lung fibroblast cells (WI-38, ATCC,~30,000 cells/ml). PSA or peptides were added as indicated, and the assay was monitored for 7 days with a change in medium every other day. Each condition was repeated in two wells. Images of the beads were captured using an inverted photo-microscope and the sprouts were counted using angiogenic add on tool in NIH Image J software.

RNA Extraction and Reverse Transcription Real-Time Quantitative PCR (Q-PCR)

Total cellular RNA was extracted from HUVEC treated with PSA or PSA-derived peptide, and untreated HUVEC, using an acid guanidium thiocyanate–phenol–chloroform method [37] using TRIzol reagent (Invitrogen, Carlsbad, CA). DNA contamination was removed by treatment of the RNA preparation with DNAse (1.0 IU/mg of RNA, Promega, Inc., Madison, WI) for 30 min at 37°C. Protein contamination was removed from the RNA isolates by Proteinase K digestion at 37°C for 15 min, followed by extraction with phenol/chloroform, and precipitation with NH_4OAc/ETOH. The final RNA pellet was dried and re-suspended in diethyl-pyrocarbonate-treated (DEPC) water. The amount of RNA was quantitated using a Nano-Drop ND-1,000 spectrophotometer (Nano-DropTM Wilmington, DE), and the RNA was stored at −80°C until used. Q-PCR was used to quantitate the effect of PSA-derived peptides on expression of cancer-related genes in HUVEC cultures. Approximately 1×10^6 HUVEC cells were treated with PSA or PSA-derived peptide for 48 hr, cells harvested, and RNA extracted. Purified RNA was reverse transcribed to cDNA using a commercial reverse transcriptase kit from Promega (Promega, Inc). Relative abundance of each mRNA species was quantitated with real time quantitative PCR using specific primers and the Brilliant1 SYBR1 green Q-PCR master mix from Stratagene (Stratagene, Inc., La Jolla, CA; Cat #600548-51).

Statistical Analysis

All experiments were repeated a minimum of three times. Values are expressed as the mean ± SD. The significance of the difference between the control and each experimental test condition were analyzed by unpaired *t*-test, and a value of $P < 0.05$ was considered statistically significant.

RESULTS

Enzymatic Activity of PSA-Derived Peptides

The enzymatic activity of f-PSA and the five PSA-derived peptides (Fig. 1) was determined using the PSA-specific fluorogenic substrate Mu-His-Ser-Ser-Lys-Leu-Gln-AFC (Calbiochem, San Diego, CA). As reported previously [29], f-PSA isolated by our standard protocol demonstrated significant enzymatic activity for the PSA-specific substrate. The generation of the fluorescent reaction product by intact PSA increased linearly over the 20 min reaction time (Fig. 2). In contrast, none of the five PSA-derived peptides demonstrated PSA-like serine protease activity capable of enzymatic hydrolysis of the PSA-specific substrate. The five PSA-derived peptides are comprised of between 11 and 17 amino acids that are contiguous in the primary sequence of PSA, however, none of the peptides contained all three amino acids that comprise the active site of the serine protease. The inset in Figure 2 demonstrates that pre-incubation of f-PSA with 50 μM Zn2+ produced complete inhibition of PSA enzymatic activity for the PSA-specific test substrate. Addition of PSA-P1L and PSA-P3L to the reaction mixture had no effect in modifying the enzymatic activity of f-PSA.

PSA-Derived Peptides Alter Expression of Pro-and Anti-Angiogenic Growth Factors

Intact f-PSA modulated expression of genes associated with proliferation and angiogenic activity in both

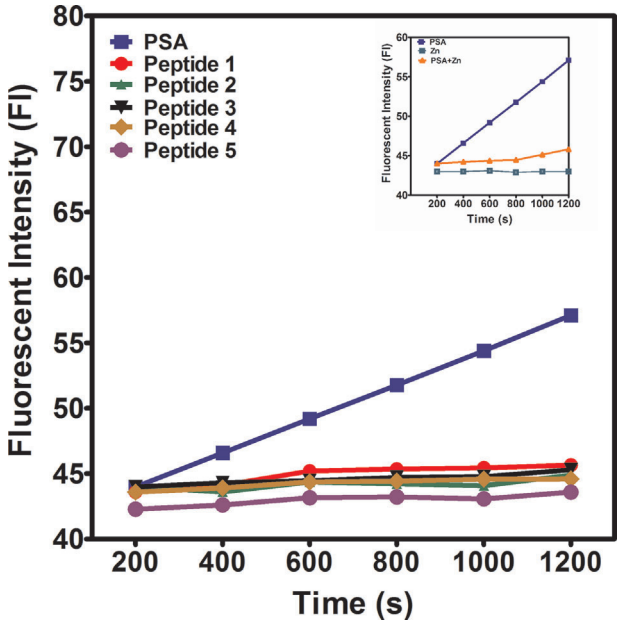

Fig. 2. Comparison of the enzymatic activity of f-PSA and the five PSA-derived peptides measured using a PSA specific substrate. The inset figure shows the enzymatic activity of PSA and PSA inactivated by treatment of Zn^{2+}. The data presented are the mean of three independent experiments.

human prostate cancer cells (PC-3M) and in human endothelial cells (HUVEC) [6,15]. Consistent with the hypothesis that f-PSA gene-regulatory activity is dependent on signaling induced by interaction of exogenous f-PSA with a cellular target, not PSA enzymatic activity, peptides derived from the primary sequence of intact PSA inhibited expression in HUVEC cells of multiple genes whose expression is modulated by intact f-PSA (Fig. 3). While the linear form of peptide three (PSA-P3L) elicited moderate reductions in expression of the majority of the genes modulated by incubation with f-PSA, the constrained (circularized) form of Peptide three (PSA-P3C) produced levels of inhibition of expression comparable to, or greater than, intact f-PSA for three of the five target genes. Consequently, the bioactivity associated with modulation of gene expression patterns in human endothelial cells was associated with signaling mechanism(s) initiated by PSA interaction with the cell, and did not require either full-length PSA protein or PSA enzymatic activity.

PSA-Derived Peptides Inhibit In Vitro Angiogenesis.

The human umbilical vein endothelial cell based assay of endothelial tube formation in Matrigel basement membrane matrix was utilized as an in vitro surrogate of in vivo angiogenesis. The requirement for PSA enzymatic function for anti-angiogenic activity was evaluated by comparison of the anti-angiogenic activity of intact f-PSA to that of the five PSA-derived peptides. Figure 4 presents representative images of endothelial cell tube formation in Matrigel by HUVEC in the absence and presence of intact PSA (10 μM), PSA derived peptides in a linear form, PSA-P3L (10 μM and 100 μM), and PSA derived peptide in a "circularized" form, PSA-P3C (10 μM and 100 μM) . Scrambled peptide and chymotrypsin treatment of HUVEC cells at concentrations comparable to the PSA/PSA mimetic peptides did not show any anti-angiogenic activity (Fig. 4). In HUVEC tube formation assay, addition of 5 μM of PSA-P3L peptide did not show any significant change in level of anti-angiogenic activity expressed by 10 μM of f-PSA. This lack of additive and or synergistic response in anti-angiogenic assay, when PSA and peptide were combined, could be due to the fact that f-PSA concentration used were at maximal. In order to see to see any additive effect it may be necessary to use PSA at suboptimal doses.

Inhibition of HUVEC tube-formation in Matrigel by f-PSA and the five linear peptides was consistent with the hypothesis that serine protease activity was not required for anti-angiogenic activity. Two of the five PSA-derived peptides in the linear conformation (PSA-P1L and PSA-P3L) demonstrated significant anti-angiogenic activity in the in vitro angiogenesis assay (Fig. 5). Intact f-PSA was reported previously to produce maximal inhibition at 10–20 μM, therefore, PSA at 10 μM was used in these studies as the positive control. PSA-P1L at 100 μM and PSA-P3L at 10 μM concentrations gave 57.99% and 49.7% inhibition of HUVEC tube-formation in Matrigel relative to intact f-PSA (10 μM), respectively. Three different scrambled peptides and another serine protease inhibitor, Chymotrypsin, were evaluated as additional controls. None of these controls had any biological activity (Fig. 4). Furthermore, three of the PSA-derived peptides tested in this study had little/no biological activity, suggesting the specificity of the inhibitory activity of the two selected PSA-derived peptides (Fig. 5). Subsequently, the two linear peptides that demonstrated marked anti-angiogenic activity in the Matrigel tube formation assay were re-synthesized with cysteine residues flanking the coding sequence of the peptide, with the cysteine residues connected through a di-sulfide bond. Figure 6 compares the inhibition of endothelial tube formation by HUVEC by the linear and "circularized" forms of Peptide three. PSA-P3C produced greater inhibition of tube formation than PSA-P3L, with the level of inhibition comparable to that by intact PSA. Inhibition of tube

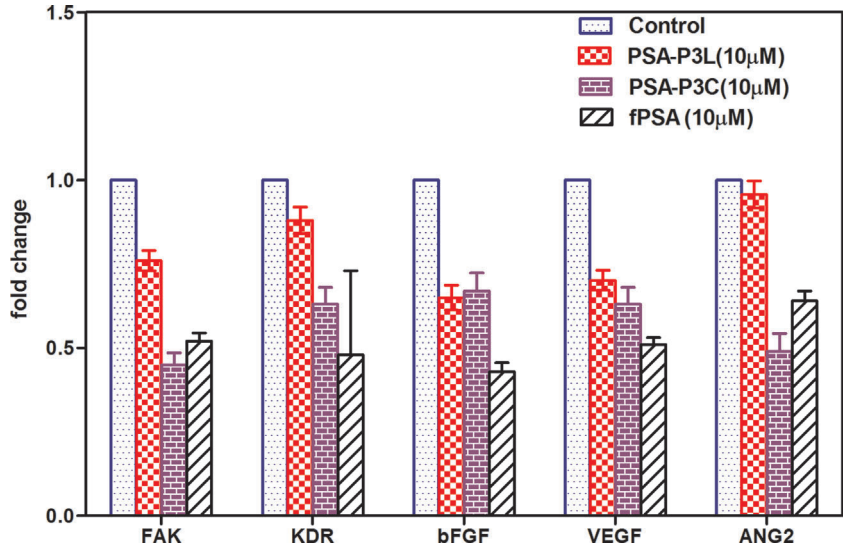

Fig. 3. Gene expression levels of angiogenesis-related genes FAK, KDR, bFGF, VEGF, and ANG2 in HUVEC treated with f-PSA or PSA-derived peptides PSA-P3L and PSA-P3C. Nearly confluent monolayers were treated with 10 μM f-PSA or peptide; RNA was extracted and reverse transcribed. The c-DNA was amplified by real-time QPCR using gene-specific primers. The results shown are the mean of three independent experiments.

formation by intact f-PSA generally increased with peptide concentration over the range tested, however, as shown in Figure 5, for some peptides increasing concentrations resulted in decreased inhibition of tube formation. The potency of inhibition by the linear form of Peptide three at a concentration of 10 μM approached that of f-PSA, but the level of inhibition decreased at 100 μM. In contrast, while the "circular-

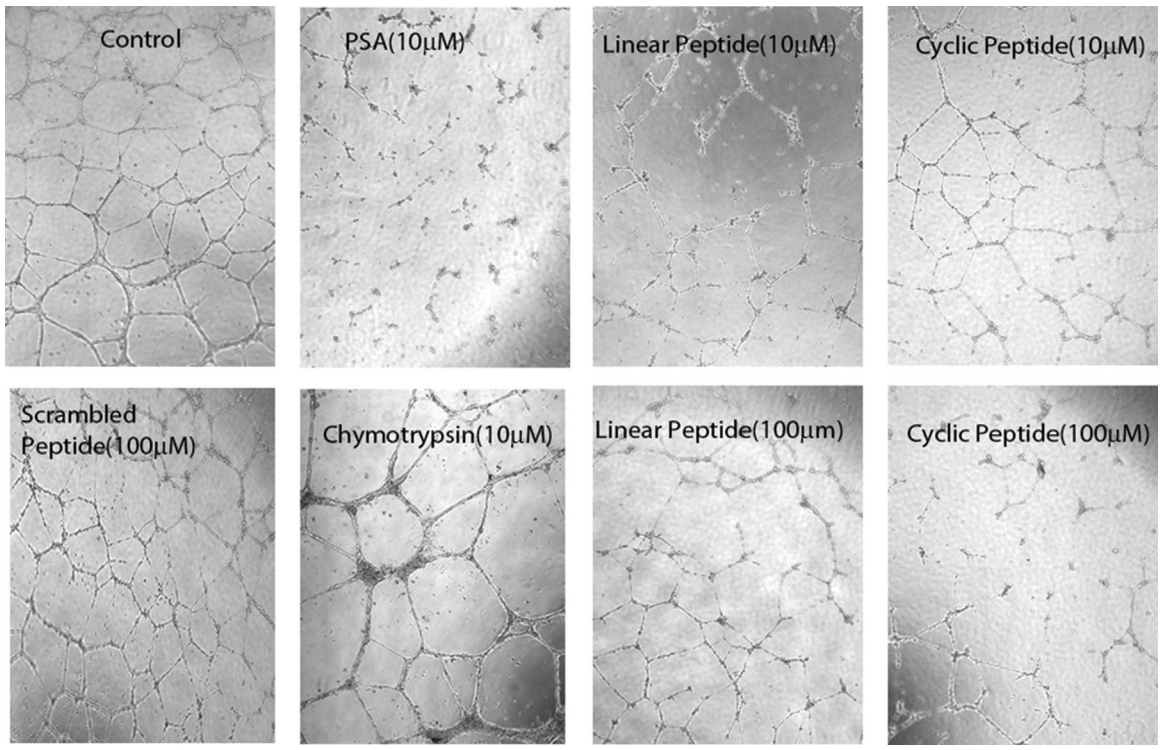

Fig. 4. Representative figure showing in vitro inhibition of angiogenesis by enzymatically active f-PSA, and PSA-derived peptides PSA-P3L and PSA-P3C. Controls used were media control, chymotrypsin (10 μM) and scrambled peptide (100 μM) HUVEC tube formation assay was performed in a Matrigel matrix. Images were processed using Analyze 7.0 and Angioquant v 1.33 to obtain average tube length in pixels for each image. All experiments were performed in triplicate.

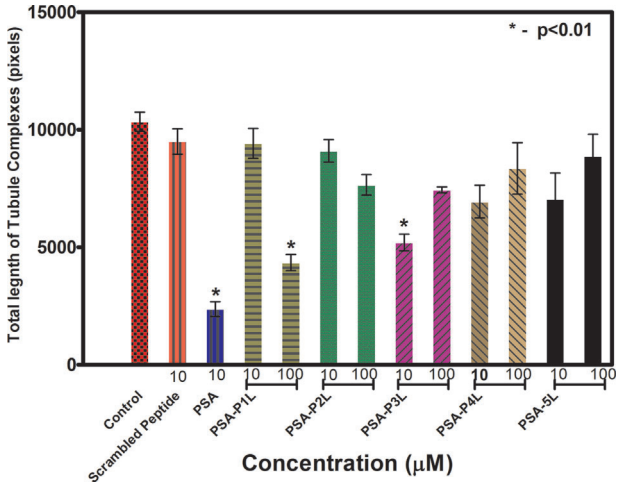

Fig. 5. Inhibition of in vitro angiogenesis by HUVECs in Matrigel using f-PSA, scrambled peptide of PSA-P3L and five PSA-derived linear peptides identified using MODELLER7: PSA-P1L, PSA-P2L, PSA-P3L, PSA-P4L, and PSA-P5L. Out of five peptides used, P1L and P3L exhibited significant inhibition of HUVEC tube formation compared to Control/scrambled peptide.

ized" form of Peptide three had slightly more inhibitory activity than the linearized form at both concentrations, inhibition by the "circularized" form of Peptide three was greatest at a 100 μM concentration, where the level of inhibition was comparable to f-PSA. The sequence within PSA-P3C was scrambled

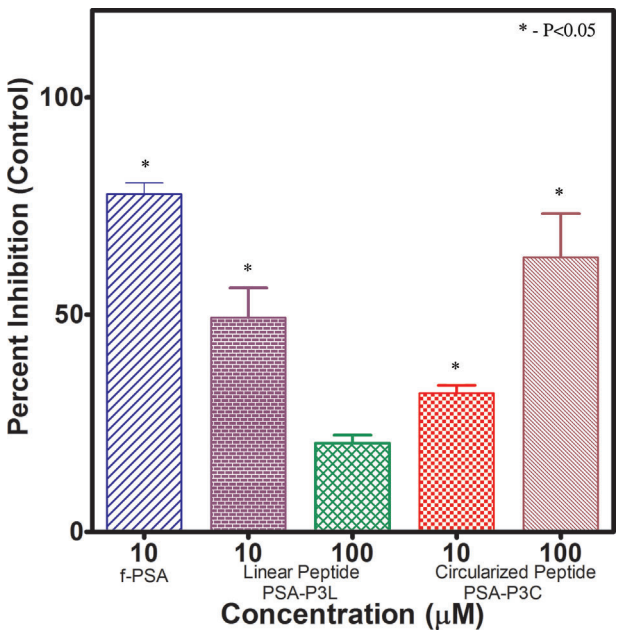

Fig. 6. Comparison of inhibition of in vitro angiogenesis by enzymatically active f-PSA versus the peptides PSA-P3L and PSA-P3C at concentrations of 10 and 100 μM.

and it resulted in loss of anti-angiogenic activity in HUVEC tube formation assay suggesting that a unique sequence is essential in expressing anti-angiogenic activity.

PSA and PSA-Derived Peptide Inhibits Angiogenesis Sprout Assay

The formation of new blood vessels during classic angiogenesis requires nascent endothelial cells to invade the extracellular matrix. To identify the biological activities of PSA and PSA mimetic peptides associated with inhibition of new blood vessel formation we used an in vitro sprouting assay. Sprouting from the bead is a recapitulation of Branching, the surface of which can be thought of as a segment of a vessel wall. HUVEC were cultured on dextran coated cytodex-3 beads and the beads embedded in fibrin gel during sprout formation. After 2–3 days HUVEC structures began to sprout from the beads, forming short, narrow cordlike structures. Subsequently, many single cells migrated away from the beads, and after 4–5 days the cords tended to disintegrate into disorganized groups of cells or single, highly migratory cells (Fig. 7). Lumens formed behind the lead cell, as previously described in vivo [38] and appeared to be surrounded by cells, as opposed to being the result of coalescing intracellular vacuoles [39,40]. PSA (10 μM) and PSA-derived cyclic peptide (PSA-P3C, 100 μM) efficiently and significantly suppressed tube formation (Fig. 7a and 7b), consistent with the anti-angiogenic or anti-proliferative effects of PSA and PSA mimetic peptide. After 4 days (Fig. 7a), the HUVEC in the treatment groups demonstrated less sprouts of shorter lengths, compared to controls, and the vessels regressed more rapidly and demonstrated numerous migrating HUVEC surrounding the bead. By Day 7, HUVEC sprouts in treatment group are markedly un-stable compared to sprouts in controls.

PSA- Derived Peptide Inhibits Cell Migration and Cell Invasion by HUVEC

The anti-angiogenic effect of PSA and PSA-derived peptides could be due to inhibition of expression of proliferation/survival related genes that results in inhibition of endothelial cell migration and association. Importantly, as reported earlier, inhibition of tube formation by HUVEC is independent of effects on endothelial cell proliferation since cell proliferation is not required for tube formation in the Matrigel assay [15]. Evaluation of the effects of f-PSA and PSA-derived peptides on migration was conducted in vitro using commercially available invasion and wound healing assay systems. Figure 8 presents images of a

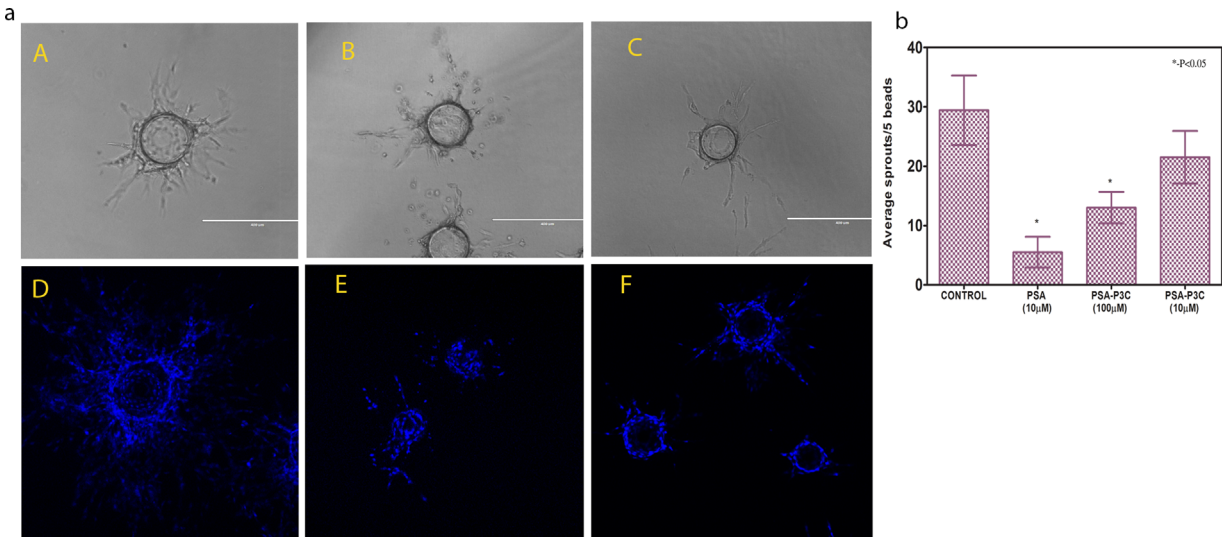

Fig. 7. PSA and PSA derived peptides inhibit capillary-like sprouting by HUVEC grown on dextran coated Cytodex beads in fibrin gels. a. Representative image shows at day 4 (A) Controls, (B) PSA, (C) PSA-P3C peptide and DAPI stained images at day 7 (D) Control, (E) PSA, (F) PSA-P3C peptide b. Average number of HUVEC sprouts per five beads on treatment of PSA and PSA derived peptides.

"wound-healing" assay, and quantitative evaluation of the extent of inhibition of cell migration into the "scratch" (repair) by the linear and cyclic forms of peptides one and three. The incubation interval was selected to allow closure of the "scratch" in control cultures. Incubation with intact f-PSA significantly inhibited repair (gap = 0.47 mm) of the damage (scratch) to the test monolayer. Incubation with the linear or cyclic forms of Peptides one and three resulted in marked inhibition of wound closure, with Peptide three producing greater levels of inhibition than Peptide one. The "circularized" form of Peptide three produced levels of inhibition of wound healing comparable to that observed for f-PSA. Similarly, both the linear and cyclic conformations of Peptide three inhibited HUVEC cell migration in the trans-well assay, with the "circularized" form of Peptide three producing inhibition comparable to that induced by f-PSA (Fig. 9). The anti-proliferative effects of f-PSA and PSA-derived peptide (PSA-P3L) were investigated by an MTT assay. Both PSA and the PSA-P3L peptide had anti-proliferative effects compared to untreated controls, however, the anti-proliferative effect was not significant between controls and peptide treated samples ($P = 0.23$) (Data not shown).

Inhibition of Anti-Angiogenic Activity of f-PSA and PSA-Derived Peptides by Complexing With ACT

In humans, the vast majority of PSA in circulation is complexed with serine proteinase inhibitors, primarily α1-anti-chymotrypsin (ACT). Consistent with the hypothesis that complexing of PSA with ACT blocks the enzymatic function of f-PSA, PSA in circulation lacks enzymatic activity [41]. Complexing of purified, enzymatically active PSA with ACT ex vivo was demonstrated to inhibit the enzymatic activity of PSA, suggesting that ACT binds in proximity to, and/or sterically hinders access, to the serine proteinase active site (Fig. 10). In the HUVEC tube formation assay, ACT alone demonstrated no inhibitory activity against tube formation (Fig. 11). Intact f-PSA reduced tube formation by almost 80%, whereas, pre-incubation of f-PSA with ACT before addition to the tube formation assay resulted in a significant reduction of the ability of f-PSA to inhibit tube formation. In contrast, pre-incubation of PSA-P3L with ACT before introduction of the peptide into the Matrigel tube formation assay had no effect on the ability of the peptide to inhibit tube formation. The lack of modulation by ACT of peptide-mediated inhibition of tube formation resulted from the lack of formation of complexes between the peptide and ACT. ACT locally produced within human prostate tissue would be available for complexing and inactivation of endogenous PSA in benign or prostate cancer tissue, providing a possible mechanism for regulation of PSA activity at the prostate tissue level.

Structural Modeling of PSA-ACT Complex

Crystal structures of cleaved and un-cleaved serine protease inhibitor (serpin) bound to trypsin,

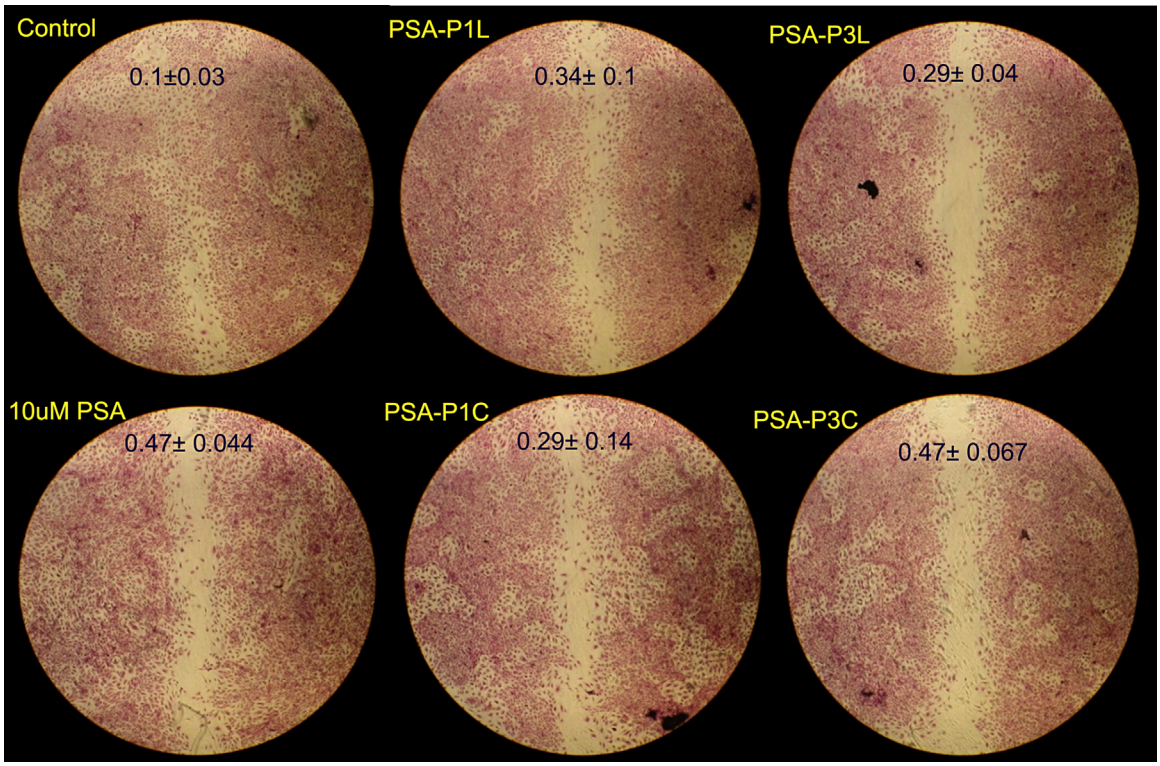

Fig. 8. Inhibitory effect of PSA and PSA-derived peptides on the migratory property of HUVEC. The commercially available CytoSelect 24-well Wound Healing Assay kit was used. Monolayers were disrupted by a plastic insert to produce a linear wound, and wound fields were measured at 0 and 24 hr using phase contrast microscopy to measure wound size.

as in 1K9O and 1EZX (Protein Data Bank), indicates substantial changes in the conformation of the trypsin post-cleavage as a result of the insertion of the serpin's reactive central loop (RCL) into the trypsin active site [42]. To understand the interaction of PSA with ACT at the molecular and atomic level, we generated structural models of free and substrate bound PSA with MODELLER7 [43,44] using the high-resolution X-ray crystal structure of KLK3 (1GVZ, 2ZCH, 2ZCL, and 2ZCK as template), and of the structure of ACT (3DLW as template), with the structure of the reactive central loop modeled from 1 K9O, and of the loop inserted into the active site of PSA modeled from 1EZX. The resulting models were subjected to molecular dynamics simulations and energy minimization using the DISCOVER module of Insight II (Accelrys, Inc). The Michaelis complex of PSA-ACT (Fig. 12) provides structural clues for the partial inactivation of PSA's anti-angiogenic activity due to complexing with ACT. RCL residues 355-ITLLSALV-362 of the ACT molecule occupy the S4-S4 protease pockets of hPSA with molecular interactions similar to that of the modelled PSA-substrate, with Leu358 occupying P1 and Ser 359 the P1' sites. The RCL is cleaved by PSA, with Ser213 of the PSA active site becoming covalently linked to Leu 358 of the RCL. Mapping the interaction zone of the RCL loop and the active site of PSA (as in Figs. 12, 13) provides a close look at the influence of PSA-ACT complex formation on the availability of the five candidate PSA-mimetic peptides explored in this study. More than 75% of peptide one is overlapped by the ACT interaction zone, whereas, less than 10% of peptide peptide three is covered reflecting the greater maintenance of PSA anti-angiogenic activity in the presence of ACT. The loop inserted model generated from 1EZX post molecular dynamics showed significant conformational changes in the PSA structure, as observed by Huntington et al [42]. In spite of this conformational change, the coverage of the candidate peptides by ACT in similar in both models. The modeling provides two significant observations. First, the primary sequence of peptides one and three in the intact PSA molecule are partially/completely covered in the PSA-ACT complex. Second, because peptides lack the tertiary structure of the active site in PSA, the peptides P1-5 should have significantly lower or no affinity to ACT in comparison with intact PSA.

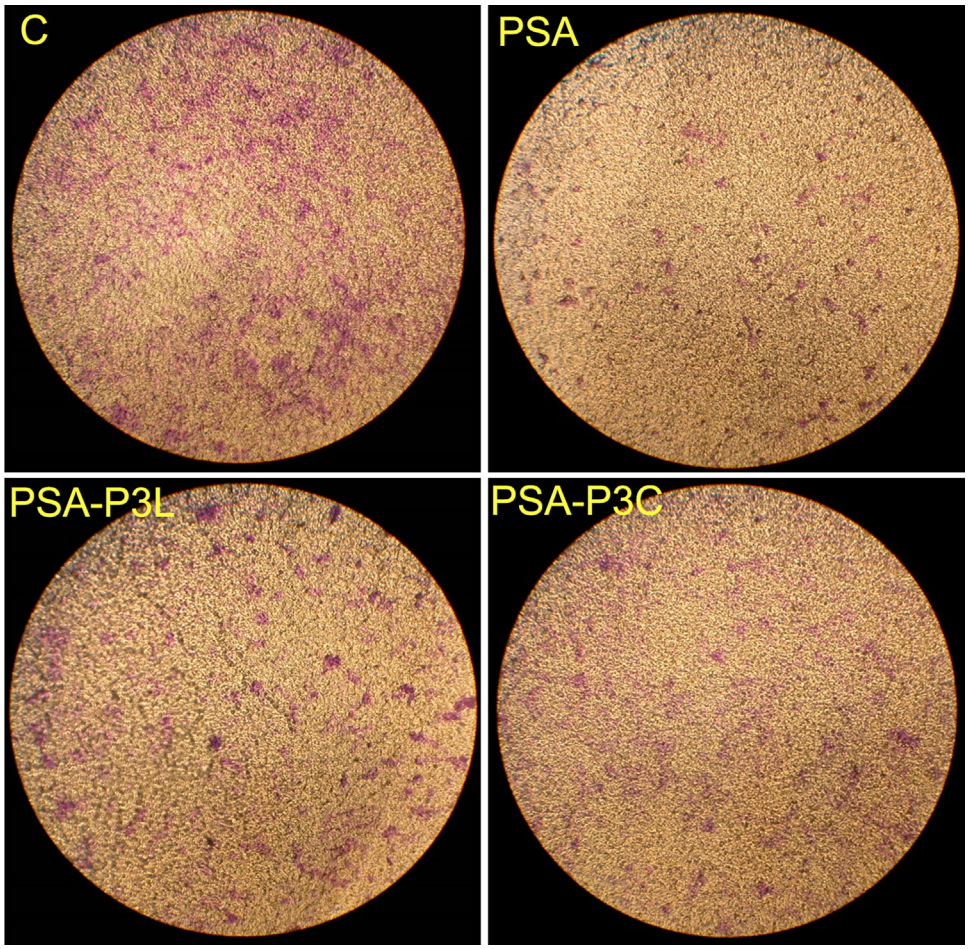

Fig. 9. Evaluation of the extent of inhibition of cell migration by the linear and circularized forms of PSA-derived peptide three (PSA-P3L & PSA-P3C) in a trans-well assay. Representative image depict invasive potential of HUVEC in the presence/absence of PSA and PSA peptides.

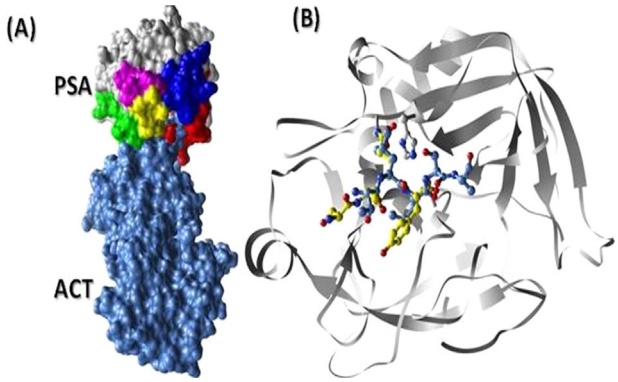

Fig. 10. Computer modeling of PSA-ACT complex (A) Predicted model of the PSA-ACT complex. (B) A portion of the ACT molecule (rendered in blue) mimics a prototypical substrate for PSA (yellow) localized in the catalytic pocket of PSA. Amino acids of ACT molecule form a covalent complex with the Ser213 residue of the active site after cleavage of the Leu358-Ser359 bond.

DISCUSSION

Prostate-specific antigen (PSA), also known as gamma-seminoprotein or kallikrein-3 (KLK3), is a serine protease enzyme encoded in humans by the *KLK3* gene [45]. The protein product of the KLK3 gene is secreted predominantly by the epithelial cells of the human prostate gland. PSA is present in nanogram range (0–4 ng/ml) in the serum of men with healthy prostates, but serum levels often are elevated in the presence of prostate cancer (CaP) or other prostate disorders (>10 ng/ml). As such, PSA has become a widely accepted biomarker for clinical diagnosis and management of prostate cancer [2,3]. However, PSA is present at the level of micrograms per gram of tissue in prostate, about 1000X the level in serum, suggesting a mechanistic role in the prostate tissue microenvironment. The function of PSA in normal prostate tissue, and in CaP tissue, is not known. While the elevated serum levels of PSA in CaP patients suggest

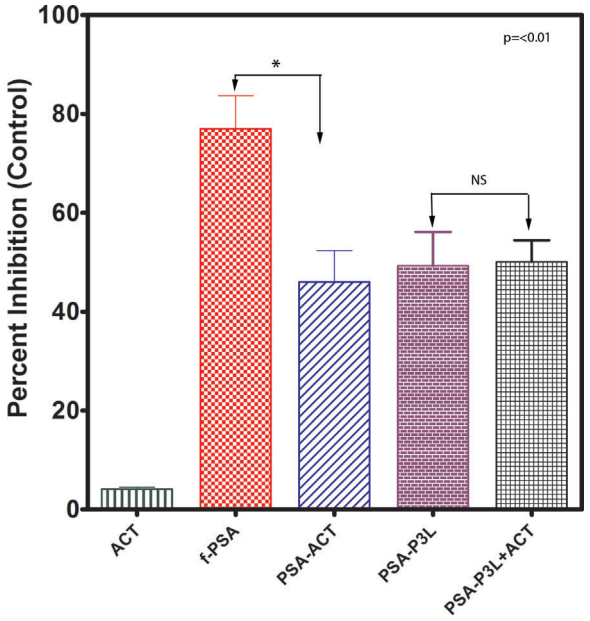

Fig. 11. Inhibition of in vitro angiogenesis by enzymatically active f-PSA, PSA-P3L and their respective complexes with ACT. PSA complxed with ACT results in significant loss in PSA anti-angiogenic activity in HUVEC Matrigel tube formation assay. On the contrary, no loss in anti-angiogenic activity was seen when PSA-P3L was mixed with ACT under identical conditions.

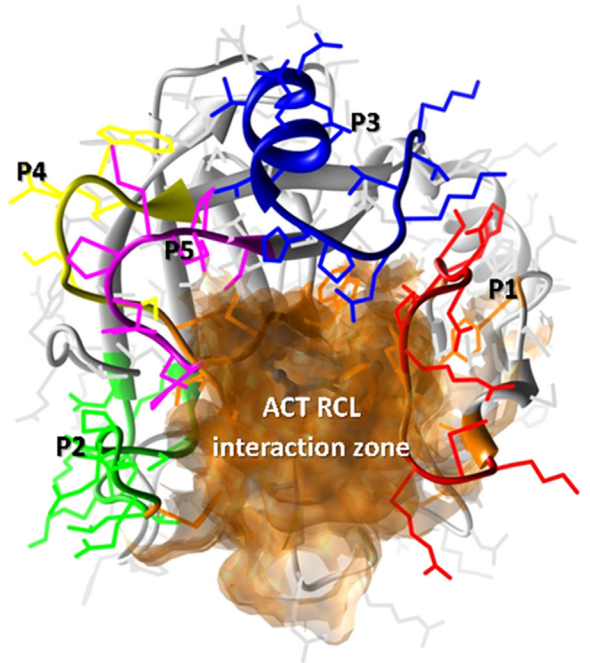

Fig. 12. Modeling of the PSA-ACT binding interface. PSA-derived candidate peptides P1, P2 and P5 are overlapped significantly by the ACT interaction zone (highlighted in orange).

a role for circulating PSA in the pathobiology of CaP, even in patients with hormone refractory cancer PSA serum levels rarely approach the level of PSA in benign prostate tissue.

The anti-angiogenic activity of PSA was first reported by Fortier et al [7]. In this study anti-angiogenic activity of PSA was linked to its enzymatic function. Mattsson et al [27] also reported that enzymatic activity of PSA is essential in inhibition of tube formation by HUVECs. They used either pro-PSA that is enzymatically "inactive" to begin with or by using monoclonal anti-PSA antibody to neutralize enzymatic activity of PSA [27]. Processing of pro-PSA by peptidases is an essential step in converting enzymatically "inactive" pro-PSA into enzymatically "active" PSA. This post translational processing results in creating an "active site" on the surface of PSA molecule required for enzymatic activity. The loss of anti-angiogenic activity of enzymatically active PSA when exposed to anti-PSA antibody may be due to loss of accessibility of the "active site" on PSA-antibody complex required for its anti-angiogenic activity. In our previous studies, we have shown that enzymatically "active" PSA and enzymatically "inactive" PSA, where enzymatic function was inhibited by Zinc, have comparable anti-angiogenic activity [15], suggesting that enzymatic function of PSA is not required for its anti-angiogenic activity. In addition, recombinant PSA mutated to ablate enzymatic activity for chromogenic PSA-specific substrate has been reported to have significant anti-angiogenic activity [8].

Importantly, the data in this study argues that enzymatic and biologic activity are not synonymous. A potential regulatory role for the f-PSA sequestered in the human prostate tissue microenvironment is suggested further by the observations that PSA expression is reduced in prostate cancer cells compared to benign prostate epithelial cells, is reduced further in poorly differentiated cancers and in castration-resistant prostate cancers, and is significantly reduced in response to anti-androgen therapies [18,21,22]. These observations suggest that PSA in the tissue microenvironment may have a tumor suppressive role, and that the complexed, biologically inactive PSA in serum cannot replace the biological function of PSA sequestered in prostate tissue that is lost with age, disease progression and iatrogenic intervention. The data presented demonstrates that complexing of PSA with ACT destroys the enzymatic function of PSA. In addition, the complexing of PSA with ACT also appears to sterically block regions of the external surface of the PSA, such as the areas of peptides one and three, that are essential to the initiation of signaling (Fig. 13). Therefore, PSA in

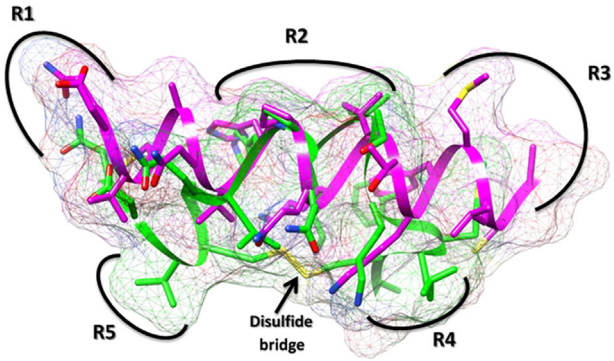

Fig. 13. Superposition of PSA linear peptide P3 (magenta) and disulfide circularized P3 (green) reveals that preserving PSA's native fold may be a requirement for enhanced anti-angiogenic activity of PSA peptides.

circulation, regardless of the level, may have little impact on the biology of a prostate cancer.

Intact f-PSA in the tissue microenvironment, in contrast, may have mechanistic roles in inhibition of, growth, invasion and metastasis of prostate cancer, as well as other human neoplasms, including breast cancer. We reported that purified, enzymatically active and enzymatically inactive, free-PSA (un-complexed to chaperone proteins) equivalently down-regulated expression of pro-angiogenic factors/cancer-related genes, including VEGF, EphA2, CYR61, Bcl2, uPA, and Pim-1 oncogene, and up-regulated expression of anti-angiogenic genes, including interferons and interferon-related genes in prostate cancer epithelial cells [6]. Furthermore, f-PSA inhibited human umbilical vein endothelial cell (HUVEC) proliferation, migration and invasion in vitro [15], and angiogenesis both in vitro and in vivo [6]. Transfection of PC-3M human prostate cancer cells with PSA before transplantation into nude mice prolonged their doubling time, and reduced their tumorigenicity and metastatic potential [11]. Lastly, PSA over-expression by cancer cells caused release of anti-angiogenic fragments by proteolytic digestion of extracellular matrix and plasminogen [12]. Therefore, PSA appears to represent both an autocrine and paracrine signal that elicits multiple biological responses, including modulation of gene/protein expression of pro-and anti-angiogenic growth factors in both prostate epithelial and endothelial cells that results in inhibition of angiogenesis and tumor growth.

To characterize further the role of PSA in the pathobiology of prostate cancer, and to evaluate the possible clinical potential of PSA-replacement as a therapeutic modality, it was important to have a reliable source of high quality PSA. At present, PSA used in research, or in clinical diagnostic laboratories, is purified from seminal plasma. These resources for generation of PSA are limited, and are associated with biological variability. Several biologically active proteins, including interferons, produced by expression in mammalian system have been evaluated clinically [46]. However, attempts to clone and express PSA, have resulted in poor expression levels that are unable to generate PSA in large enough quantities for clinical evaluation. Further, PSA systemically introduced into the blood potentially would complex rapidly with circulating protease inhibitors, primarily α1 anti-chymotrypsin and α2 macroglobulin, and by enzymatically/biologically inactivated. Consequently, this study focused on evaluation of candidate PSA-mimetic peptides derived from the primary structure of PSA as potential therapeutic modalities. Peptides that sustain substantial portions of the critical biological activity of intact PSA could be chemically synthesized at minimal cost, would be of consistent clinical quality, and since they represent a native protein sequence, should be minimally antigenic.

Five candidate PSA-mimetic peptides (PSA-P1L to PSA-P5L) were designed based upon their localization on the surface of native PSA in aqueous solution. These peptides were hypothesized to represent regions of the PSA molecule capable of interacting with target(s) on a cell surface, and independent of the active enzymatic site of PSA. None of five PSA-mimetic peptides, ranging between 11–17 contiguous amino acids in the primary sequence of PSA, demonstrated serine protease activity capable of hydrolysis of the test PSA-specific substrate. PSA-mimetic peptides (PSA-P1L and PSA-P3L) had no influence on enzymatic activity of f-PSA. Consistent with our previous report that f-PSA that was enzymatically inactivated by pre-incubation with Zn^{2+} maintained anti-angiogenic activity, two of the five PSA-derived peptides (PSA-P1L and PSA-P3L) had significant anti-angiogenic activity both in terms of modulation of expression of genes involved in blood vessel development, including FAK, KDR, bFGF, VEGF and ANG2, and in terms of inhibition of formation of endothelial cell tubes in MATRIGEL, endothelial cell migration/invasion, and in the anti-angiogenic sprouting assay. The stability of a linear peptide can be increased by introduction of cysteine residues at the flanking regions for the creation of a circularized peptide by generating a di-sulfide bond [47]. While the linear form of peptide three (PSA-P3L) produced moderate levels of inhibition relative to f-PSA, the circularized form of peptide three (PSA-P3C) produced levels of inhibition of gene expression, and inhibition of endothelial cell tube formation, comparable to that achieved by f-PSA. The native structural motif of P3

may be required for its anti-angiogenic activity, and the structure constrained by the artificial disulfide bond that circularized P3, may partially/largely preserve a "native" conformation (Fig. 13) and presents hydrophobic regions R2, R4 and R5 comparable to f-PSA, suggestive of potential hydrophobic interactions with its binding target. While the regions R1 and R2 are required for the basal activity of P3, regions R4 and R5 (absent in linear P3) may be required for higher affinity interaction with its binding target. R2-R4-R5 pharmacophore holds structural clues towards designing peptido-mimetic/small molecular anti-angiogenic compounds

These data support our hypothesis that the enzymatic activity of PSA is not required for the inhibitory action of PSA, and that the anti-angiogenic/anti-tumorigenic action of PSA is mediated through binding of a hydrophilic domain on the surface of the PSA molecule to a target on the cell membrane of prostate endothelial cells and prostate cancer epithelial cells. Consequently, PSA replacement therapy via systemic administration of PSA-mimetic peptides may provide an effective treatment to counteract the reduction/loss of the endogenous anti-tumorigenic/anti-angiogenic regulatory protein, PSA, with age, disease progression and/or ADT.

ACKNOWLEDGMENTS

This work was supported by Alliance Foundation, Roswell Park Cancer Institute, Buffalo, NY and by Margaret Duffy and Robert Cameron Troup Memorial Fund for Cancer Research of Kaleida Health and the Kaleida Health Foundation. We are thankful to Dr. Latif Kazim for his input in many aspects of this study and to Ashley Orillion for technical assistance.

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