

Detection of prostate-specific membrane antigen on HUVECs in response to breast tumor-conditioned medium

TIANCHENG LIU¹, MOHAMED JABBES², JESSIE R. NEDROW-BYERS¹,
LISA Y. WU¹, JEFFREY N. BRYAN² and CLIFFORD E. BERKMAN^{1,3}

Departments of ¹Chemistry and ²Veterinary Clinical Sciences, Washington State University,
Pullman, WA 99164; ³Cancer Targeted Technology, Woodinville, WA 98072, USA

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Abstract. Prostate-specific membrane antigen (PSMA), a well-known biomarker of prostate cancer, has also been found to be highly expressed in the neovasculature of multiple non-prostatic solid tumors. As a consequence, it has the potential to become a biomarker for tumor-associated vasculature. Herein, we describe an *in vitro* model for assessing PSMA expression associated with tube formation by primary human umbilical vein endothelial cells (HUVECs) cultured in Matrigel and induced by tumor-conditioned medium (TCM) derived from human breast cancer cells (MDA-MB-231). In contrast to vascular endothelial growth factor (VEGF)-containing endothelial cell medium, TCM induced higher expression of PSMA in HUVECs. The vessel-like tubes were detected by imaging with fluorescent PSMA inhibitors. Consequently, this *in vitro* model is expected to enable subsequent studies aimed at determining the role of PSMA in angiogenesis and factors that induce it.

Introduction

The cell-surface enzyme prostate-specific membrane antigen (PSMA) is an important biomarker and target in prostate cancer research (1). PSMA is up-regulated and strongly expressed on prostate tumor cells and as a consequence, it has attracted significant attention as both an imaging and therapeutic target for prostate cancer (2-4). In addition, PSMA is selectively expressed in the neovasculature of multiple non-prostatic solid tumors, including breast, kidney, bladder, lung, colon, renal, transitional cell, pancreas, neuroendocrine, and gastric cancers, but it is not expressed in normal vascular endothelium (5-8). The recent successful vascular targeting of metastatic

tumors in patients with advanced solid tumors using the humanized anti-PSMA monoclonal antibody J591 radiolabeled with ¹¹¹Indium, serves as proof-of-concept that PSMA is a valid biomarker for targeted imaging and therapy of non-prostatic tumors (9,10).

The unique enzymatic activities of PSMA (11-13) and the resolution of its crystal structure (14-16) have enabled the development of various chemical inhibitor scaffolds for this enzyme-biomarker (17-24). Recently, the successful deployment of PSMA inhibitors as targeting motifs for imaging and therapeutic agents suggests that such constructs can serve as pharmacokinetic alternatives to antibodies (25-30). These studies also support the concept that such compounds may also be applied as diagnostic and therapeutic agents targeted to PSMA-positive (PSMA+) tumor-associated vasculatures of various non-prostatic tumors.

Although PSMA expression in tumor neovasculature of patients has been identified by immunohistochemical methods, there are limited examples of *in vitro* neovasculature models that can be used to evaluate the effectiveness of PSMA-targeted imaging or therapeutic agents. In addition, there are equally few *in vitro* studies that have examined the tumor factors that induce PSMA expression in vascular cells. Therefore, we set out to address these gaps in the current study. First, the tube formation capability of HUVECs (human umbilical vein endothelial cells) cultured with or without Matrigel was determined with VEGF-containing endothelial cell medium or tumor cell conditioned medium (TCM) derived from human breast cancer cells (MDA-MB-231 and MCF-7). Visualization of tube formation was obtained by phase contrast light microscopy. Second, the presence of PSMA on HUVECs cultured under the same conditions was detected by immunofluorescence analysis and further analyzed by quantitative real-time RT-PCR. We also examined the effectiveness of two fluorescent PSMA inhibitors [Fig. 1: FAM-X-CTT-54 (IC₅₀ = 0.35 nM) (26) and Cy5.5-CTT-54 (IC₅₀ = 1.6 nM) to selectively label HUVECs cultured in TCM derived from MDA-MB-231 cells]. Our data suggest that factors secreted by highly metastatic MDA-MB-231 breast tumor cells potentiate PSMA expression in Matrigel-cultured HUVECs. These results are consistent with the observations that greater PSMA expression was limited to the tumor-associated neovasculature of various non-prostatic metastatic lesions.

Correspondence to: Dr Clifford E. Berkman, Department of Chemistry, Washington State University, Pullman, WA 99164-4630, USA
E-mail: cberkman@wsu.edu

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Materials and methods

Preparation of Cy5.5-CTT-54. The general procedure followed the protocol previously described (26,27,31). In brief, a solution of 1 μ mol Cy5.5 mono-reactive NHS Ester (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) in 120 μ l DMSO was added to a stirred solution of the inhibitor core 1 (3 μ mol, 150 μ l of 20 mM in H₂O), and 30 μ l of 1 M NaHCO₃. The reaction mixture was stirred for 8 h in the dark at room temperature. The pH of the resulting solution was then adjusted to 9.3 by the addition of 6 μ l of 1 M Na₂CO₃. The unreacted inhibitor core 1 was scavenged by stirring with 50 mg of Si-Isocyanate resin (SiliCycle, Inc., Quebec, Canada) overnight at room temperature. The solution was subsequently centrifuged (9,000 rpm, 10 min) and the supernatant was lyophilized in a 2 ml microcentrifuge tube. Unreacted and/or hydrolyzed Cy5.5-NHS was removed by successively triturating the lyophilized solid with 1 ml portions of DMSO and centrifuging the mixture (1 min at 13,000 rpm) after each wash; this process was repeated 10 times. The Cy5.5-CTT-54 was dissolved in 50 mM Tris buffer (pH 7.5) to give a final concentration of 1 mM (approximately 800 μ l).

IC₅₀ determination for Cy5.5-CTT-54. Inhibition studies were performed as described previously with only minor modifications (17,26). Working solutions of the substrate (N-[4-(phenylazo)-benzoyl]-glutamyl- γ -glutamic acid, PABGgG) and inhibitor were made in TRIS buffer (50 mM, pH 7.4). Working solutions of purified PSMA were diluted in TRIS buffer (50 mM, pH 7.4 containing 1% Triton X-100) to provide from 15 to 20% conversion of substrate to product in the absence of inhibitor. A typical incubation mixture (final volume 250 μ l) was prepared by the addition of either 25 μ l of an inhibitor solution or 25 μ l Tris buffer (50 mM, pH 7.4) to 175 μ l Tris buffer (50 mM, pH 7.4) in a test tube. PABGgG (25 μ l, 10 μ M) was added to the above solution. The enzymatic reaction was initiated by the addition of 25 μ l of the PSMA working solution. In all cases, the final concentration of PABGgG was 1 μ M while the enzyme was incubated with five serially diluted inhibitor concentrations providing a range of inhibition from 10 to 90%. The reaction was allowed to proceed for 15 min with constant shaking at 37°C and was terminated by the addition of 25 μ l methanolic TFA (2% trifluoroacetic acid by volume in methanol) followed by vortexing. The quenched incubation mixture was quickly buffered by the addition of 25 μ l K₂HPO₄ (0.1 M), vortexed, and centrifuged (10 min at 7,000 g). An 85 μ l aliquot of the resulting supernatant was subsequently quantified by HPLC as previously described (32,33). IC₅₀ values were calculated using Kaleida Graph 3.6 (Synergy Software).

Endothelial cell culture. Primary human umbilical vein endothelial cells (HUVECs) were obtained from Lifeline Cell Technology (Walkersville, MD), and cultured in complete Vasculife medium (Lifeline Cell Technology) containing 2% fetal bovine serum (FBS), 10 mM L-glutamine, 5 ng/ml recombinant human vascular endothelial growth factor (rh-VEGF), 5 ng/ml recombinant human epidermal growth factor (rh-EGF), 5 ng/ml recombinant human basic fibroblast growth factor (rh-bFGF), 15 ng/ml recombinant human insulin-like growth factor 1 (IGF-1), 50 μ g/ml ascorbic acid, 1 μ g/ml hydrocortisone

hemisuccinate, and 0.75 units/ml heparin sulfate, and maintained in a humidified incubator at 37°C and 5% CO₂. HUVECs may be sub-cultured by detachment of 0.05% Trypsin-0.02% EDTA solution once the culture is 80-90% confluent and actively proliferating. HUVECs passaged 3-6 times were successfully archived with the freezing medium (10% DMSO, 10% FBS in Vasculife medium) for future use.

Preparation of tumor cell conditioned medium. This protocol was based on a previous report with minor modifications (34). The breast cancer cell lines MCF-7 and MDA-MB-231 were respective gifts from Dr Margaret E. Black and Dr David W. Koh (Washington State University-Pullman). The human prostate cancer cell lines LNCaP and PC-3 were obtained from the American Type Culture Collection (Manassas, VA). The breast cell lines were cultured in Eagle's minimum Essential medium (EMEM) with 10% FBS in T75 flasks in a humidified incubator at 37°C and 5% CO₂. The prostate cancer cell lines were cultured under the same conditions except in RPMI-1640 medium. At 100% confluence of cell monolayer, the old medium was removed, and cells were washed three times in serum-free RPMI-1640 and incubated with 10 ml serum-free RPMI-1640 each flask for 24 h. Tumor cell conditioned medium (TCM) was centrifuged for 10 min at 3,000 g (4°C) and the supernatants were collected, filtered through a 0.22 μ m filter, and stored at -20°C until use.

Tube formation on Matrigel. The day before the fluorescence-labeling experiments, growth factor-reduced Matrigel (BD Biosciences, Bedford, MA) was thawed overnight at 4°C on ice and, the day of experiment, plated on the bottom of BD Falcon 2-well Cultureslides at 200 μ l/each well, and left at 37°C for 30 min for gelification. Approximately 10⁵ cells/well were seeded on Matrigel-coated 2-well Cultureslides and incubated for 18 h with 2 ml complete Vasculife medium or TCM in a humidified incubator at 37°C and 5% CO₂. The tube formation was visualized using a compound light microscope (Olympus BH-2, Olympus Optical Co. Ltd., Tokyo, Japan) at x20 magnification (objective lens numerical aperture = 1.25). Digital images were obtained using a digital camera system (Jenoptik ProgRes Camera, C12plus, JENOPTIK Laser, Optik, System GmbH, Jena, Germany) mounted on the micro-scope.

Immunocytochemistry detection of PSMA. The mouse monoclonal antibody 7E11 was provided by Cytogen Corporation (Princeton, NJ). The goat anti-mouse secondary antibody-TRIC was obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Immunofluorescence detection of PSMA was conducted as we previously described (24). As a positive control for this method of detection, LNCaP cells were cultured for 3 days on the slides under normal conditions (24). HUVECs cultured on the slides with or without Matrigel were washed twice in 1X PBS buffer (phosphate buffered saline), fixed in 4% paraformaldehyde in PBS for 20 min at room temperature, and permeabilized with cold-methanol for 5 min at -20°C. The fixed cells were blocked with 1% BSA, PBS for 30 min and incubated with primary antibody 7E11 (300X diluted in PBS containing 1% BSA) for 60 min at room temperature. After washing, the cells were incubated with a secondary antibody

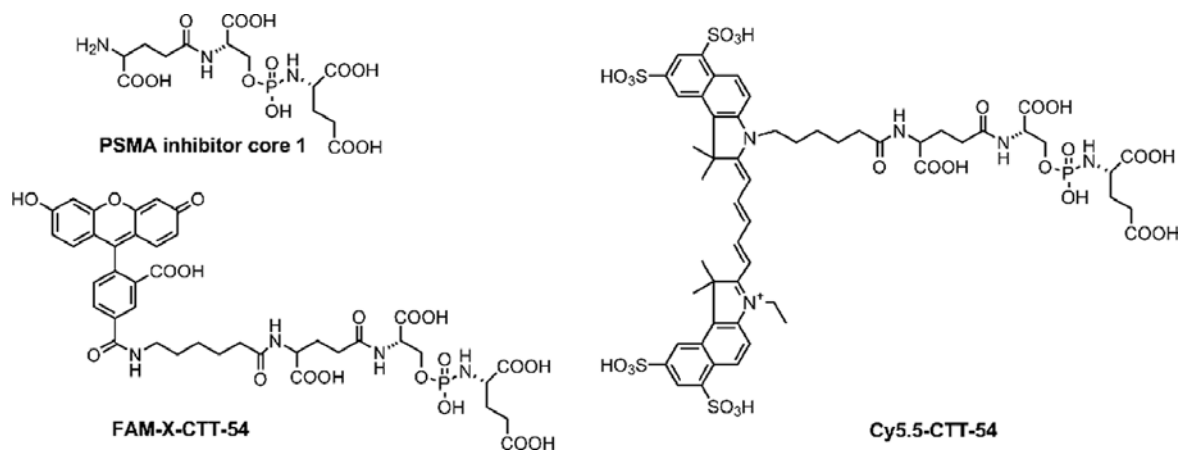


Figure 1. Structures of phosphoramidate peptidomimetic inhibitor core 1, and its fluorescent dye conjugates: FAM-X-CTT-54 and Cy5.5-CTT-54.

(goat anti-mouse IgG-TRITC, 50X diluted in 1% BSA, PBS) for 60 min, counterstained with Hoechst 33342 (Invitrogen-Molecular Probes, Carlsbad, CA), and mounted in Vectashield® Mounting Medium (Vector Laboratories, Inc., Burlingame, CA) for confocal microscopy.

Cell-labeling with fluorescent PSMA inhibitors. Cells cultured on the 2-well Culture slides with or without Matrigel were washed twice with warm medium A (phosphate-free RPMI-1640 containing 1% FBS), then incubated with 1 ml of either fluorescent inhibitor (10 μ M) in warm medium A for 2 h in a humidified incubator at 37°C and 5% CO₂. All the above treated cells were washed three times with cold-KRB buffer pH 7.4 (mmol/l: NaCl 154.0, KCl 5.0, CaCl₂ 2.0, MgCl₂ 1.0, HEPES 5.0, D-glucose 5.0) and fixed with 4% paraformaldehyde in KRB for 20 min at room temperature. The cellular nuclei were counterstained with Hoechst 33342, then the cells were mounted in Vectashield Mounting Medium for microscopy.

Fraction of tumor cell conditioned medium. The MDA-MB-231 TCM was fractionated through 30, 10 and 3 kDa molecular weight cut-off centrifugal filter devices (Millipore Corporation, Bedford, MA) according to the manufacturer's instructions. The fractions: >30, 10-30, 3-10 and <3 kDa were collected and stored at -20°C until use. The effects on tube formation and PSMA expression of HUVECs in the presence of the individual fractions with Matrigel were evaluated.

Confocal laser scanning microscopy. Cells were visualized under 25x (for FAM-X-CTT-54) or 40x (for Cy5.5-CTT-54) oil immersion objective using an LSM 510 META laser Scanning Microscope. Hoechst 33342 was excited with a Diode laser (405 nm) and the emission collected with a BP420-480 nm filter. FAM-X-CTT-54 staining was excited using 488 nm from an Argon laser, and the emission collected with a LP505 nm filter. PSMA immunofluorescence was excited using 543 nm from a HeNe laser, and the emission collected with a BP560-615 nm filter. Cy5.5-CTT-54 staining was excited using 633 nm from a HeNe laser, and the emission collected with a LP 650 nm filter. To reduce interchannel crosstalk, a multi-tracking technique was used and images were taken at a

resolution of 1,024x1,024 pixels. Confocal scanning parameters were set up so that the control cells without treatment had no fluorescent signal from background. The imaging colors of fluorescent Dyes (Hoechst 33342, FAM and TRITC) were defined as blue, green and red, respectively. As the emission wavelength of the near-infrared fluorescent dye Cy5.5 is beyond visible ranges, fluorescence pseudocolor of Cy5.5 was assigned as red. The pictures were edited by National Institutes of Health (NIH) Image J software (<http://rsb.info.nih.gov/ij>) and Adobe Photoshop CS2.

Quantitative real-time RT-PCR. HUVECs cultured in Matrigel were released without damage and recovered using BD Matri-Sperse Cell recovery solution (BD Biosciences, Franklin Lakes, NJ). Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA) from HUVECs cultured under experimental conditions in MCF-7 or MDA-MB-231 cell TCM, or VasculLife media with VEGF added and from LNCaP cells as a positive control. Contaminating DNA was removed using TURBO DNA free kit (Ambion, Austin, TX). RNA was cleaned to yield high purity. RNA was converted to cDNA using the SuperScript Vilo cDNA synthesis kit (Invitrogen, Carlsbad, CA). The PSMA primers and TaqMan MGB probes were designed with primer Express 3.0 (Applied Biosystems, USA). In addition the PSMA primers were also designed to span first intron region to avoid DNA contamination and differentiate PSMA from PSM' (35). cDNA was amplified by PCR using the primers PSMA151F (5'-CCCTTCATTGACCTCAACTACATGGT-3') and PSMA472 R (5'-TGATGTTCTCAGCTTTCAA TTCATCC-3'). The product was cloned into competent vectors using the Topo-TA cloning kit (Invitrogen, Carlsbad, CA). The sequence of the insert in the clones was confirmed with dideoxynucleotide sequencing. Quantitative real-time PCR was performed as previously described (36). A standard curve for the PSMA gene was constructed with five 10-fold serial dilutions of the plasmid from 3.0x10⁷ to 3.0x10³. The experimental samples were amplified with following primer pair PSMA343F (5'-TGGCGGGTGGCTTCTTT-3') and PSMA423R (5'-AGG AGTAATGTTAGTAGCTTCATTGGAGG-3'), and the PCR products were further quantified with the probe PSMA173P containing a fluorophore and quencher (5' FAM-CTCTTCGG

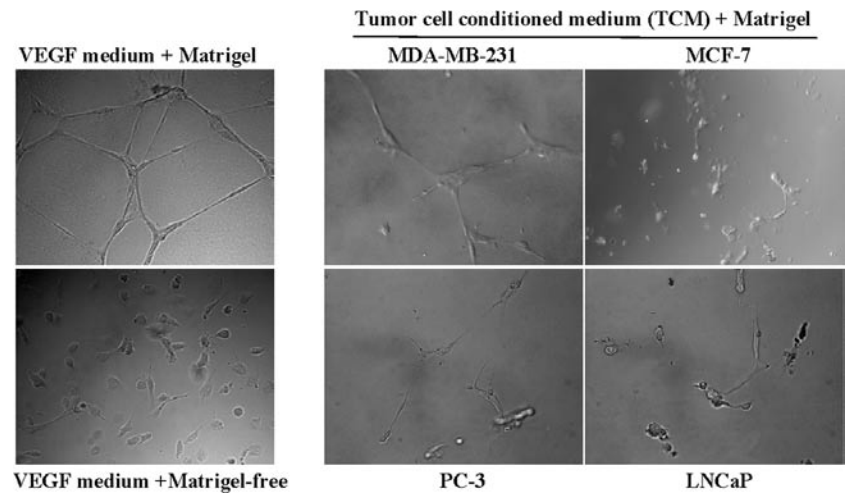


Figure 2. Tube formation by HUVECs on Matrigel. HUVECs were plated on Matrigel in the presence of VEGF-containing Vasculife medium, tumor cell conditioned medium (from MDA-MB-231, MCF-7, PC-3 and LNCaP, respectively). HUVECs cultured in the presence of VEGF-containing Vasculife medium but in the absence of Matrigel served as control samples. The phase contrast photographs were captured by an inverted light microscope at magnification x20.

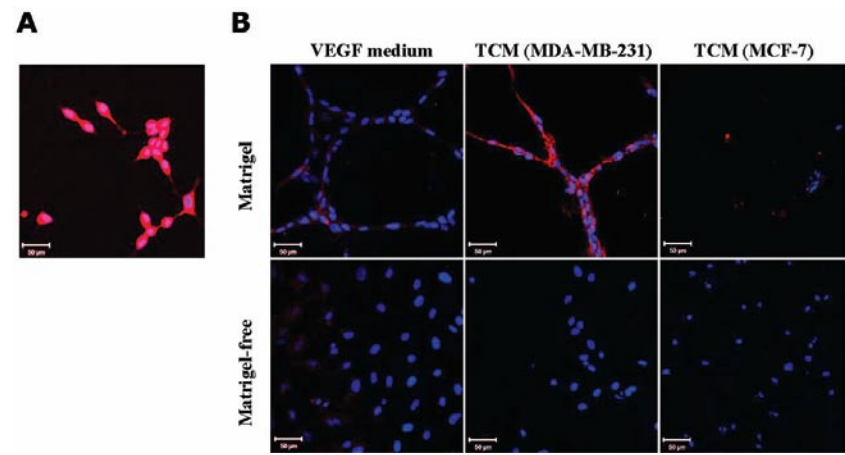


Figure 3. Immunofluorescence detection of PSMA on HUVECs. (A) LNCaP cells as a PSMA-positive control sample. (B) HUVECs plated with or without Matrigel in the presence of VEGF-containing Vasculife medium or tumor cell conditioned medium from MDA-MB-231 and MCF-7 cells. Immunofluorescence signals (red) for PSMA were clearly detected on the tube-like formations by HUVECs cultured in TCM from MDA-MB-231 cells with less PSMA detected on HUVECs cultured in either Matrigel in VEGF-contained Vasculife medium or TCM from MCF-7 cells. No PSMA was detected on HUVECs cultured in the absence of Matrigel. Cell nuclei were counterstained with Hoechst 33342 (blue). The cellular imaging was visualized by confocal microscopy; distance scale is 50 μ m.

GTGGTTTAT-MGB 3'). A standard threshold was set and CT values were converted to number of copies per μ g of RNA. Differences were tested for significance using a one-way ANOVA with significance set at $P<0.05$.

Results

TCM-induced tube formation. HUVECs seeded on Matrigel formed vessel-like tube formation networks in VEGF-containing Vasculife medium. The tube formation also was observed for HUVECs cultured in TCM from MDA-MB-231 cells. In contrast, HUVECs formed only incomplete, short tube-like structures in TCM from MCF-7, PC-3, and LNCaP cells, or cultured separately without Matrigel in VEGF-containing Vasculife medium (Fig. 2).

PSMA expression in TCM-induced vessel-like tubes. The mouse monoclonal anti-PSMA antibody 7E11, specifically binds to the cytoplasmic domain of PSMA and has been widely utilized to detect PSMA by Western blotting or by immunofluorescence (11,37). In a positive control study, a strong fluorescence signal for PSMA on LNCaP cells was detected using 7E11-based immunofluorescence (Fig. 3A). This same protocol was further employed to detect PSMA on HUVECs. Compared with little or no detectable PSMA on HUVECs cultured in VEGF-containing Vasculife medium or MCF-7 TCM, with or without Matrigel, considerable expression of PSMA in MDA-MB-231 TCM-induced vessel-like tubes was detected by immunofluorescence. On the other hand, only MDA-MB-231 TCM without Matrigel cannot induce PSMA expression on HUVECs (Fig. 3B). It also was noted that HUVECs were not viable in

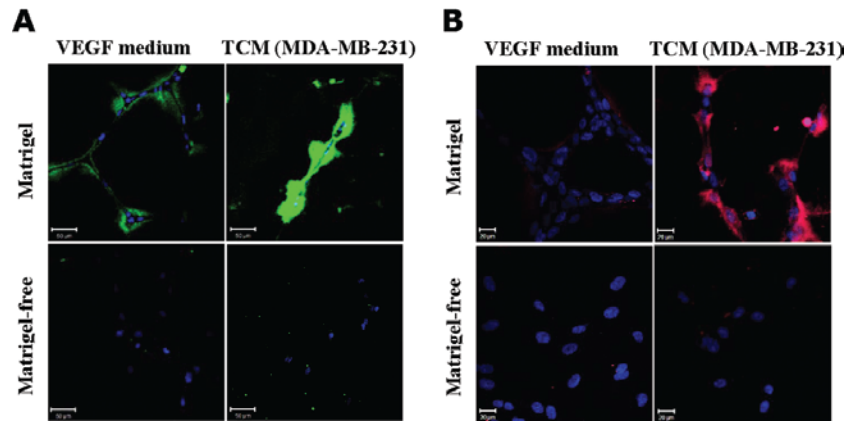


Figure 4. Fluorescent inhibitor-affinity labeling of PSMA in HUVECs. HUVECs were plated with or without Matrigel in the presence of VEGF-contained Vasculife medium or tumor conditioned medium from MDA-MB-231 cells. Both FAM-X-CTT-54 labeling (green, A) and Cy5.5-CTT-54 labeling (red, B) of PSMA was clearly detected in TCM-induced tubes with less on HUVECs cultured in Matrigel in the presence of VEGF-contained Vasculife medium, and none observed on HUVECs cultured in Matrigel-free conditions. Cell nuclei were counterstained with Hoechst 33342 (blue). Cellular imaging of FAM-X-CTT-54 labeling was visualized at magnification x25 by confocal microscopy, distance scale is 50 μ m. Cellular imaging of Cy5.5-CTT-54 labeling was visualized at magnification x40 by confocal microscopy, distance scale is 20 μ m.

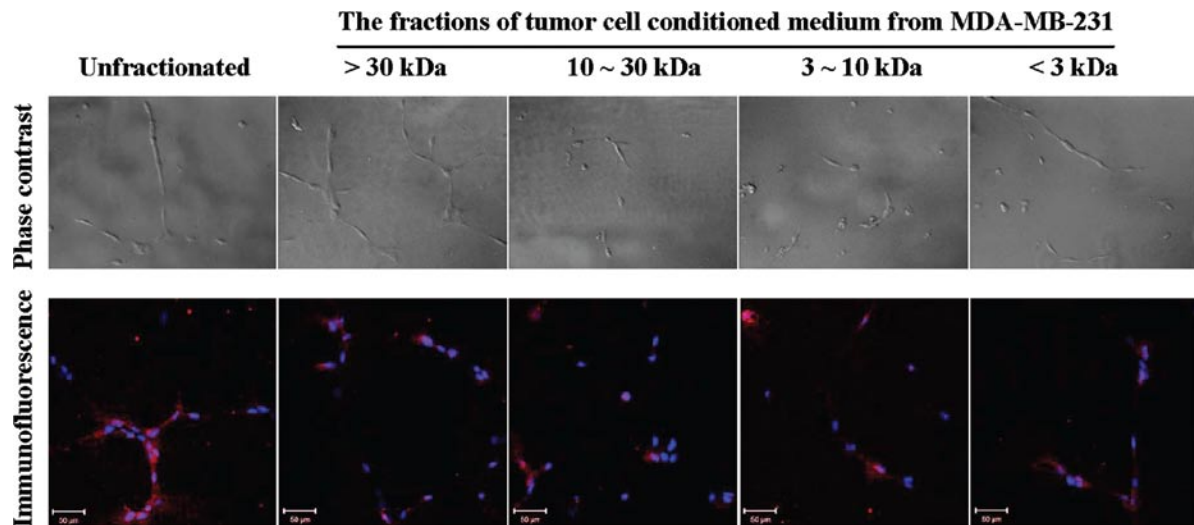


Figure 5. The effects on tube formation and PSMA expression with fractions of tumor conditioned medium from MDA-MB-231 cells. HUVECs were plated on Matrigel in the presence of TCM from MDA-MB-231 (unfractionated) and decreasing size fractions. (Top) Phase contrast photographs were captured by an inverted light microscope at magnification x20. (Bottom) Cellular immunofluorescence of PSMA was visualized at magnification x25 by confocal microscopy. The nuclei were counterstained with Hoechst 33342 (blue). The distance scale is 50 μ m.

MCF-7 TCM without Matrigel, which were characterized by nuclear shrinking and fragmentation (Fig. 3B). Based on the above results, it is clear that presently unknown factors secreted by MDA-MB-231 induces both tube formation and PSMA expression for HUVECs cultured on Matrigel, and also support the viability of HUVECs cultured in the absence of Matrigel.

Affinity labeling of PSMA in TCM-induced vessel-like tubes. FAM-X-CTT-54, a specific PSMA fluorescent inhibitor was designed and evaluated for PSMA-targeted fluorescence imaging of LNCaP cells in our previous studies (24,26). In this study, a new, near infrared fluorescence inhibitor Cy5.5-CTT-54 was synthesized and its high inhibition against PSMA

was revealed with IC_{50} value at 1.6 nM. Furthermore, FAM-X-CTT-54 and Cy5.5-CTT-54 were employed for fluorescence imaging of PSMA-positive HUVECs. Consistent with the results for the immunofluorescence study above, the greatest labeling by these fluorescent PSMA inhibitors was observed for MDA-MB-231 TCM-induced vessel-like tubes with Matrigel, weak signals for HUVECs cultured on Matrigel in VEGF-containing Vasculife medium, and no signal detected for matrigel-free VEGF-containing Vasculife medium or MDA-MB-231 TCM cultured HUVECs (Fig. 4).

The cellular effects of fractioned TCM. The MDA-MB-231 TCM-induced vessel-like tube formation was dependent on factors from two fractions from the TCM: >30 and more

interestingly, <3 kDa (Fig. 5). Furthermore, the effect of two fractions is additive in inducing PSMA expression (Fig. 5).

Quantitative real-time RT-PCR. The experimental and standard curve samples amplified with 92.1% efficiency. HUVECs cultured in MDA-MB-231 TCM expressed 3.72×10^5 (SEM 1.42×10^4) copies of PSMA per μg of RNA. HUVECs cultured in VEGF-containing VascuLife medium expressed 2.11×10^5 (SEM 1.80×10^4) copies of PSMA per μg of RNA. HUVECs cultured in MCF-7 TCM expressed 1.60×10^5 (SEM 1.11×10^4) copies of PSMA per μg of RNA. LNCaP cells expressed 8.31×10^7 (SEM 2.53×10^6) copies of PSMA per μg of RNA. Differences between groups were significant ($P < 0.001$).

Discussion

Estrogen receptor negative (ER⁻) breast cancer has been clinically recognized as being more aggressive and having a poorer prognosis than ER⁺ breast cancer. In our current study, MDA-MB-231 (ER⁻) and MCF-7 (ER⁺) cells were chosen as *in vitro* models to represent these two different progressive stages of breast cancer. Based on our data, MDA-MB-231 (ER⁻) cells promoted greater angiogenesis with higher PSMA mRNA transcription and protein expression in HUVECs cultured in Matrigel than MCF-7 (ER⁺) cells. These results are consistent with the higher levels of VEGF and MMP-9 secreted by MDA-MB-231 cells compared to MCF-7 cells (34,38).

With respect to the enzyme-biomarker PSMA, an interesting question now remains as to what in TCM from MDA-MB-231 is correlated with greater expression of PSMA in HUVECs. Based on the present PSMA immunofluorescence and affinity-labeling data for HUVECs cultured in Matrigel with VEGF-containing medium, it is apparent that VEGF levels are not mainly correlated with PSMA expression as only weak signals were observed for these conditions. Interestingly, TCM from MDA-MB-231 cells was unable to induce any detectable level of PSMA on HUVECs cultured in the absence of Matrigel. Therefore, our current data suggest that factors both >30 and <3 kDa from TCM secreted by MDA-MB-231 cells in combination with the Matrigel environment induces PSMA expression on HUVECs.

Inducible PSMA specific expression on angiogenic vasculature suggests that PSMA participates in neovessel growth in developing tumors. Experimental evidence to further support this hypothesis is based on results from Shapiro's group in which they observed that in either PSMA-null mice, or in PSMA wild-type mice treated with a PSMA inhibitor, angiogenesis was significantly impaired in the Matrigel implants (39). In our current study, *in vitro* PSMA expression on cultured HUVECs was specifically limited to growth conditions with MDA-MB-231-TCM in Matrigel. Together these data suggest that PSMA may also be a clinically relevant biomarker for tumors undergoing vascularization and consequently a predictive marker for those tumors likely to respond to antiangiogenic therapy. The successful *in vitro* model described herein is expected to enable further studies that elucidate the relationship between PSMA expression and tumor angiogenesis with a goal to develop tumor-vascular targeting agents for imaging and therapeutic applications.

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