Expression of Prostate-Specific Membrane Antigen (PSMA), Increases Cell Folate Uptake and Proliferation and Suggests a Novel Role for PSMA in the Uptake of the Non-Polyglutamated Folate, Folic Acid

Veronica Yao,[1] Clifford E. Berkman,[2] Joseph K. Choi,[2] Denise S. O'Keefe,[1] and Dean J. Bacich[1]*

[1]*Department of Urology, University of Pittsburgh, Pittsburgh, Pennsylvania*
[2]*Department of Chemistry, Washington State University, Pullman, Washington*

BACKGROUND. Prostate specific membrane antigen (PSMA) is a unique folate hydrolase that is significantly upregulated in prostate cancer. In a mouse model, PSMA is able to facilitate prostate carcinogenesis, however, little is known about the mechanism by which this occurs. As PSMA is able to hydrolyze polyglutamated folates, and cancer cells proliferate directly in response to available folate, we examined if expression of human PSMA in PC-3 cells confers a proliferative advantage in a microenvironment with physiologically relevant folate levels.
METHODS. Proliferation and folate uptake of PC-3 prostate cancer cells expressing human-PSMA or vector alone was assessed in media containing low (LF; 1 nM), physiological (PF; 25 nM), or high (HF; 2.3 μM) folate with or without poly-γ-glutamated folate (Pte-Glu_5) or folic acid, and a specific inhibitor of the enzymatic activity of PSMA, 2-(phosphonomethyl)-pentanedioic acid (2-PMPA). Folic acid was tested for its ability to competitively inhibit the enzymatic activity of PSMA.
RESULTS. Proliferation of PC-3-PSMA cells grown in the presence of poly-γ-glutamated folate, was significantly higher than that of PC-3-vector cells, an advantage which was attenuated by the addition of 2-PMPA. In media containing physiologic levels of folate, PSMA expression increased folic acid uptake approximately twofold over non-expressing cells. Folic acid was able to inhibit hydrolysis of *N*-[4-(phenylazo)-benzoyl]-glutamyl-γ-glutamic acid (PABGgG) by PSMA in a competitive inhibition assay.
CONCLUSION. These findings implicate PSMA in both the metabolism of polyglutamated folates, and in the uptake of monoglutamated folates. Under conditions of LF or PF levels, PSMA gives cells expressing it a proliferative advantage. *Prostate* © 2009 Wiley-Liss, Inc.

KEY WORDS: **folic acid; prostate cancer; 2-(phosphonomethyl)-pentanedioic acid; one-carbon metabolism**

Denise S. O'Keefe and Dean J. Bacich contributed equally to this work.

Grant sponsor: Department of Defense Prostate Cancer Research Program; Grant numbers: W81XWH-050-1-0015, W81XWH-07-1-0146; Grant sponsor: American Foundation for Urological Diseases/American Urological Association Education and Research (AFUD/AUAER); Grant sponsor: NIH; Grant number: 5R21CA124892; Grant sponsor: Wendy Will Case Cancer Foundation.

*Correspondence to: Dr. Dean J. Bacich, PhD, Department of Urology, University of Pittsburgh, 5200 Center Avenue, Room G-39, Pittsburgh, PA 15232. E-mail: bacichdj@upmc.edu
Received 21 May 2009; Accepted 1 September 2009
DOI 10.1002/pros.21065
Published online in Wiley InterScience (www.interscience.wiley.com).

INTRODUCTION

The American Cancer society predicts that there will be nearly 200,000 new cases of prostate cancer diagnosed and almost 30,000 deaths from this disease in 2009 [1]. Relatively little is known about the molecular events that underlie the development and progression of prostate cancer. Prostate specific membrane antigen (PSMA), is a type II membrane protein that is highly expressed in prostatic intraepithelial neoplasia (PIN) and in primary and metastatic prostate cancers [2]. Normal and hyperplastic prostate tissues predominantly express a truncated form of PSMA designated PSM′ [3]. Expression of PSMA in cancer tissues correlates with the stage of disease and Gleason score [4]. PSMA expression is also higher in prostate cancer cells from hormone-refractory patients [4,5] and increased PSMA expression has been shown to be an independent marker of disease recurrence [6,7].

Upregulated PSMA expression in aggressive tumors implies a role for PSMA in cancer progression, and a selective advantage for cells expressing PSMA. Indeed, the majority of transgenic mice expressing human PSMA in their prostates at a level comparable to that of human prostate tissues developed PIN-like lesions, while their wild-type siblings did not [8]. In addition, when PSMA-transgenic prostates were subjected to tissue recombination and grafted under the renal capsule of immune-deficient mice, the PIN-like lesions progressed to form adenocarcinoma [9]. Cells isolated from these recombinants were able to form colonies in semi-solid agar indicating their tumorigenic potential. In contrast, tissue recombinants derived from wild-type prostates did not exhibit adenocarcinoma. Progression from PIN-like lesions to adenocarcinoma in PSMA transgenic prostates suggests that PSMA expression facilitates prostate carcinogenesis.

Although the physiologic function of PSMA in the prostate is unknown, PSMA has been shown to cleave α-linked glutamate from *N*-acetylaspartyl glutamate (known as NAALADase activity) [10] and γ-linked glutamates from polyglutamated folates (known as folate hydrolase activity) [11,12]. Prostate cancer tissues have significantly elevated levels of PSMA enzymatic activity as compared with normal and hyperplastic prostate tissues, suggesting that the enzymatic activity of PSMA contributes to tumor progression [13]. Furthermore, Conway et al. [14], found that angiogenesis is severely impaired in PSMA-null mice and that the enzymatic activity of PSMA was required for endothelial cell invasion in vitro. This strongly suggests that the enzymatic activity of PSMA plays a key role in prostate carcinogenesis and progression.

Recently, Ghosh et al. [15], found that expression of PSMA in PC-3 cells reduced the invasiveness of these cells, whereas knockdown of PSMA expression increased the invasiveness of LNCaP cells, and the expression of PSMA mutants lacking enzymatic activity reduced the impact of PSMA expression on invasiveness. However, these findings contradict the association between PSMA expression and severity of the disease as well as studies using PSMA-transgenic and PSMA-null animals. In addition, Ghosh et al., performed their experiments in standard RPMI 1640 media, which contains about 100× the level of folate found in normal human serum [16]. When we carried out similar experiments but in media containing physiologically relevant folate levels, PSMA-expressing PC-3 cells were more invasive relative to their vector alone counterparts, and the level of invasion was decreased in response to folic acid addition to the media, suggesting that folic acid is able to block the enzymatic site of PSMA [9]. We have also previously found that PSMA expression provides LNCaP cells with a growth advantage in media containing LF (<1 nM) and PF (25 nm), supplemented with 100 nM poly-γ-glutamated folate (Pte-Glu_5) [16], and this advantage was lost when the cells were exposed to the excess folate levels found in regular media [17]. We speculated that the growth advantage in folate-limiting media potentially occurred via the ability of PSMA-expressing cells to hydrolyze the gamma-glutamyl tail of folate polyglutamates, resulting in folates that can be taken up by cells for replication. Indeed, inhibition of the folate hydrolase activity of PSMA with 2-(phosphonomethyl)-pentanedioic acid (2-PMPA) reduced the growth advantage of LNCaP cells. Therefore, the microenvironment in which cells are grown is an important consideration for analysis. This is especially true for folate, a compound integral for the metabolic processes of the body [18], as the prostate has a particularly high requirement for folate [19].

In our previous study, we utilized LNCaP and DU-145 cells to investigate if the folate hydrolase activity of PSMA provides PSMA-expressing cells with a growth advantage in a microenvironment containing a physiologically relevant level of folate [17]. However, there are many differences between LNCaP and DU-145 cells aside from PSMA expression. In this study, we demonstrate that, at physiologically relevant folate levels and in the presence of poly-γ-glutamated folate, PC-3 cells expressing human PSMA (PC-3-PSMA) possess a growth advantage as compared to PC-3 cells transfected with vector only (PC-3-vector). Using 2-PMPA, we were able to show that PSMA provides this growth advantage through its folate hydrolase activity. Surprisingly, PSMA expression resulted in a nearly twofold increase in uptake of tritiated folic acid,

a non-polyglutamated folate, suggesting a novel mechanism for PSMA action. These findings are particularly interesting in light of the recent report that men supplemented with 2.5 times the recommended daily allowance for folate are statistically significantly more likely to develop prostate cancer than their non-supplemented counterparts [20].

MATERIALS AND METHODS

Materials

The human prostate cancer cell line PC-3 was obtained from the American Type Culture Collection (Rockville, MD). Cell culture media were purchased from Invitrogen Corporation (Carlsbad, CA). Pteroyl-pentaglutamic acid (Pte-Glu_5; cat. No. 16.255) was obtained from Schircks Laboratory (Jona, Switzerland). 2-PMPA was kindly provided by MGI Pharma (formerly Guildford Pharmaceuticals) (Bloomington, MN). MTT Assay Kit was purchased from Chemicon (Temecula, CA).

Plasmid Construction and Cell Transfection

The pLNCX-PSMA plasmid was constructed on the basis of pLNCX2 vector (Clontech, Mountain View, CA) and contained a Col E1 origin of replication, a neomycin resistance gene, and DNA encoding for PSMA under the regulation of the immediate early CMV promoter.

PC-3 cells were transfected with the pLNCX-PSMA plasmid or vector alone using the FUGENE6 transfection reagent (Roche Diagnostics, Indianapolis, IN). Briefly, PC-3 cells were transfected with 3 μg of plasmid DNA pre-incubated with 2 μl of FUGENE 6 in serum-free RPMI 1640 medium for 30 min at room temperature. Cells were then grown for 72 hr in standard RPMI 1640 medium containing 5% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. Stable clones were selected for by the addition of 800 μg/ml neomycin to the media.

Tissue Culture

PC-3-PSMA or PC-3-vector cells were maintained in standard RPMI 1640 medium (Invitrogen Corporation; containing 2.3 μM folic acid, referred to as ''high folate'' (HF)) supplemented with 10% FBS (1.8–2 ng/ml folate; Atlanta Biologicals, Lawrenceville, GA), L-glutamine (2 mM), and penicillin/streptomycin. Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2. Folate-free RPMI 1640 medium (Invitrogen Corporation) supplemented with 10% FBS, L-glutamine, and penicillin/streptomycin was referred to as ''low folate'' (1 nM folic acid, LF) medium. Folate-free RPMI 1640 media supplemented with 10% FBS, L-glutamine, and penicillin and streptomycin was mixed with standard RPMI 1640 media to produce a final folate concentration of 25 nM, and was referred to as ''physiological folate'' (PF) media [18]. PC-3-PSMA and PC-3-vector cells were cultured in LF, PF, and HF media.

Western Blot for PSMA Expression

PSMA expression in PC-3-PSMA and PC-3-vector cells was determined by Western Blot. Whole cell lysates were prepared with lysis buffer containing Triton X-100 and complete EDTA-free protease inhibitor cocktail (Roche, Nutley, NJ). Approximately 15 μg of protein lysate was run on an 8% SDS–PAGE gel and transferred onto a PVDF membrane for 15 min at 15 V. The membrane was blocked with TBST containing 3% casein overnight at 4°C and incubated with PSMA (CYT-351) (Cytogen Corp., Princeton, NJ) then β-actin (Sigma–Aldrich, St Louis, MO) monoclonal primary antibodies for 1 hr at room temperature. The primary antibody was detected by sequential incubation of the membrane with goat anti-mouse IgG secondary antibody (Pierce Biotechnology, Rockford, IL), then chemiluminescent reagents (ECL Western Blot Kit; Amersham, Piscataway, NJ), and exposure to X-Omat LS film (Kodak, Rochester, NY) until bands were visualized. LNCaP cells, which express high levels of PSMA, were used as a positive control for PSMA expression.

PSMA NAALADase Activity

To determine that the PSMA enzymatic activity was functional in PC-3-PSMA cells, NAALADase activity was assayed using [^{3}H]-*N*-acetyl-aspartyl-glutamate (NAAG) as previously described with some modifications [12]. Cell membranes were prepared from PC-3-PSMA cells by homogenization in 50 nM Tris (pH 7.4) and centrifugation at 1,000*g* for 5 min, followed by ultracentrifugation of the supernatant at 70,000*g* for 35 min. The pellet was then resuspended in 50 nM Tris (pH 7.4) buffer with 0.1% Triton X-100 and homogenized. The assay sample contained approximately 5 μg of membrane protein, 200 nM [^{3}H] NAAG, and Tris–HCl pH 7.4. After incubation at 37°C for 30 min, the sample was loaded onto a chromatography column containing AG 1×8 Resin 200–400 mesh (formate form) (Bio-Rad Laboratories, Hercules, CA) pre-conditioned with distilled water. The column was washed with 1 M formic acid into scintillation mixture and total counts determined using a Beckman LS 8100 scintillation counter. Parental PC-3 cells and PC-3-vector cells were used as negative controls whereas LNCaP cells were used as a positive control.

Folate Uptake and Retention

PC-3-PSMA or PC-3-vector alone cells were grown in RPMI 1640 (folate-free; Invitrogen Corporation) containing limiting folic acid (2 nM) for 1 week to deplete them of their intracellular folate stores. After this time, the media was replaced with RPMI 1640 (folate-free) containing 25 nM [^{3}H] folic acid (folic acid, diammonium salt, [3′,5′,7,9-^{3}H]-; specific activity 47.9 Ci/mmol, Moravek Biochemicals, Brea, CA), which is not polyglutamated, and cells were grown for a further 24 hr. The cells were extensively washed with PBS, counted, and isotopic content determined using a liquid scintillation counter (Wallac 1409 DSA liquid scintillation counter).

Growth Assays

To determine the effect of PSMA on cell growth, PC-3-PSMA, and PC-3-vector cells, which had been maintained in LF medium for 3 weeks (to deplete endogenous folate), were seeded in 96-well plates and grown in LF, PF, or HF medium with or without 100 nM Pte-Glu_5 for 21 days. To determine if the folate hydrolase activity of PSMA is the mechanism by which PSMA confers a growth advantage, cells were grown in LF, PF, or HF medium containing 0, 1, 10, 25, and 50 μM 2-PMPA, with or without 100 nM Pte-Glu_5 for 21 days. Cells were re-fed weekly with fresh media and inhibitor and did not require splitting. To determine if PSMA gives a growth advantage when folic acid is the sole source of folate, PC-3 cells expressing PSMA or vector alone were grown in media containing 25 nM folic acid for 1 week to clear them of stored folates. They were then plated into 96 well plates (675 cells/well), in media containing 25, 50, or 75 nM folic acid for 8 days. Cell proliferation was analyzed using an MTT assay Kit according to the manufacturer's instructions (Chemicon, Temecula, CA). Briefly, 10 μl MTT solution was added to each well and incubated at 37°C for 4 hr. The reaction was stopped by adding 100 μl HCL/isopropanol solution and absorbance measured on a Bio-Rad ELISA plate reader (Bio-Rad Laboratories) at a wavelength of 595 nm and a reference wavelength of 630 nm. Data are expressed as percentage growth, relative to absorbances of cells grown in HF media (100% growth). All experiments were performed in quintuplet wells.

IC_{50} Determination for Folic Acid

Inhibition studies were performed as described previously with only minor modifications [21–23]. Working solutions of the substrate (*N*-[4-(phenylazo)-benzoyl]-glutamyl-γ-glutamic acid, PABGgG) and folic acid (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 μl) was prepared by the addition of either 25 μl of an inhibitor solution or 25 μl Tris buffer (50 mM, pH 7.4) to 175 μl Tris buffer (50 mM, pH 7.4 containing 1% Triton X-100) in a test tube. PABGgG (25 μl, 10 μM) was added to the above solution. The enzymatic reaction was initiated by the addition of 25 μl of the PSMA working solution. In all cases, the final concentration of PABGgG was 1 μM 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 μl methanolic TFA (2% trifluoroacetic acid by volume in methanol) followed by vortexing. The quenched incubation mixture was quickly buffered by the addition of 25 μl K_2HPO_4 (0.1 M), vortexed, and centrifuged (10 min at 7,000*g*). An 85 μl aliquot of the resulting supernatant was subsequently quantified by HPLC as previously described. IC_{50} values were calculated using KaleidaGraph 3.6 (Synergy Software).

Statistical Analysis

Data are presented as mean ± standard deviation (SD), unless otherwise stated. Statistical analyses were carried out using SigmaStat version 3.5 and are *t* tests unless otherwise noted. Differences were considered significant only when the *P*-value was <0.05.

RESULTS

Protein Expression and Enzymatic Activity

Western Blot and NAALADase assays were performed on lysates from PC-3-PSMA clones to confirm that they express enzymatically functional PSMA protein. Approximately 15 μg of whole cell lysates from PC-3-PSMA clones, a PC-3 vector clone, and LNCaP cells were examined for their PSMA protein expression. The LNCaP cell line, which endogenously expresses PSMA, was used as a positive control for PSMA expression. As shown in Figure 1A, PC-3-PSMA clones B9, B11, and B16 and LNCaP cells expressed PSMA, but non-transfected and PC-3-vector cells did not express PSMA. The level of PSMA expressed in PC-3-PSMA cell clones B9 and B11 was similar to the level expressed in LNCaP cells. NAALADase assays were performed on membrane preparations from PC-3-PSMA clone B9, PC-3-vector B3, and LNCaP cells utilizing an established quantitative assay that measures the liberation of [^{3}H]-glutamate from [^{3}H]-NAAG

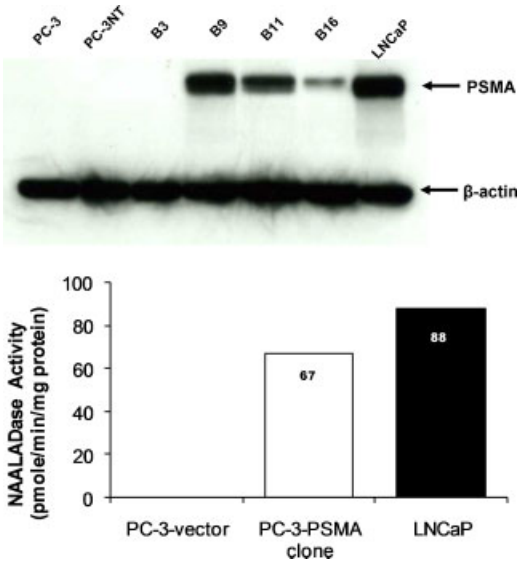

Fig. 1. PSMA protein expression (**A**) and enzymatic activity (**B**) in PC-3-PSMA clones (B9, B11, B16), PC-3-vector (B3), and LNCaP cells. A: Whole cell lysates were isolated and PSMA protein expression was determined by Western blot. B: Membrane lysates from the PC-3 PSMA B9 and PC-3 B3 (vector) clones were isolated and NAALADase activity was assayed using by measuring the liberation of [^{3}H]-glutamate from [^{3}H]-NAAG, as described in the Materials and Methods Section. Clones B9 and B11 were used for further experiments as they express similar levels of PSMA to the LNCaP prostate cancer cell line. NT = not transfected.

[12]. As shown in Figure 1B, enzymatic activity of PC-3-PSMA cells was similar to that of LNCaP cells at 67 and 88 pmol/min/mg of protein, respectively. There was no detectable NAALADase activity in PC-3-vector cells.

PC-3-PSMA Cells Have a Growth Advantage in the Presence of Poly-γ-Glutamated Folate

The effect of PSMA on cell proliferation was assessed by adding 100 nM of Pte-Glu$_5$ to either PC3 vector control cells or to PC3-PSMA cells that had been grown in medium supplemented with either LF (1 nM), PF (25 nM), or HF (2.3 μM). To clear the cells of excess stored folates, they were first grown in low-folate media for 3 weeks, and then placed in the experimental media. The proliferation rate for each cell type grown in HF was set at 100% (Fig. 2A).

PC-3-vector cells grown in LF and PF proliferated at 4.2% and 3.6%, respectively, of the rate of the cells in HF (Fig. 2B) ($P < 0.001$). The addition of Pte-Glu$_5$ resulted in a marked increase in the proliferation of PC-3-vector cells grown in LF and PF. However, these rates were still significantly lower than that of PC-3-vector cells grown in HF ($P < 0.01$ for LF and $P < 0.05$ for PF, Fig. 2B).

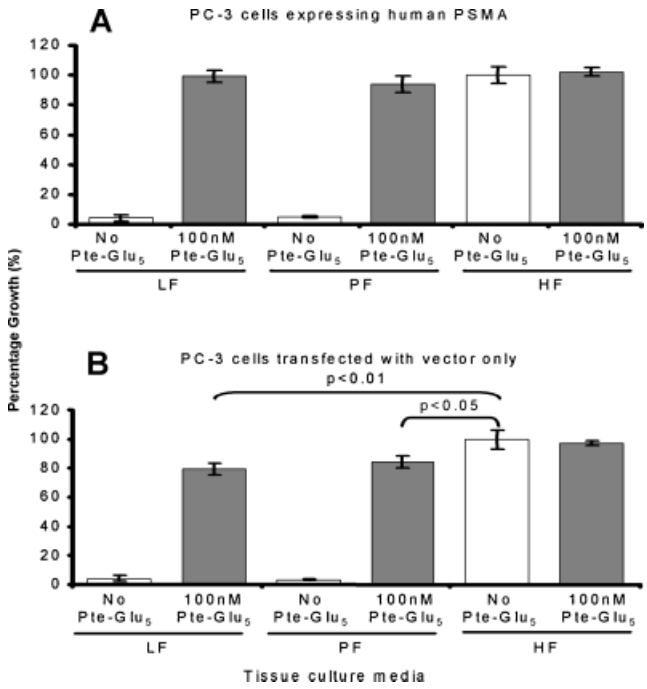

Fig. 2. PSMA expression is able to rescue cell proliferation under conditions of limiting or PF. (**A**) The growth of PSMA expressing cells was not limited relative to regular media when the cells were placed in media with limiting folic acid, and 100 nM Pte-Glu$_5$. (**B**) In contrast, PC-3 cells with vector alone had statistically significantly less growth when under limiting folate conditions relative to the same cells grown in unlimited folic acid (regular media). Shown as percentage growth of PC-3-PSMA and PC-3-vector cells in low folate (LF), physiological folate (PF), and high folate (HF) media in the presence or absence of Pte-Glu$_5$. Cells were grown in LF for 3 weeks and then seeded onto 96-well plates, at 2,000 cells per well, in quintuplet. Cells were cultured in LF, PF, and HF media in the absence (white) or presence (shaded) of 100 nM Pte-Glu$_5$ for 21 days. Proliferation was measured by MTT assay and normalized to cells grown in HF medium without Pte-Glu$_5$. Results are normalized as mean ± standard deviation.

Similar to the vector control cells, PC-3-PSMA cells grown in LF and PF proliferated at 4.1% and 4.9%, respectively, of the rate of the cells in HF ($P < 0.001$) (Fig. 2A). The addition of Pte-Glu$_5$ in the cell culture media significantly increased the proliferation of PC-3-PSMA cells grown in LF and PF media by 24.1- and 19.2-fold, respectively. In HF, addition of Pte-Glu$_5$ did not significantly alter the growth of either vector control or PC-3-PSMA cells. In contrast to PC-3 vector control cells, the addition of Pte-Glu$_5$ to PC-3-PSMA in LF or in PF increased proliferation rates to that of PC-3-PSMA cells grown in HF medium.

These results demonstrate that PSMA expression is able to fully compensate for the limit of available folates in cells grown in LF and PF levels, in the presence of Pte-Glu$_5$. Although the vector-transfected cells had a growth increase with the addition of

polyglutamated folate, the growth is statistically significantly less than when folate is not limiting.

2-PMPA Reduces the Growth Advantage Shown by PC-3-PSMA Cells in the Presence of Poly-γ-Glutamated Folate

If PSMA confers a growth advantage by hydrolyzing polyglutamated folate, then inhibition of this activity should abrogate the growth advantage of PC-3-PSMA cells. Cell proliferation was assayed in the presence of Pte-Glu_5 and the folate hydrolase inhibitor, 2-PMPA. Growth of cells with Pte-Glu_5 was set at 100%. 2-PMPA significantly reduced the proliferation of PC-3-PSMA cells grown in LF medium containing Pte-Glu_5 ($P < 0.01$) (Fig. 3A). Proliferation of PC-3-PSMA cells declined by 31.9%, 30.8%, 41.2%, and 61.6% with the addition of 1, 10, 25, and 50 μm 2-PMPA, respectively. In PF medium and Pte-Glu_5, PC-3-PSMA proliferation also declined in the presence of 2-PMPA, but only reached significance at concentrations of 25 and 50 μm ($P < 0.01$). 2-PMPA did not inhibit growth of PC-3-PSMA in HF medium, presumably due to the abundance of folic acid in this media. In contrast, 2-PMPA did not inhibit the proliferation of PC-3-vector cells grown in Pte-Glu_5, regardless of the level of folic acid (Fig. 3B). In addition, in the absence of Pte-Glu_5, 2-PMPA did not significantly alter the proliferation of either PC-3-PSMA or PC-3-vector cells (Fig. 3C,D). These results suggest that PSMA confers a growth advantage to the cells via its enzymatic activity.

PSMA Expression Confers a Growth Advantage in Medium Containing Physiologically Relevant Levels of Folate, and Increases Cellular Uptake of the Non-Polyglutamated Folate, Folic Acid

The role of PSMA as a folate hydrolase is well established. However it is also known that PSMA undergoes constitutive internalization, and that this process is significantly accelerated in the presence of an antibody targeted at the enzymatic site of PSMA [24]. We therefore investigated if PSMA might internalize the monoglutamated (alpha-linked) folate form after hydrolyzing its polyglutamated tail. We first tested this by growing PC-3-PSMA and PC-3-vector cells for 1 week in 25 nM folic acid, which is not polyglutamated, to deplete the cells' intracellular stores of folate. The cells were then plated into 25, 50, or 75 nM folic acid-containing media, grown for 8 days, and

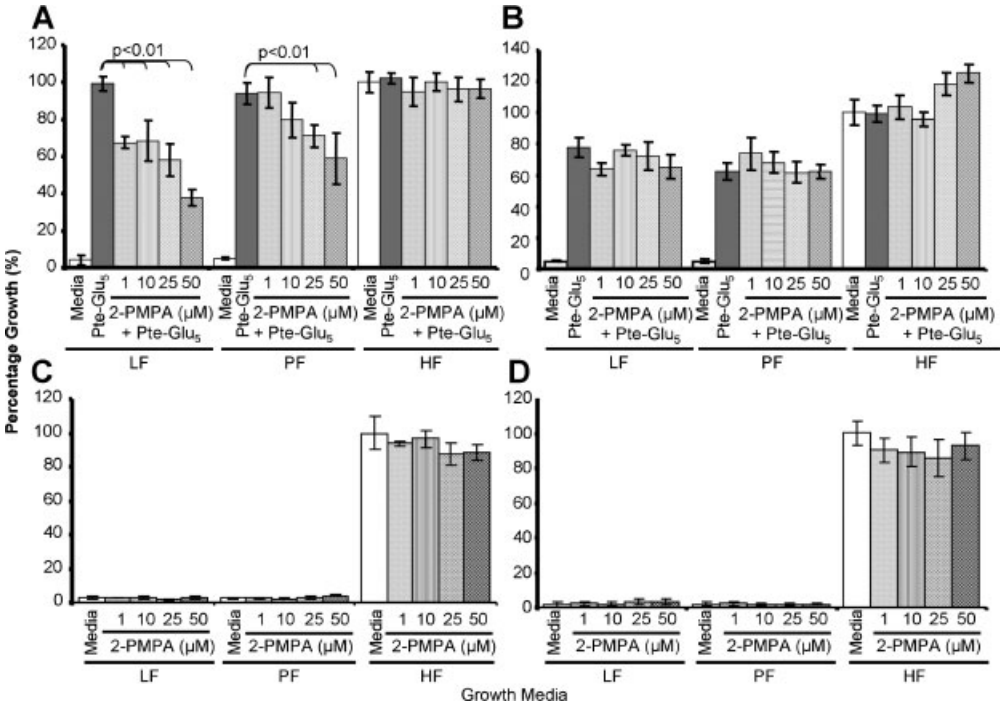

Fig. 3. The proliferative advantage conferred by PSMA expression under conditions of limiting folate is reversed by treatment with 2-PMPA. 2-PMPA is a potent and specific inhibitor of the enzymatic activity of PSMA. The effects of 2-PMPA on the proliferation of PC-3-PSMA (**A**,**C**) and PC-3-vector (**B**,**D**) cells under conditions of limited folate exposure are shown. Cells, initially grown in low folate (LF) medium, were seeded in quintuplet onto 96-well plates. Cells were then grown in LF, physiological (PF), or high folate (HF) media plus 2-(phosphonomethyl)-pentanedioic acid (2-PMPA, 0 – 50 μM), in the presence (A,B) or absence (C,D) of 100 nM Pte-Glu_5 for 21 days. Proliferation was measured by MTT assay and normalized to cells grown in HF medium alone. Results are reported as the mean ± standard deviation.

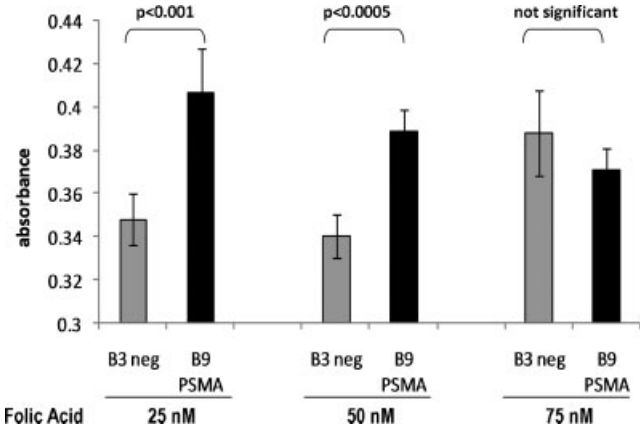

Fig. 4. Comparison of the growth of PC-3 cells +/− PSMA expression in media containing only monoglutamyl folate, folic acid as the sole folate. Cells were grown in 25 nM folate for 1 week to clear them of stored folates. They were then plated into 96-well plates, 675 cells per well, in media containing 25, 50, or 75 nM folic acid. After 8 days growth was determined by MTT assay. PC-3-PSMA clone B9 cells are represented by the black bar, PC-3-vector clone B3 cells are the gray bar. Growth was significantly increased in the PSMA expressing cells when the media contained 25 or 50 nM folic acid, however this growth advantage was lost when the level of folic acid was increased to 75 nM.

proliferation measured by MTT assay (Fig. 4). Relative to their vector-alone counterparts, PSMA expressing cells had a significant growth advantage, however, this effect was lost when the level of folic acid was increased to 75 nM. This level of folate is the minimum level required for PC-3 growth without PSMA expression, as has been shown by others [19]. Next, we examined if PSMA expressing cells were able to take up more folate than their vector alone counterparts. As shown in Figure 5, two independently derived clones of PC-3-PSMA cells were able to accumulate approximately twice as much radiolabeled folic acid as two independent clones of PC-3-vector cells ($P < 0.001$) over a 24 hr period. These findings implicate PSMA in folate uptake, and specifically in the uptake or retention of folic acid, the monoglutamyl, fully bioavailable form of the vitamin.

The Enzymatic Activity of PSMA is Inhibited by Folic Acid

PSMA expression has previously been shown to facilitate migration of PC-3 cells transfected with the PSMA cDNA through matrigel-coated membranes [9]. This effect, however, was not seen in media containing high levels of folate, and was gradually diminished under conditions of increasing folic acid [9]. These findings suggest that folic acid may be able to enter the active site of PSMA, and compete with its other substrates. Based on this and the fact that PSMA expressing cells had an increased uptake of tritiated folic acid, we wanted to determine if PSMA was directly binding to folic acid, or indirectly increasing folic acid uptake by either causing an increase in folate transporter expression or decreasing folate loss from the cell. We tested the ability of folic acid to inhibit the enzymatic activity of purified PSMA. At pH 7.4, at the IC_{50} of folic acid was determined to be 56 μM, s.e.m.

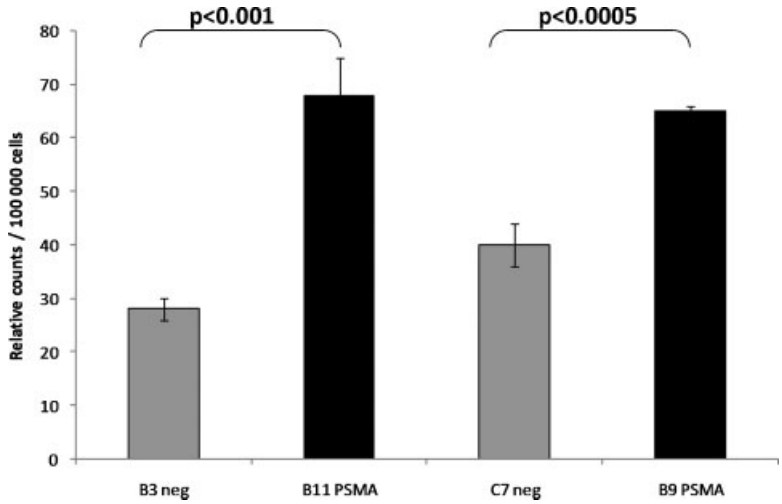

Fig. 5. PSMA expression increases cell retention of tritiated folic acid. Cells were grown for 1 week in media containing limiting folic acid (2 nM), then for 24 hr in media containing 25 nM tritiated folic acid. Intracellular retention of radiolabeled folic acid was determined by liquid scintillation analysis of cell pellets and is shown per 100,000 cells. PC-3-PSMA ($n = 6$; three experiments each clone) expressing cells imported or retained significantly more tritiated folic acid than PC-3-vector cells ($n = 6$; three independent experiments each clone). Data shown are from six independent experiments utilizing two independently derived clones for each of PC-3-vector alone and PC-3-PSMA (mean ± SD). Clone B11 was paired with clone B3, clone B9 was paired with clone C7 for the assays.

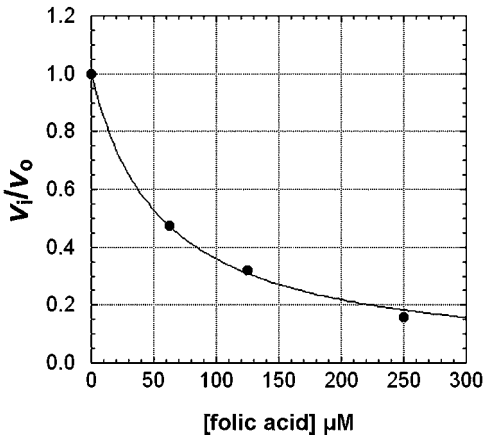

Fig. 6. IC_{50} determination for folic acid with PSMA. The ability of folic acid to inhibit hydrolysis of PABGgG by purified PSMA was measured and the IC_{50} determined to be 56 ± 2.4 μM.

2.4 μM (see Fig. 6). While these findings suggest that at least at pH 7.4 folic acid can bind PSMA, albeit weakly, it is possible that under different pH or when exposed to different monoglutamyl folate-based structures, PSMA can significantly increase folate uptake.

DISCUSSION

The prostate has a high requirement for folate that is integral to the metabolic processes within the body, such as DNA, RNA, and protein methylation, and formation of methionine for polyamine synthesis as well as for replication and growth [18,19]. In prostate cancer cells, polyamine production is significantly upregulated, and under conditions of folate deprivation, polyamine synthesis is maintained at the expense of other folate/methylation requiring reactions [19]. Although, poly-γ-glutamated folates are not typically found in the extracellular space, dead or dying prostate cells could liberate them within the prostate tumor microenvironment. As prostate cells become cancerous, they express more membrane bound PSMA, resulting in folate hydrolase activity outside the cell [2]. Since only non-polyglutamated (monoglutamated) folates can enter cells, we propose that in the LF tumor microenvironment, tumor cells with increased surface PSMA may have greater rates of proliferation as they can convert extracellular poly-γ-glutamated folate to a form that cells can import. LNCaP cells, which endogenously express PSMA, have a growth advantage in medium containing limiting folate and poly-γ-glutamated folate, but this is ameliorated by the addition of 2-PMPA, which is an inhibitor of PSMA enzymatic activity [17]. Unexpectedly, we also observed that PC-3 cells ectopically expressing PSMA had a significant growth advantage when grown under conditions of limited folate in the absence of polyglutamated folate, suggesting that PSMA may increase the uptake of monoglutamated folates.

PSMA Expression Confers a Growth Advantage in the Presence of Physiologically Relevant Levels of Mono- and Polyglutamated Folates

In this study, we assessed the proliferation of PC-3-PSMA and PC-3-vector cells in LF (1 nM), PF (25 nM), and HF (2.3 μM)-containing media, in the presence or absence of Pte-Glu_5 and the PSMA inhibitor 2-PMPA. The PC-3-PSMA clones used for this study express enzymatically functional human PSMA protein at a similar level to that endogenously expressed by the prostate carcinoma derived LNCaP cells, which is consistent with that seen in human prostate tumors.

Since rapidly dividing cancer cells depend on an abundant supply of folate, a lack of folic acid, or other folates results in an impairment of cell proliferation. Similar to LNCaP and DU-145 cells [17], the proliferation of both PC-3-PSMA and PC-3-vector cells declined when grown in LF or PF containing media as compared with cells grown in regular, high-folate media. The proliferation of PC-3-PSMA and PC-3-vector cells decreased and stabilized after 2–3 weeks of culture under low or physiologic conditions, suggesting that it takes this amount of time to deplete intracellular stores of folate within these cells (data not shown). As expected, the proliferation of PC-3 PSMA cells grown in PF medium for 2 weeks was approximately threefold greater than when they were grown in LF medium. Unexpectedly, the growth of PC-3 PSMA cells was significantly greater than PC-3-vector cells grown in PF (25 nM) for 2 weeks (data not shown). This observation may be due to the fact that it could take longer for PC-3-PSMA cells to deplete their intracellular stores of folates, and/or the presence of PSMA helps in the acquisition of folate from the extracellular environment. As the LF and PF media contain non-polyglutamated folate (in the form of folic acid) as the sole folate source, these findings hint that PSMA may increase cellular uptake of monoglutamated folates.

To examine these findings in further detail, we grew the PC-3 lines for 1 week in media containing 25 nM folic acid to decrease their intracellular folate stores, and then in 25, 50, or 75 nM folate for 1 week. Growth in media containing 25 or 50 nM folic acid was significantly increased in the PSMA expressing cells versus the PC-3 vector alone cells. The growth advantage conferred by PSMA expression was lost in media containing 75 nM folic acid (Fig. 4), which is also within the range at which the folic acid concentration is considered non-limiting for growth of PC-3 cells [19].

For this experiment the growth rate of both cell types was significantly greater than that seen in the previous two growth experiments (Fig. 4 vs. Figs. 2 and 3). This was likely due to the fact that the cells had higher intracellular folate levels at the beginning of the experiment as they were grown in media containing 25 nM folic acid for 1 week prior, whereas the cells used in the first two growth experiments were grown in 1 nM folic acid for 3 weeks prior to initiation of the experiment.

As we had predicted from our original hypothesis, the proliferation of PC-3-PSMA cells was also significantly increased when they were grown in LF and PF media containing Pte-Glu_5, compared to cells grown without it (Figs. 2 and 3). This suggests that cells expressing PSMA are able to hydrolyze extracellular Pte-Glu_5 and take up the resulting monoglutamated folate into the cell for proliferation. The presence of Pte-Glu_5 did not enhance the proliferation of PC-3-PSMA cells grown in regular, high-folate media, implying that levels of bioavailable folate, while abundant in high-folate medium, are a rate-limiting factor for proliferation of these cells in the low and physiologic media. 2-PMPA, a potent and selective competitive inhibitor of the enzymatic activity of PSMA [25], reduced the enhanced proliferation of PC-3-PSMA cells cultured in LF or PF media supplemented with Pte-Glu_5 (Fig. 3). These results suggest that the folate hydrolase activity of PSMA enables PC-3-PSMA cells to use extracellular poly-γ-glutamated folate for proliferation when bioavailable folate is growth limiting.

Although the data suggests that PSMA provides cells with the ability to hydrolyze extracellular poly-glutamated folates in order to acquire folate, it is also possible that the enhanced proliferation seen in PC-3-PSMA cells may in part, be due to the transport of Pte-Glu_5 by PSMA itself. Liu and colleagues used a quantitative cell surface biotinylation assay to demonstrate that PSMA is constitutively internalized [26]. Internalization of PSMA is mediated by the five N-terminal amino acids present in the cytoplasmic tail, and the enzymatic activity of PSMA seems not to be necessary for the internalization of PSMA [27]. The finding that an anti-PSMA antibody significantly increases the rate of internalization suggests that PSMA might function as a receptor mediating the transport of a putative ligand [26]. It is interesting to note that PSMA is a member of the M28 peptidase family, and although it is no longer enzymatically active, the transferrin receptor has 50% homology to PSMA at the amino acid level, including throughout the peptidase domain [28]. Therefore from an evolutionary point of view, the comparative structure of PSMA and the transferrin receptor suggest that it is possible either of the proteins could simultaneously possess both an enzymatic and receptor function.

Although PC-3-vector cells do not express PSMA, they also demonstrated increased proliferation when grown in folate-limited media containing Pte-Glu_5. This increase in proliferation was significantly less than that of PC-3-PSMA cells under the same culture conditions. This may have been due to contamination of the Pte-Glu_5 with de-glutamated folates in the original preparation, although this is not likely as we have previously shown that our Pte-Glu_5 is relatively pure (>95%) by HPLC analysis [17]. Alternatively, the Pte-Glu_5 may have become de-glutamated from hydrolysis by gamma glutamyl-hydrolase contributed to the media from the fetal calf serum [29], or from secreted gamma-glutamyl hydrolase from the PC-3 cells. This enzyme is expressed at very low levels in the normal prostate and in prostate cancers [30]. Gamma-glutamyl hydrolase has been shown to cleave successive end-terminal gamma-glutamyl groups from poly-L-γ-glutamyl derivatives of folate [31]. It can cleave intracellular stores of poly-γ-glutamated folates, or when secreted it can cleave extracellular poly-γ-glutamated folates [32]. While the level of glutamyl hydrolase activity has not been reported for fetal bovine serum, it is significant in mouse plasma which is capable of hydrolyzing penta-γ-glutamated folate analogs by 78% in the first 18 hr of incubation [33]. Regardless, addition of the poly-γ-glutamated folate to the PC-3 vector cells was not able to restore growth to the level seen in media not limiting in folate, while for PC-3 PSMA cells, growth was restored to the same level as when folate was not limiting.

Expression of PSMA Increases Cellular Uptake of a Non-Polyglutamated Folate, Folic Acid

The finding that PSMA expression significantly increased cell uptake of [^{3}H] folic acid under conditions of limiting folate is intriguing (Fig. 5). It is already known that PSMA increases internalization when the enzymatic site is bound by an antibody [26]; it may be possible that PSMA can bind folic acid, which is the chemically produced, bioavailable form of folate found in tissue culture media, multivitamins, and also used to fortify grains and flours for the U.S. food supply. In fact, since fortification of the food supply and the number individuals consuming supplements containing folic acid has increased, overall serum folate levels have significantly increased and unmetabolized folic acid has been found in the peripheral circulation of over 75% of subjects studied [34,35]. In our experiments, when the only folate source was folic acid and it was limiting, the PSMA-expressing cells proliferated more quickly than their non-expressing counterparts. Thus PSMA

expression increases the transport of extracellular folic acid into the cell, likely leading to the increased retention of total folates. To determine if PSMA is capable of binding folic acid, we next utilized purified PSMA to see if folic acid could inhibit the enzymatic activity. At pH 7.4, the addition of folic acid was able to inhibit PSMA substrate hydrolysis by 50% at $56 \pm 2.4\,\mu M$. It therefore seems possible that the increased uptake of tritiated folic acid by cells expressing PSMA may indeed be mediated directly by the protein itself. It is interesting to note that although the neuropeptide NAAG binds PSMA much more strongly than folic acid, there is no evidence in the literature or elsewhere that NAAG is ever found in the peripheral circulation. Likewise, although high-affinity folate receptors exist (e.g., folate receptors α, β, and γ), none of these are expressed in PC-3 cells [36]. Thus in the absence high-affinity receptors or other substrates, including polyglutamated folates, PSMA may bind and possibly transport folic acid, or other folates, into the cell.

In this study, we provide proof of principle that PSMA can bind folic acid, a folate with no additional gamma-glutamates, at pH 7.4. However there are more than 40 different folate species [37], and binding may be affected by pH as well as other factors. PSMA is known to have two pH maxima, pH 5 and 8, for its enzymatic activity, and it is known that another folate analog, methotrexate, has an IC_{50} of 10 μM against the NAAG-hydrolyzing activity of PSMA [38]. The IC_{50} of penta-γ-glutamated folate against the NAAG-hydrolyzing activity of PSMA is 13 nM [38] indicating a significantly higher affinity for PSMA than the monoglutamated form. Therefore PSMA may bind polyglutamated folates, and instead of releasing them after hydrolysis, internalize the monoglutamated form. We intend to explore the role of PSMA as a folate transporter more completely in future studies. It is interesting to note, however, that the reduced folate carrier, one of the major folate transporters, has a binding capability for folic acid in the 200 μM range at pH 7.4 [39]. In addition, at pH 7.4, the ability of the proton-coupled folate transporter (PCFT) to transport folates is low (influx K_m $\sim$56 μM [40]), but is much more active at pH5.5 (influx K_m $\sim$1.3 μM [40]) where PCFT becomes the predominate folate transporter, and transport by the RFC disappears altogether [41]. Both these major folate transporters have similar affinities for folic acid under these conditions as PSMA.

Given that PSMA resides on the luminal membrane of the cell, it will come into contact with monoglutamated folates present in prostatic secretions [42]. Therefore, the possibility exists that PSMA may also act as a transporter for monoglutamated folate, at least in the prostate. This hypothesis is supported by the discovery of an unidentified folate-binding protein in membrane solubilized prostate homogenate that is the same size as PSMA (100 kDa) [43]. Another piece of evidence supporting the potential role of PSMA as a folate transporter comes from its expression in the Bowman's capsule and proximal tubules of the murine (data not shown) and human kidneys [44]. Folate is filtered through the glomeruli of the kidney, and reabsorbed via the renal tubules. In the mouse, this process is mediated by folate receptor alpha [45]; however, there appears to be another mechanism by which folate can be reabsorbed by the kidney under conditions of low dietary folate. Folate receptor alpha/folate-binding protein 1 knockout mice exhibit 20% clearance of folate relative to creatinine, when subjected to a LF diet [45]. If folate receptor alpha were the only mechanism by which folate was reabsorbed, one would expect this number to be closer to 100%. In addition, the same study showed that folate receptor beta/folate-binding protein 2 knockout mice had no effect on folate re-absorption, implicating an as yet unknown mechanism for folate re-absorption in the kidney [45]. If the function of PSMA was solely as a folate hydrolase, it is hard to understand why it is expressed in the region of the kidney involved in folate re-uptake, as these cells should not come into contact with polyglutamated forms of folate. Thus, we propose that PSMA also functions to bind and import folate as part of the resorptive process in the renal tubules, and likely in other epithelial tissues as well. In the context of these other data, our findings are especially interesting because they suggest a new function for PSMA involving monoglutamated folates, and potentially implicate it as a novel folate receptor.

The membrane localization of PSMA and its high expression in prostate cancer cells makes PSMA an ideal therapeutic target. PSMA inhibitors are currently being investigated for their neuroprotective effects in neurological disorders such as stroke, ischemia, and other neurodegenerative diseases [46]. Inhibitors to the enzymatic activity of PSMA may offer promise in the field of oncology. Studies on the physiological role of PSMA have provided support for this idea. Conway et al. [14] reported that PSMA plays an important role in angiogenesis as a cell surface enzyme by modulating integrin signal transduction. Furthermore, treatment with 2-PMPA was able to abrogate angiogenesis; suggesting that inhibitors to the enzymatic activity of PSMA could be effective as a new anti-angiogenic therapy [14]. This study and our previous study [17] demonstrate that inhibitors to the enzymatic activity of PSMA may represent a novel approach for slowing prostate cancer growth. However more importantly, this study also highlights the importance of considering folate levels in the extracellular environment when

studying the function of PSMA in cell culture, and especially when analyzing the effectiveness of PSMA inhibitors as therapeutic agents for patients with different serum folate levels.

CONCLUSION

The results of this study suggest that PSMA provides prostate cancer cells with a growth advantage in the tumor microenvironment, possibly through its folate hydrolase activity. In addition, we may have identified a novel function for PSMA as a folate transporter, potentially resulting in increased cellular folate levels in PSMA expressing cells, and subsequently increased proliferation. In light of the recent findings that moderate levels of supplemental folic acid increases the incidence of prostate cancer [20], and the fact that prostate cancers almost universally over-express PSMA, our study may help shed light on the mechanism by which this occurs.

ACKNOWLEDGMENTS

This research was supported by the Department of Defense Prostate Cancer Research Program under the awards numbered W81XWH-050-1-0015 (DJB) and W81XWH-07-1-0146 (DJB). Views and opinions of, and endorsements by the authors do not reflect those of the US Army or the Department of Defense. This research was also partly supported by the American Foundation for Urological Diseases/American Urological Association Education and Research (AFUD/AUAER, VY and DJB), and by NIH 5R21CA124892 (DSOK) and the Wendy Will Case Cancer foundation (DSOK). We would also like to thank Diana Harya for technical assistance and Stanley Wang, for assistance with the preparation of figures for the manuscript, Dr. Cyril Barinka and Professor Joe Neale for helpful discussions regarding the potential of PSMA for binding folates, and Dr. Julie Brown for her assistance with proof-reading the manuscript.

REFERENCES

1. Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ. Cancer Statistics, 2009. CA Cancer J Clin 2009;59(4):225–249.
2. Bostwick DG, Pacelli A, Blute M, Roche P, Murphy GP. Prostate specific membrane antigen expression in prostatic intraepithelial neoplasia and adenocarcinoma: A study of 184 cases. Cancer 1998;82(11):2256–2261.
3. Su SL, Huang IP, Fair WR, Powell CT, Heston WD. Alternatively spliced variants of prostate-specific membrane antigen RNA: Ratio of expression as a potential measurement of progression. Cancer Res 1995;55(7):1441–1443.
4. Kawakami M, Nakayama J. Enhanced expression of prostate-specific membrane antigen gene in prostate cancer as revealed by in situ hybridization. Cancer Res 1997;57(12):2321–2324.
5. Wright GL Jr, Grob BM, Haley C, Grossman K, Newhall K, Petrylak D, Troyer J, Konchuba A, Schellhammer PF, Moriarty R. Upregulation of prostate-specific membrane antigen after androgen- deprivation therapy. Urology 1996;48(2):326–334.
6. Ross JS, Sheehan CE, Fisher HA, Kaufman RP Jr, Kaur P, Gray K, Webb I, Gray GS, Mosher R, Kallakury BV. Correlation of primary tumor prostate-specific membrane antigen expression with disease recurrence in prostate cancer. Clin Cancer Res 2003;9(17):6357–6362.
7. Mitsiades CS, Lembessis P, Sourla A, Milathianakis C, Tsintavis A, Koutsilieris M. Molecular staging by RT-pCR analysis for PSA and PSMA in peripheral blood and bone marrow samples is an independent predictor of time to biochemical failure following radical prostatectomy for clinically localized prostate cancer. Clin Exp Metastasis 2004;21(6):495–505.
8. Bacich DJ, Harsch KM, Heston WD. Transgenic mice expressing human Prostate-Specific Membrane Antigen (PSMA) in their prostate: A potential model for prostate cancer initiation, development and therapy; 2002 April 6-10; San Francisco.
9. Yao V, Parwani A, Maier C, Heston WD, Bacich DJ. Moderate expression of prostate-specific membrane antigen, a tissue differentiation antigen and folate hydrolase, facilitates prostate carcinogenesis. Cancer Res 2008;68(21):9070–9077.
10. Carter RE, Feldman AR, Coyle JT. Prostate-specific membrane antigen is a hydrolase with substrate and pharmacologic characteristics of a neuropeptidase. Proc Natl Acad Sci USA 1996;93(2):749–753.
11. Pinto JT, Suffoletto BP, Berzin TM, Qiao CH, Lin S, Tong WP, May F, Mukherjee B, Heston WD. Prostate-specific membrane antigen: A novel folate hydrolase in human prostatic carcinoma cells. Clin Cancer Res 1996;2(9):1445–1451.
12. Tiffany CW, Lapidus RG, Merion A, Calvin DC, Slusher BS. Characterization of the enzymatic activity of PSM: Comparison with brain NAALADase. Prostate 1999;39(1):28–35.
13. Lapidus RG, Tiffany CW, Isaacs JT, Slusher BS. Prostate-specific membrane antigen (PSMA) enzyme activity is elevated in prostate cancer cells. Prostate 2000;45(4):350–354.
14. Conway RE, Petrovic N, Li Z, Heston W, Wu D, Shapiro LH. Prostate-specific membrane antigen regulates angiogenesis by modulating integrin signal transduction. Mol Cell Biol 2006; 26(14):5310–5324.
15. Ghosh A, Wang X, Klein E, Heston WD. Novel role of prostate-specific membrane antigen in suppressing prostate cancer invasiveness. Cancer Res 2005;65(3):727–731.
16. Chanarin I. The folates. In: Baker BN, Bender DA, editors. Vitamins in Medicine. London: Heinemann Medical Books; 1980. pp 247–314.
17. Yao V, Bacich DJ. Prostate specific membrane antigen (PSMA) expression gives prostate cancer cells a growth advantage in a physiologically relevant folate environment in vitro. Prostate 2006;66(8):867–875.
18. Eto I, Krumdieck CL. Role of vitamin B12 and folate deficiencies in carcinogenesis. Adv Exp Med Biol 1986;206:313–330.
19. Bistulfi G, Diegelman P, Foster BA, Kramer DL, Porter CW, Smiraglia DJ. Polyamine biosynthesis impacts cellular folate requirements necessary to maintain S-adenosylmethionine and nucleotide pools. FASEB J 2009;23(9):2888–2897.
20. Figueiredo JC, Grau MV, Haile RW, Sandler RS, Summers RW, Bresalier RS, Burke CA, McKeown-Eyssen GE, Baron JA. Folic acid and risk of prostate cancer: Results from a randomized clinical trial. J Natl Cancer Inst 2009;101(6):432–435.

21. Wu LY, Anderson MO, Toriyabe Y, Maung J, Campbell TY, Tajon C, Kazak M, Moser J, Berkman CE. The molecular pruning of a phosphoramidate peptidomimetic inhibitor of prostate-specific membrane antigen. Bioorg Med Chem 2007;15(23):7434–7443.
22. Maung J, Mallari JP, Girtsman TA, Wu LY, Rowley JA, Santiago NM, Brunelle AN, Berkman CE. Probing for a hydrophobic a binding register in prostate-specific membrane antigen with phenylalkylphosphonamidates. Bioorg Med Chem 2004;12(18): 4969–4979.
23. Anderson MO, Wu LY, Santiago NM, Moser JM, Rowley JA, Bolstad ES, Berkman CE. Substrate specificity of prostate-specific membrane antigen. Bioorg Med Chem 2007;15(21): 6678–6686.
24. Liu H, Moy P, Kim S, Xia Y, Rajasekaran A, Navarro V, Knudsen B, Bander NH. Monoclonal antibodies to the extracellular domain of prostate-specific membrane antigen also react with tumor vascular endothelium. Cancer Res 1997;57(17):3629–3634.
25. Slusher BS, Vornov JJ, Thomas AG, Hurn PD, Harukuni I, Bhardwaj A, Traystman RJ, Robinson MB, Britton P, Lu XC, Tortella FC, Wozniak KM, Yudkoff M, Potter BM, Jackson PF. Selective inhibition of NAALADase, which converts NAAG to glutamate, reduces ischemic brain injury. Nat Med 1999;5(12): 1396–1402.
26. Liu H, Rajasekaran AK, Moy P, Xia Y, Kim S, Navarro V, Rahmati R, Bander NH. Constitutive and antibody-induced internalization of prostate-specific membrane antigen. Cancer Res 1998; 58(18):4055–4060.
27. Rajasekaran SA, Anilkumar G, Oshima E, Bowie JU, Liu H, Heston W, Bander NH, Rajasekaran AK. A novel cytoplasmic tail MXXXL motif mediates the internalization of prostate specific membrane antigen. Mol Biol Cell 2003;3:3.
28. Rawlings ND, Barrett AJ. Structure of membrane glutamate carboxypeptidase. Biochim Biophys Acta 1997;1339(2):247–252.
29. Ndaw S, Bergaentzle M, Aoude-Werner D, Lahely S, Hasselmann C. Determination of folates in foods by high-performance liquid chromatography with fluorescence detection after pre-column conversion to 5-methyltetrahydrofolates. J Chromatogr A 2001;928(1):77–90.
30. Diehn M, Sherlock G, Binkley G, Jin H, Matese JC, Hernandez-Boussard T, Rees CA, Cherry JM, Botstein D, Brown PO, Alizadeh AA. SOURCE: A unified genomic resource of functional annotations, ontologies, and gene expression data. Nucleic Acids Res 2003;31(1):219–223.
31. Waltham MC, Lin S, Li WW, Goker E, Gritsman H, Tong WP, Bertino JR. Capillary electrophoresis of methotrexate polyglutamates and its application in evaluation of gamma-glutamyl hydrolase activity. J Chromatogr B Biomed Sci Appl 1997;689(2): 387–392.
32. Yao R, Rhee MS, Galivan J. Effects of gamma-glutamyl hydrolase on folyl and antifolylpolyglutamates in cultured H35 hepatoma cells. Mol Pharmacol 1995;48(3):505–511.
33. Mhaka A, Gady AM, Rosen DM, Lo KM, Gillies SD, Denmeade SR. Use of methotrexate-based peptide substrates to characterize the substrate specificity of prostate-specific membrane antigen (PSMA). Cancer Biol Ther 2004;3(6):551–558.
34. Kalmbach RD, Choumenkovitch SF, Troen AM, D'Agostino R, Jacques PF, Selhub J. Circulating folic acid in plasma: Relation to folic acid fortification. Am J Clin Nutr 2008;88(3):763–768.
35. Kelly P, McPartlin J, Goggins M, Weir DG, Scott JM. Unmetabolized folic acid in serum: Acute studies in subjects consuming fortified food and supplements. Am J Clin Nutr 1997;65(6):1790–1795.
36. Hattori Y, Maitani Y. Folate-linked nanoparticle-mediated suicide gene therapy in human prostate cancer and nasopharyngeal cancer with herpes simplex virus thymidine kinase. Cancer Gene Ther 2005;12(10):796–809.
37. Bagley PJ, Selhub J. Analysis of folate form distribution by affinity followed by reversed-phase chromatography with electrical detection. Clin Chem 2000;46(3):404–411.
38. Luthi-Carter R, Barczak AK, Speno H, Coyle JT. Hydrolysis of the neuropeptide N-acetylaspartylglutamate (NAAG) by cloned human glutamate carboxypeptidase II. Brain Res 1998;795(1–2):341–348.
39. Zhao R, Qiu A, Tsai E, Jansen M, Akabas MH, Goldman ID. The proton-coupled folate transporter: Impact on pemetrexed transport and on antifolates activities compared with the reduced folate carrier. Mol Pharmacol 2008;74(3):854–862.
40. Qiu A, Jansen M, Sakaris A, Min SH, Chattopadhyay S, Tsai E, Sandoval C, Zhao R, Akabas MH, Goldman ID. Identification of an intestinal folate transporter and the molecular basis for hereditary folate malabsorption. Cell 2006;127(5):917–928.
41. Zhao R, Matherly LH, Goldman ID. Membrane transporters and folate homeostasis: Intestinal absorption and transport into systemic compartments and tissues. Expert Rev Mol Med 2009; 11:e4.
42. Wallock LM, Tamura T, Mayr CA, Johnston KE, Ames BN, Jacob RA. Low seminal plasma folate concentrations are associated with low sperm density and count in male smokers and nonsmokers. Fertil Steril 2001;75(2):252–259.
43. Holm J, Hansen SI, Hoier-Madsen M. High-affinity folate binding in human prostate. Biosci Rep 1993;13(2):99–105.
44. Al-Ahmadie HA, Olgac S, Gregor PD, Tickoo SK, Fine SW, Kondagunta GV, Scher HI, Morris MJ, Russo P, Motzer RJ, Reuter VE. Expression of prostate-specific membrane antigen in renal cortical tumors. Mod Pathol 2008;21(6):727–732.
45. Birn H, Spiegelstein O, Christensen EI, Finnell RH. Renal tubular reabsorption of folate mediated by folate binding protein 1. J Am Soc Nephrol 2005;16(3):608–615.
46. Arias C, Becerra-Garcia F, Tapia R. Glutamic acid and Alzheimer's disease. Neurobiology (Bp) 1998;6(1):33–43.