Prostate-specific Membrane Antigen Directed Selective Thrombotic Infarction of Tumors

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ABSTRACT

Prostate-specific membrane antigen (PSMA), a glutamyl preferring carboxypeptidase, is found in prostate and other carcinomas present on both tumor cells and associated microvascular lining cells. We find that the channel structures delineated by PSMA-expressing cells in human and rat prostate tumors are in functional continuity with the vasculature and thus form part of tumor microvasculature. The PSMA-positive cell-outlined channels are CD31 negative and mutually exclusive of CD31-positive cell-lined channels elsewhere in the tumor consistent with tumor cells adapted to a pseudovascular phenotype in vascuagenic mimicry. To assess the functional potential of such PSMA-lined microvasculature to selectively direct infarctive tumor therapy, we coupled the soluble extracellular domain of tissue factor to a PSMA catalytic site inhibitor to create a PSMA-directed selective tumor vascular thrombogen (STVT). This protein produced selective local in vivo infarctive necrosis of the rat Mat Lu prostate tumor when administered i.v. The combined administration of this STVT with low-dose doxorubicin produced a significant tumoroidal effect, resulting in complete eradication of some tumors. This is consistent with the therapeutic potential for a PSMA-directed STVT and expands the potential for selective infarctive ablation of tumors.

INTRODUCTION

The PSMAα protein is a marker of prostate epithelial cells that are more highly expressed by CaP, especially in more advanced tumors (1). First identified by monoclonal antibody 7E11C-5 where it was found to be up-regulated in poorly differentiated, metastatic, and recurrent CaP (1–3). The cDNA was cloned and found to encode a type II transmembrane protein (1, 2), a glutamyl preferring carboxypeptidase that releases glutamate by hydrolysis of γ or α linkages (1, 4). This protein also has been described in brain as a neuropeptidase (1, 5), as well as in small intestine as folate hydrolase (1, 4). In normal prostatic epithelium, a cytosolic form of PSMA is found, whereas in CaP, there is a nearly 100-fold increase of the membrane form (6). There is evidence that indicates PSMA expression is up-regulated not only in tumor cells but also is found associated with local, what appear to be, microvascular lining cells in CaP and other tumors (1, 7, 8). We here explore whether PSMA-positive cells constitute, in part, intratumoral vasculature structures and what have been thought to be PSMA-expressing endothelial cells may be tumor cells adapted to vascuagenic mimicry (9).

The inappropriate expression of a novel gene product on the luminal surface of intratumoral microvascular lining cells provides a potential target to localize and assemble molecules for imaging or therapy. We have previously demonstrated the feasibility of localizing TF to tumor microvasculature to induce local tumor vasculature thrombosis (10). This strategy has successfully induced selective infarctive necrosis of tumors and frequent complete eradication in a proof of principle murine tumor model and without undesirable effects (10–12). We have characterized PSMA expression in both the human LuCap tumor model and the rat Mat Lu prostate tumor model. Using a STVT incorporating a PSMA catalytic site inhibitor as the selective targeting element, i.v. administration induced selective local infarction of Mat Lu tumors. Combined therapy with doxorubicin significantly enhanced tumor eradication and prolonged the tumor-free status.

MATERIALS AND METHODS

Reagents. Murine monoclonal antibody J591, specific for the extracellular domain of PSMA, was kindly provided by Dr. N. Bander (School of Medicine, Cornell University). Monoclonal antibodies against mouse CD31 (MEC 13.3) and rat CD31 (TLD-3A12) were from PharMingen (La Jolla, CA). Biotinylated 7E11C-5 antibody was from Dr. J. Murphy (Pacific Northwest Cancer Foundation, Seattle, WA). Purified human plasma factor VIIa was from Hematologic Technologies (Essex Junction, VT). Cell lines LuCap and the Mat Lu were from American Type Culture Collection. Lipoamino-incorporated doxorubicin (Doxil) was from ALZA Corporation (Mountain View, CA). Streptomyces avidini was from American Type Culture Collection and grown for isolation of DNA using the QIAmp kit method (Qiagen, Valencia, CA).

Immunohistochemistry. Immunohistochemical analysis was performed on formalin fixed as well as fresh frozen 5-μm tissue sections mounted on polylysine-coated slides. For endothelial identification, biotinylated murine antirat CD-31 monoclonal antibody (TLD-3A12) or biotinylated rat antimonoclonal CD-31 monoclonal antibody (MEC 13.3) was used at 1 μg/ml as first antibody, then the reaction was developed with fluorescein-conjugated streptavidin. For identification of PSMA in frozen sections, reaction of mouse monoclonal antibody J591 was followed by biotinylated rabbit antimouse IgG, and the reaction was visualized with Texas-red-conjugated streptavidin. Staining of PSMA in formalin-fixed tissue was performed with biotinylated 7E11C-5 antibody. The tissue sections were analyzed with the aid of laser scanning confocal microscopy (Bio-Rad, Hercules, CA).

Strep-TF Fusion Protein. TF cDNA containing amino acids 3–311 was obtained by PCR of a human cDNA library (Clontech, Palo Alto, CA) with primers BM21: 5'-ACTCAAAATACGTGCGACA-3' and BM33: 5'-TTAagttTCACGTGCCCCATACTCCTACCGG-3'. The resulting 639-bp fragment was isolated by gel electrophoresis and subjected to a second PCR following overlapping oligos were annealed and inserted into the BamHI and HindIII sites of the vector pTrHisC (Invitrogen, Carlsbad, CA). The BM51 oligo also encodes a thrombin cleavage site (Val-Pro-Arg-Gly-Ser) for selective proteolytic deletion of the His tag from the expressed protein. This plasmid (NuV120) was further modified to contain a linker sequence with three repeats of Gly-Ser between the thrombin cleavage sequences and those of TF. The following overlapping oligos were annealed and inserted into the BamHI and Aval sites of NuV120: nuv20-1: 5'-ATAGTCTTAGGCCACTAAATTACG-3'; nuv20-2: 5'PO4--GACCTGCTAAACTGACG-3'; nuv20-3: 5'PO4--GATCACCAGAGG-GGCGTTCAGTGTTCAAGTGTTCA-3'; nuv20-4: 5'PO4--GGAGGTGG-AGGTCTCG-3'; nuv20-5: 5'PO4--TCTGCGGATCCCCAGA-3';
nuv20–6: 5′-PO4-AGGTATTGACTGAATTAGCGAGGATGGATTG-3′; nuv20–7: 5′-PO4-CCACCCTCAGACCGCCTGACCTGGATATT-3′; and nuv20–8: 5′-CGCGGGAACCTCACCCTCTGAACCTTCCA-3′.

The resulting plasmid (Nuv127) encodes a His-tag, a thrombin cleavage site, three repeats of the spacer Gly3Ser, and TF residues 3–211. This vector can be used to create expression vectors for various STVT molecules by inserting a cDNA sequence encoding the derived amino acids into the unique BamHI and KpnI sites. The streptavidin gene was amplified by PCR with Pfu polymerase (Stratagene) and oligonucleotides strept1: 5′-ACCACGGTCTCGATTACCGGC-3′ and strept2: 5′-ACTACTGTGAACGCGCGTCCAGG-3′ resulting in a 514-bp fragment. The 514-bp fragment was purified and used as template for a second PCR amplification, this time with the oligonucleotides strept3: 5′-CACACAGATGCTCGCGAGGCGC-3′ and strept4: 5′-CACACAGATGCTCGCGAGGCGC-3′. BamHI and KpnI sites, respectively, are underlined, and extra nucleotides, in italics, were added for efficient enzyme digestion. The resulting DNA fragment of 486 bp was purified, digested with BamHI and KpnI, and cloned into the BamHI and KpnI sites of Nuv127. The resulting plasmid Nuv159 expresses a protein shown diagrammatically in Fig. 2A. BL21 transformed with Nuv159 plasmid were grown in Super Medium (25 g of tryptone, 15 g of NaCl/liter) supplemented with biotin. Cells were induced with 1 mM isopropyl-1-thio-

Preparation of STVT. Biotinylated DβE peptide (biotin-GSGSDβE) was synthesized using Fmoc chemistry and validated by mass spectrometry. Biotin-DβE was mixed at a 10:1 molar ratio with strep-TF fusion protein at 1 mg/ml in physiological saline for 30 min. The resultant DβE-strep-TF conjugate was freed of free biotin-DβE by dialysis. Immediately before injection, the DβE-strep-TF conjugate was mixed with an equimolar concentration of factor VIIa for 10 min to generate DβE-strep-TF-VIIa complex.

Cell Culture. The LuCap cells were cultured in RPMI 1640 supplemented with 10% FCS, glutamine (2 mM), HEPES (10 mM), sodium pyruvate (1 mM), and glucose (4.5 g/liter). Mat Lu cells were cultured in RPMI 1640 with 10% FCS, glutamine (2 mM), and 250 μM dexamethasone.

Factor Xa Generation Assay. Factor Xa generation assays were performed as described previously (12) with modification provided for association of the STVT constructs to PSMA-expressing LnCap cells. Cells were plated at 8 × 10^5/well in 96-well plates and allowed to attach for 4 h in medium above. Medium was replaced with HBSA buffer [0.15 M NaCl, 5 mM CaCl_2, 0.5% BSA, and 20 mM HEPES (pH 7.4)], and serial concentrations of DβE-strep-TF-VIIa or Strep-TF-VIIa complex were added to the wells. After 5 min of incubation, factor X was then added to a final concentration of 1 μM. After 5 min at 37°C, the limited proteolytic conversion of factor X to factor Xa was measured using biotin-labeled D127 (NuV127) as described previously (11) and solubilized in 6 M GuHCl (pH 8.0). The protein construct was then purified in a Ni-NTA column equilibrated and washed with 6 M GuHCl (pH 8.0) and eluted with 0.5 M NaCl. The protein construct was purified in two steps with a Source 15Q 16/10 column followed by a Sephacryl S-200 gel filtration.

Animal Models. The LuCap human prostate tumor was carried as a xenograft in WEHI nude mice (The Scripps Research Institute Breeding Facility) (13). The tumors were passaged by implantation of ~2-mm³ fragments in the s.c. tissue of the back of the mice. The rat Mat Lu prostate carcinoma, carried in male Copenhagen rats ages 4–6 weeks (Harlan Sprague Dawley, Germantown, NY), was inoculated with 5 × 10^3 Mat Lu cells s.c./site in the back of the rats. Treatment was initiated once tumors reached 200 mm³ through bolus i.v. injection of the STVT or control protein (0.1 mg/kg based on strep-TF protein) and repeated twice at 2-day intervals. For combination therapy,
liposomal doxorubicin (Doxil) at 2 mg/kg was separately injected i.v. Tumor
growth and other physical signs were monitored daily, including gross evi-
dence of tumor necrosis, local tumor ulceration, as well as evidence of toxicity,
including mobility, response to stimulus, eating, and weight of each animal.
The studies have been reviewed and approved by the Institutional Animal Care
and Use Committee of The Scripps Research Institute. The work was con-
ducted in the Scripps Research Institute facilities, which are accredited by the
Association for the Assessment and Accreditation of Laboratory Animal Care.
The Scripps Research Institute maintains an assurance with the U.S. Public
Health Service and is registered with the United States Department of Agri-
culture and is in compliance with all regulations relating to animal care and
welfare.

Statistical Analysis. Statistical significance was determined by the two-
tailed Student’s t test, except for statistical significance of survival curves,
which used the logrank test using GraphPad Prism version 3.00 (GraphPad
Software, San Diego, CA).

RESULTS

PSMA-positive Cells Delineate some Intratumoral Microchan-
nels. Immunohistochemical analysis of the human LuCap tumors
clearly identified PSMA-positive cells that line and thereby delineate
the microscopic channels with structural characteristics not unlike
microvascular channels (Fig. 1A). The lumens of these channels are
formed by tumor cells, and PSMA expression is more intense on the
aspect of tumor cell membranes that constitute the lumenal surface of
the channels (Fig. 1B). Double staining of the LuCap tumor with
antimouse CD31 antibody and PSMA-specific antibody suggests
these PSMA-delineated microchannels are distinct and mutually ex-
clusive of microvascular channels lined by CD31-positive cells (Fig.
1C). Similar microchannels lined by PSMA-positive cells were also
observed in syngeneic rat Mat Lu tumors (Fig. 1D).

To address the issue of whether the PSMA-positive microchannels
are valid elements of the intratumoral microvasculature and directly
continuous with the general vasculature, we infused bacteriophage i.v.
as a vascular marker in tumor-bearing rats. Tumors were harvested
~2 min after infusion, rapidly frozen, and sectioned. Double immu-
nostaining for PSMA and bacteriophage revealed that the PSMA-
positive cell-lined microchannels contained phage (Fig. 1D) indicative
of immediate functional continuity with the general vasculature.

The STVT Functionally Associates with PSMA-positive Cells.
To confer cell surface assembly of the designed STVT to PSMA
positive cells, a known suicide inhibitor of PSMA glutamyl car-
boxypeptidase, namely DβE, was incorporated (14–16). The biotin-
GSGSDβE inhibitor structure was coupled to Strep-TF protein (Fig.
2, A and B) through the high affinity binding of biotin to the strepta-
vidin domain. However, targeting alone is not sufficient for function
because the STVT must also align properly on an anionic cell mem-
brane surface and associate with factor X substrate that has localized
to the same locus. The specific activity of the assembled DβE:strep-
TF:VIIa complex on PSMA-expressing LnCap cells (Fig. 2C) was
analyzed in a factor Xa generation assay that requires the functional
assembly. Unlike most tumor cells, LnCap cells do not express TF as
indicated by the coagulation assays and examined by Western blot
data not shown) and do not directly activate substrate factor X to the
active product factor Xa and thereby drive the thrombogenic cascade
(Fig. 2D). The dose dependent increase of factor Xa generation in the
presence of LnCap cells was striking in comparison to the control
Strep-TF lacking the PSMA-targeting element, indicating that the
PSMA-directed STVT functionally assembles on the cell surface via
binding of DβE to PSMA (Fig. 2D) and functionally initiates the
thrombogenic cascade.

Tumor Infarction in Vivo. The control Strep-TF protein was not
toxic in rats over a wide range of concentrations, thereby permitting
evaluation of the potential for selective tumor thrombosis and infarc-
tive necrosis in tumor-bearing rats. i.v. administration of the PSMA-

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Fig. 2. A, schematic of Strep-TF fusion protein. After
folding and purification the His tag was removed with
thrombin. B, LnCap cells stained with J591 PSMA-specific
antibody; note strong surface positivity for PSMA. C,
silver-stained SDS gel electrophoresis of purified Strep-TF
complex in the factor Xa generation assay, demonstrating
that the PSMA-directed STVT retains substrate recognition
and conversion activity when in the presence of PSMA-
positive cells. ■, Strep-TF; ○, STVT.
directed STVT was associated with a rapid wave of microthrombosis and resultant infarction of Mat Lu tumors (Fig. 3A) with significant retardation in tumor growth (Fig. 3B). An average 70% reduction in tumor mass was observed compared with controls (Fig. 3C). The center of the tumors in the experimental group showed gross signs of ischemic necrosis. In contrast, there was no microthrombosis or areas of necrosis in these highly cellular tumors from the control group (Fig. 4A). Occluded tumor microvessels were widespread in the experimental group (Fig. 4B), with platelet aggregates, packed erythrocytes, and fibrin (Fig. 4C). The tumor interstitium that commonly contained a few erythrocytes was infiltrated with inflammatory cells (Fig. 4D). After the standard three infusions at 2-day intervals, tumors showed very extensive necrosis with liquefaction of the entire central region of the tumors. However, at the growth edge of tumors from the treated animals, a rim of viable tumor tissue remained.

**Combined STVT Plus Doxorubicin Therapy**. To address the potential to enhance selective tumor microvascular thrombosis and infarction of tumors, infusions of both STVT and low-dose liposomal doxorubicin (2 mg/kg) were conducted. Three infusions of each were administered at 2-day intervals as above. There was a virtually complete arrest of tumor growth and even gross eradication of tumors in some rats (Fig. 5). This same combination therapy had a significant beneficial effect on survival of the tumor-bearing animal hosts ($P < 0.001$). The prolongation of survival of rats treated with STVT alone was modest but was significant. Therapy with low-dose liposomal doxorubicin alone had no measurable benefit (Fig. 6).

**DISCUSSION**

PSMA protein is synthesized by normal prostate epithelial cells; however, it is more highly expressed after neoplastic transformation of these cells (1). The protein is a glutamyl, preferring carboxypeptidase that hydrolyzes γ or α linkages to release glutamate (1, 4). Whereas normal prostate epithelial cells produce a cytosolic form of PSMA, transformation results in a nearly 100-fold increase of the membrane isoform (6). Some recent studies have suggested that PSMA expression is up-regulated not only in prostate carcinomas but also is associated with the local endothelial cells in prostate carcinomas and even in association with other tumors (1, 7, 8).

We have demonstrated in this study that PSMA-positive cells are found lining intratumoral microchannels that are not lined by conventional CD31-positive endothelial cells. Tumor cell surface expression of PSMA is more intense on that aspect of tumor cell membranes that delineate the luminal surface of these tumor cell-lined channels. These PSMA-delineated microchannels are continuous with the general vasculature based on the very rapid entry of bacteriophage into these channels after infusion by the tail vein. Additional studies demonstrate that extensive thrombotic infarction occurs after administration of a PSMA-localizing STVT. It is reasonable to hypothesize that the PSMA-positive cell delineated channels, which lack the usual endothelial marker CD31, are likely tumor cells adapted to a pseudovascular phenotype. Such adaptation has been described as vasulogenic mimicry (17–23) wherein tumor cells, rather than endothelial cells, adapt and line intratumoral microvascular channels. A recent study showed heterogeneous-invasive prostate carcinoma cell lines have the potential to form perfusable vasulogenic-like networks in culture (9). Existence of such networks in aggressive rat and human tumors in vivo, similar to this study, were observed (9). Although a current topic of some interest and debate (24–27), a greater degree of elucidation of the intrinsic cell biology and vasulogenic characteristics remain to be developed (28–30). However, in addition to the vasulogenic mimicry hypothesis, tumor cell surface molecules may be directly accessed by molecules in blood, including therapeutic agents, through direct transmigration of tumor cells through the microvascular lining cells to the lumen and subsequent detachment into the circulation during metastasis. These tumor cells initially localize to endothelial cells locally before releasing into circulation (29). In one study, it was estimated that ~15% of perfused vessels of a colon carcinoma xenograft were mosaic vessels with focal regions where tumor cells appeared to contact the microvessel lumen (30). Tumor cells accounted for ~4% of the total vascular surface area in these colon carcinomas. Similar numbers of mosaic vessels were found in human colon carcinoma biopsies (30), underscoring the complexity of...
intratumoral microvasculature and differences from normal vasculogenic rules.

A selective tumor microvascular thrombotic infarction strategy was used to determine whether tumor cell surface-expressed molecules, in this case PSMA, could both localize and properly assemble the cell surface TF:VIIa:X:membrane complex to initiate the thrombogenic cascade in vivo. Using this PSMA-directed STVT, we observed robust and highly selective tumor microvascular thrombosis and infarctive necrosis of syngeneic prostate tumors in the rats. The gross and histopathological changes observed were similar to those previously described by us and others for selective infarctive therapy of tumors (10, 31). Signs of tumor vasculature thrombosis occurred immediately after initial infusion of the STVT followed by infarction and necrosis. The adopted three-dose protocol was without any general adverse effect on the rats. However, despite the rapid tumor destruction, there remained viable tumor cells at the tumor periphery. However, host survival was significantly extended.

Combination therapy of the PSMA-directed STVT with low-dose liposomal doxorubicin was far more effective. Doxorubicin alone had no observable effect on tumor growth or survival. However, separate bolus infusions of doxorubicin at the time of STVT infusion greatly potentiated the effect on the tumors. Abrupt infarctive features of the tumors were observed. Although not directly addressed, the doxorubicin appears to facilitate eradication of tumor cells at the peripheral edge of the tumors where microthrombosis and necrosis appears to be less effective. However, the more attractive interpretation is that because doxorubicin is known to induce endothelial cell apoptosis (32), it may also have injured the tumor microvascular endothelium and thereby increasing exposure of the tumor cells to plasma proteins to potentiate the local thrombotic activity of the PSMA-directed STVT.

These experiments underscore the potential importance of tumor cell exposure and even participation in intratumoral microvasculature.

Fig. 4. A, H&E-stained section of untreated Mat Lu tumor. Tumor cells are very poorly differentiated, and the tumor microvasculature is not easily identified. B, Mat Lu tumor after treatment exhibiting extensive necrosis and frequent microvessel thrombotic occlusion (arrows). C, treated tumor at higher magnification demonstrating a thrombosed vessel containing platelet aggregates, packed RBCs, and fibrin. D, higher magnification of treated tumor illustrating characteristic infiltration of inflammatory cells (arrows).

Fig. 5. Low-dose liposomal doxorubicin augmentation of the tumoricidal effect of PSMA-directed STVT therapy. In representative experiments, combination therapy resulted in nearly complete growth arrest of tumors in the treated animals (○, n = 12) in striking contrast to those treated only with low-dose liposomal doxorubicin (■, n = 12). The data points represent mean ± SE of 12 rats (P < 0.001). The experiment was reproducible with comparable results.

Fig. 6. Survival analysis of Mt Lu tumor-bearing rats. Combination treatment with PSMA-directed STVT with low-dose liposomal doxorubicin demonstrated significantly increased survival (n = 10) of tumor-bearing hosts. Low-dose doxorubicin alone (n = 10) had no significant effect. Survival was modestly prolonged in rats treated with STVT alone (n = 10) in contrast to the control saline-treated group (n = 10) or the doxorubicin group. The statistical significance between saline treated and STVT treated as well as between STVT treated and combination STVT plus doxorubicin treatment are significant with P < 0.0001 by logrank test.
Such exposure to the blood supports the therapeutic potential to target large molecules such as proteins to tumor cell surface molecules. The success of targeting the present STVT to a tumor cell surface-specific molecule expands the possible application of this approach to include other tumor cell surface molecules as the facilitators and targets. Dual therapy of a functional STVT and a cytotoxic agent shows promise in enhancing the infarctive eradication of tumors. Infarctive tumor eradication as been reported by us and others (10, 31) has the potential to develop as an effective therapeutic tactic.

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