Correlation Between PSMA and VEGF Expression as Markers for LNCaP Tumor Angiogenesis

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Our aim is the identification and correlation of changes in tumor-associated protein expression which results from therapy. LNCaP tumors, excised from nude mice treated either by orchiectomy or with the chemotherapeutic agent paclitaxel, were evaluated for the expression of proteins and receptors associated with growth, differentiation, and angiogenesis using immunohistologic procedures. Compared to untreated control tumors, both treatments reduced the expression of vascular endothelial growth factor (VEGF), prostate-specific membrane antigen (PSMA), prostate-specific antigen (PSA), androgen receptor (AR), and epidermal growth factor receptor (EGFR). The effect of paclitaxel treatment on AR expression was the most significant ($P = .005$). Of particular interest was identifying a significant correlation ($P < .000801$) between PSMA and VEGF expression regardless of treatment modality. These altered expressions suggest that PSMA may also be a marker for angiogenesis and could represent a target for deliverable agents recognizing either prostatic tumors or endothelial development. Cell surface PSMA would then present a unique target for treatment of patients early in their development of prostatic metastases.

INTRODUCTION

Prostate-specific membrane antigen (PSMA) has gained increased attention as a marker for prostate cancer. Unlike the well-characterized and secreted prostate-specific antigen (PSA), PSMA remains cell associated and can be internalized, acting as if it were a receptor molecule. PSMA is also characterized as folate hydrolase (FOLH-1) and this activity [1] makes it a good candidate for prodrug activation [2]. In addition, its peptides have been utilized in a vaccine associated with antigen presenting dendritic cells [3]. Favorable responses have been noted in several clinical trials of these vaccines [4, 5, 6, 7]. Although the prostate has the greatest PSMA expression, it is also found in the brain, salivary glands, the small intestine [8], as well as endothelial cells associated with many solid tumors [8]. Therefore it has been suggested that PSMA may be utilized both as a marker and as a

therapeutic target [8]. In addition to its potential for prodrug activation, it has also been considered a target for radiotherapy. Indeed, the J591 monoclonal antibody, with specificity for the extracellular domain of PSMA, has been shown to deliver alpha-emitting radiation to prostate cancer cells both in vitro and in vivo using the LNCaP model [9, 10]. Other monoclonals (7E11, J415, Hybritech PEQ226.5, PM2J004.5, in addition to J591) would appear to have similar potential with reactivity against renal carcinoma, transitional cell carcinoma of the urinary bladder, testicular embryonal carcinoma, colonic and neuroendocrine carcinomas, glioblastoma multiforme, malignant melanoma, as well as pancreatic duct, nonsmall cell lung, soft tissue, and breast carcinomas [11]. In an effort to associate the expression of PSMA with additional markers associated with growth, differentiation, or angiogenesis, we utilized histologic sections prepared for a previous study [12] and evaluated further specific protein expression following two therapeutic modalities.

In a previous study [12], utilizing LNCaP tumors carried in athymic nude mice, we evaluated the effect of paclitaxel, thalidomide, and orchiectomy upon markers associated with growth, differentiation, and angiogenesis. We reported significant ($P \leq .001$) effects of paclitaxel therapy resulting in diminished tumor volume, Bcl-2, cyclin D, and PSA expression, and serum PSA levels. Thalidomide therapy also produced significant effects, but

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in fewer markers. Bcl-2 expression was elevated ($P \leq$ .011), and tissue PSA expression dropped ($P \leq$ .002). Orchiectomy also significantly ($P \leq$ .001) raised Bcl-2 immunoreactivity and was accompanied with a significant ($P \leq$ .002) decrease in tumor volume.

In this additional study the following proteins associated with growth, differentiation, or angiogenesis were further evaluated using immunohistologic techniques.

Vascular endothelial growth factor (VEGF) is a protein released by many tumors which stimulates the directed growth of endothelial cells toward malignancies, resulting in increased vascularity through the process of angiogenesis.

PSMA and PSA are both well-characterized markers for prostate tissue and are expressed in most differentiated tumors. These markers may provide suitable targets for the selective delivery of new therapeutic agents.

The androgen receptor (AR) is also a marker for prostate tumor differentiation and is currently being evaluated for efficacy in specific targeting.

The epidermal growth factor receptor (EGFR) is an important binding site for several growth stimulatory ligands, including transforming growth factor-alpha (TGF-α) and EGF. EGFR is overexpressed in many tumors and its kinase activity has become the preferential target for new therapeutic agents which utilize either monoclonal antibodies [13], antisense oligonucleotides [14], or various small domain specific molecules.

Paclitaxel (taxol) is an antimicrotubule agent which promotes the assembly of microtubules from tubulin dimers and prevents their depolymerization. This stabilization prevents the dynamic reorganization of the microtubule network necessary for vital interphase and mitotic cellular functions. Paclitaxel also induces abnormal bundles of microtubules during the cell cycle and promotes the formation of multiple microtubule asters during mitosis [15]. These processes appear to account for most of paclitaxel antitumor activity, resulting in a blockade at the transition point between metaphase and anaphase during mitosis. The cell is then directed towards an apoptotic death pathway [16] presumably accompanied with a decrease in Bcl-2 expression, as observed in our earlier study [12].

Orchiectomy is the "gold standard" for the treatment of advanced prostate cancer; however, relapse occurs in most patients following an average of 2.5 years, and the progressing metastatic tumors are usually hormone insensitive. New therapies are very much required for advancing prostate cancer and a greater understanding of the angiogenic process could produce new targets for inhibition.

METHODS

LNCaP tumor line

The LNCaP human prostate cancer cell line was purchased from the American Tissue Culture Collection (ATCC) and maintained in RPMI 1640 in a 5%-CO_2 atmosphere at 37°C. 2.4–2.6×10^6 in vitro propagated cells were suspended in 0.5 mL of RPMI and mixed with an equal volume of Matrigel. The 1-mL volume was implanted subcutaneously into the right flank of nude mice. Tumors took approximately 6 weeks to appear and had a take rate of greater than 75%.

Animals

Athymic nude mice (nu/nu) were purchased from Harlan Sprague-Dawley (Indianapolis, Ind) and maintained in a sterilized environment according to guidelines established by the US Department of Agriculture and the American Association for Accreditation of Laboratory Animal Care (AAALAC). This project was approved by the Institutional Animal Care and Utilization Committee (IACUC) of the Cook County Hospital and the Hektoen Institute.

Paclitaxel

Paclitaxel (taxol) drug [15] is derived by a semisynthetic process from *Taxus baccata,* has a molecular weight of 853.9 and an empirical formula of $C_{14}H_{51}NO_{14}$, and was obtained from Bristol-Myers Squibb.

Treatment groups

Athymic nude mice were inoculated with 2.4–2.6×10^6 in vitro propagated LNCaP cells into the right-flank position. Fifteen nonnecrotic tumors which exceeded 1 cm in diameter were randomly divided into three groups of five for treatment as follows:

(1) group 1: controls; no treatment,

(2) group 2: treated with paclitaxel (20 mg/kg × 5 days),

(3) group 3: orchiectomy.

Therapy schedule

Therapy was begun when tumors exceeded 1 cm in diameter. The experiment was terminated by euthanasia 6 weeks later when the tumors were excised and paraffin embedded for immunohistochemistry. As previously reported [12] treated tumors significantly diminished in size with therapy, whereas the controls continued to progress in volume.

Marker determination and statistical evaluation

Immunohistochemistry was performed as previously described [8] for VEGF (Santa Cruz; Santa Cruz, Calif), PSMA (Cytogen Corporation, Princeton, NJ), PSA (Santa Cruz), AR (Santa Cruz), EGFR (Santa Cruz), and graded on a 0-to-5 (greatest) immunoreactive scale. This qualitative assessment of staining took into consideration both the area stained and its intensity for the numerous fields evaluated. Each individual specimen and its derived replicate slides (at least two) were evaluated by 4–5 individuals. Evaluations were performed randomly, in the blind and

TABLE 1. Immunohistochemical staining.

Group	VEGF	PSMA	PSA	AR	EGFR
Control	2.40 ± 2.01	2.67 ± 1.98	2.00 ± 2.35	3.87 ± 1.44 ↑	2.32 ± 1.48
Orchiectomy	1.37 ± 1.13	1.80 ± 0.30	0.60 ± 0.89	2.13 ± 1.22 |	1.58 ± 0.90
Paclitaxel	1.26 ± 1.54	1.08 ± 1.12	0.10 ± 0.22	1.06 ± 0.75 ↓	1.68 ± 1.28
P value	—	—	—	.005	—

TABLE 2. Correlation* between marker expression.

Group	Statistics	PSMA	PSA	AR	EGFR
	Correlation	0.769	0.306	0.0383	0.0836
VEGF	P value	0.000801	0.267	0.892	0.767
	N	15	15	15	15
	Correlation		0.418	0.197	0.0587
PSMA	P value	—	0.121	0.481	0.835
	N		15	15	15
	Correlation			0.797	0.113
PSA	P value	—	—	0.000375	0.689
	N			15	15
	Correlation				0.296
AR	P value	—	—	—	0.284
	N				15

*Pearson product moment correlation.

on different occasions, then means and standard deviations were determined. Only nonnecrotic fields were evaluated. When possible, sequential sections prepared from the same embedded block were stained for the various markers. Statistical significance was evaluated by Sigma Stats Solutions 2.03 software (AB, Canada).

RESULTS

Table 1 lists the means and standard deviations which resulted from multiple (blind) evaluations of slides by the investigators. Control values (± SD) obtained by staining with specific antibodies were 2.40 ± 2.01 for VEGF, 2.67 ± 1.98 for PSMA, 2.00 ± 2.35 for PSA, 3.87 ± 1.44 for AR, and 2.32 ± 1.48 for EGFR. Values obtained following orchiectomy were 1.37 ± 1.13 for VEGF, 1.80 ± 0.30 for PSMA, 0.60 ± 0.89 for PSA, 2.13 ± 1.22 for AR, and 1.58 ± 0.90 for EGFR. Values obtained for the paclitaxel group were 1.26 ± 1.54 for VEGF, 1.08 ± 1.12 for PSMA, 0.10 ± 0.22 for PSA, 1.06 ± 0.75 for AR, and 1.68 ± 1.28 for EGFR. Although all values were diminished following both treatment modalities, significance ($P = .005$) was only found in AR expression between controls and those tumors treated with paclitaxel.

Table 2 lists the correlation coefficients obtained between all groups and the various protein markers. A correlation coefficient of 0.769 was obtained by Pearson product moment correlation between VEGF and PSMA. This had a significance of $P = .000801$. Variations in tumor volume or in the amount of tissue necrosis did not appear to influence expression of either protein.

DISCUSSION

Prostate cancer has the highest incidence of any human malignancy and is second only to lung cancer in male mortality [17]. Although localized prostate cancer can be cured by either radical prostatectomy or radiation therapy, regional involvement usually proceeds to become a metastatic disease. Obviously newer chemotherapeutic compounds are needed and antiangiogenic therapy could theoretically have potential efficacy. In an effort to evaluate newer agents we treated human LNCaP tumors carried in nude mice with paclitaxel, as well as the "gold standard" orchiectomy. PSMA expression in the endothelium of multiple types of solid tumors [8, 11] has been previously reported and suggests a possible role of PSMA in angiogenesis.

This study supports those by O'Keefe et al [8] and Chang et al [11] and identify a coordinated expression between PSMA and VEGF, a major stimulating protein for angiogenesis. Treatment by either chemo- or hormonal deprivation did not alter this correlation further, suggesting that PSMA could represent a new angiogenic marker for solid tumors. As previously reported [12] treatment of the hormone-sensitive LNCaP tumor with paclitaxel was more effective in inhibiting growth than thalidomide, and this could account for the significant decrease in AR expression in this study. The diminished expression of VEGF following androgen deprivation also supports the findings by Isaacs et al [18, 19] in the LNCaP model.

For prostate cancer PSMA presents a particularly attractive target, since the advanced form of this disease is

noted by extreme metastatic dissemination requiring neovascularization. PSMA would present itself as a target for prostatic malignancy as well as for angiogenesis.

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