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SHORT COMMUNICATION

Vaccination against prostate cancer using a live tissue factor deficient cell line in Lobund–Wistar rats

Julie E. Heinrich · Morris Pollard · William A. Wolter · Zhong Liang · Hui Song · Elliot D. Rosen · Mark A. Suckow

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Abstract Reducing expression of the tissue factor gene in prostate adenocarcinoma cells (PAIII) results in a cell line that, in vivo, mimics the growth of wildtype (wt) PAIII. However, instead of continuing to grow and metastasize as wt PAIII tumors do, tissue factor deficient PAIII (TFD PAIII) masses spontaneously regress after several weeks. Although whole cell vaccines are typically inactivated prior to administration to prevent proliferation within the host, numerous studies have suggested that exposure to live, attenuated, whole tumor cells, and the extracellular microenvironment they recruit, increases immunotherapeutic potential. Here, we provide support for this notion, and a strategy through which to implement it, by demonstrating that subcutaneous vaccinations with the TFD PAIII protect the Lobund–Wistar rat against subsequent wt PAIII cell challenge. TFD PAIII immunized rats suffered significantly less metastasis of wt PAIII challenge tumors compared to unvaccinated naïve controls rats. These results offer the intriguing possibility that the TFD PAIII vaccine is an effective system for the prevention and, possibly, the treatment of prostate cancer.

Keywords Prostate cancer · Vaccine · Tissue factor · Whole-tumor cell · Stroma · Metastasis

J. E. Heinrich · M. Pollard · W. A. Wolter · M. A. Suckow (✉)
University of Notre Dame Biological Sciences,
Notre Dame, IN, USA
e-mail: msuckow@nd.edu

J. E. Heinrich
e-mail: jheinric@nd.edu

Z. Liang · H. Song · E. D. Rosen
Department of Medical and Molecular Genetics,
Indiana University School of Medicine,
Indianapolis, IN, USA

Introduction

The immune system is capable of influencing the development and spread of a variety of cancers, including prostate cancer (PC). In rat and transgenic mouse models of PC, inoculations with inactivated whole tumor cells, cells transfected with co-stimulatory molecules/combined with adjuvants, and antigen-pulsed dendritic cells have provided protection against challenge and autochthonous prostate tumors [1–6]. Most importantly, immunotherapy clinical trials have shown that vaccines can enhance the survival time of men with hormone refractory PC [7, 8].

While these studies provide promise for development of an effective PC vaccine, clinical response rates in PC patients have fallen short of expectations. Many recent anti-tumor vaccines have focused on specific, static targets within the immune system, methodology that neither protects against the influence of epitope escape strategies, nor employs the possibilities of anti-stromal responses and immunity related to the necrosis of tumor tissue. Here we provide support for the notion that a live PC cell vaccine is effective in curbing the development and spread of transplanted tumors. This candidate vaccine, Tissue Factor Deficient PAIII (TFD PAIII), is comprised of live tumor cells which multiply in vivo, recruit a stromal microenvironment, and subsequently self-regress, thus increasing the antigenic profile and complexity compared to inactivated, non-proliferating tumor cell vaccines.

The TFD PAIII vaccine is a derivative of the Lobund–Wistar (L–W) rat metastatic prostate adenocarcinoma cell line PAIII [9] and is engineered to be deficient in expression of a single gene, tissue factor. This

transmembrane glycoprotein is a critical component in the cascade initiating blood coagulation/hemostasis. Hypercoagulation has been well documented in a variety of cancers and tissue factor expression, specifically, has been correlated with the aggressive growth and spread of cancer [10–15]. Interference with the anti-tissue factor pathway has resulted in increased experimental lung metastasis, indicating an important role for tissue factor in the escape and migration of neoplastic cells [16, 17].

We have found that TFD PAIII inoculations confer protection against the growth of challenge wildtype (wt) PAIII cells and their subsequent metastasis in L–W rats. After prophylactic exposure to TFD PAIII, L–W rats administered challenge wt PAIII cells exhibit significantly less metastasis, and smaller challenge tumors than unvaccinated rats.

Materials and methods

Vaccine development and cell line preparation

To stably reduce expression of tissue factor mRNA in wt PAIII cells, double stranded synthetic oligonucleotides corresponding to rat TF mRNA sequence were cloned into the *Apa*I–*Eco*RI sites of multiple cloning site adjacent to the U6 promoter of the vector pND776. In addition to the U6 promoter, the vector contained EGFP and NeoR transcription cassettes. The oligonucleotides contained inverted repeats corresponding to 19 nucleotide regions of tissue factor mRNA separated by a seven nucleotide linker, a polyT sequence (pol I termination signal) following the inverted repeat and sticky ends for ligation into the *Apa*I–*Eco*RI sites. Transcription from the U6 promoter followed by endonucleolytic digestion of the single stranded RNA generated a hairpin RNA with a double-stranded stem. After transfection of PAIII cells with the plasmid, NeoR, fluorescent colonies were picked to establish clonal cell lines. Thirteen different sets of oligonucleotides generating siRNAs corresponding to 19 bp stretches of tissue factor mRNA between positions 168 and 678 were constructed. Tissue factor mRNA levels were determined for 10 clonal cell lines for each siRNA by quantitative RT-PCR. The greatest reduction of TF mRNA (75% inhibition) was achieved with a cell line expressing the siRNA corresponding to rat TF RNA nt 260–278.

Prior to inoculations, a reduction in tissue factor mRNA in the TFD PAIII was confirmed by quantitative RT-PCR, and normalized with rat housekeeping gene PBG-D (prophobilinogen deaminase). Total RNA (100 ng) extracted from 12 clonal cell lines of PA3 and PA3-TFD cells were added to PCR mix containing 0.15 µl Multiscribe and Gold Taq DNA polymerase (Applied Biosystems, USA), 3 µl 10× buffer A (Applied Biosystems, USA), 3.6 ml 10 mM dNTP, 4.8 µl 25 mM MgCl2, 0.3 µl Eppendrof Priner RNase inhibitor (Fisher Scientifics), 0.15 mmol L^{-1} of specific forward and reverse primers and 3 mmol L^{-1}specific FAM reporter dye 5′ labeled probe (3′ TAMRA quenched) in a final reaction volume of 30 µl. TaqMan One-step Q-RT-PCR was then performed using the ABI Prism 7700 sequence detector with an initial RT step of 48°C for 30 min then with a denaturation step of 95°C for 10 min followed by 40 cycles of at 95°C for 15 s (denaturation) and 60°C for 1 min (annealing and extension). Forward and reverse primers for rat tissue factor were 5′-CCACCTTTCTCGGCTTCCTT-3′ and 5′-CTTTCCCTGGAGGACTGCC-3′, corresponding to nucleotides 107–126 and 151–169 of rat tissue factor mRNA, and sequence specific probe was 6FAM TCC TTCAGGTGGCCGTTGGTGC-TAMRA. PBG-D specific forward and reverse primers corresponding to nucleotides 1151–1171 and 1233–1252 of the rat prophobilinogen deaminase, and probe were 5′-TCAA GTGCCCTGTGCTCCTT-3′, 5′-GGCCCCAAGTT GAGGCATAT-3′ and 6-FAM CAAAGTTGTTGAG CTTCGGCCTTGGAA-TAMRA respectively. Absolute standards (0.975–250 fg) prepared from wt PAIII cell total RNA identical to real time PCR products were included on each plate to ensure equal efficiency of amplification between standards and PCR products generated in sample wells.

The wt PAIII challenge cell line, originally obtained from a autochthonous L–W rat prostate tumor at the University of Notre Dame, was cultured in Dulbecco's modified Eagle's medium (DMEM; Mediatech, Inc., Herndon, VA,), 10% heat inactivated fetal bovine serum (Fisher Scientific), 1% L-glutamine (Mediatech), 1% penicillin streptomycin (Mediatech), in 5% CO_2. Each TFD PAIII vaccination and wt PAIII challenge inoculation contained 1×10^6 cells in a volume of 0.2–0.3 cc DMEM, free of bovine serum and adjuvant. All TFD vaccinations were administered subcutaneously to the right flank, while wt PAIII challenge inoculations were administered subcutaneously to the left flank.

Animals

All animal studies were approved by the University of Notre Dame Institutional Animal Care and Use Committee and the standards described in the *Guide for the Care and Use of Laboratory Animals* [18] were followed. All studies included 2–3-month-old male Lobund–Wistar rats obtained from the breeding colony

at University of Notre Dame. These rats were originally derived from a long-term breeding colony of germfree inbred Wistar strain rats, which has since been conventionalized and maintained as a pathogen-free colony.

Experimental design

Thirty L–W rats were administered subcutaneously 1×10^6 TFD PAIII cells to the right flank followed by an identical booster at day 21. TFD PAIII injections resulted in the appearance of small (< ~2 cm in diameter) subcutaneous masses at the injection site in 30 out of 30 rats by day 10. Six vaccinated animals were sacrificed before complete regression of TFD PAIII mass for histological evaluation. On day 42, the 24 remaining vaccinated animals, together with 12 naïve age-matched controls were administered 1×10^6 wt PAIII cells to the left flank.

On day 85 (1.5 months after wt PAIII challenge, historically the approximate amount of time it takes for subcutaneous wt PAIII cells to thoroughly metastasize to the lungs) all animals were euthanized and necropsied. Body weights and tumor weights were recorded at time of necropsy. Lungs were partitioned and subpleural foci enumerated at 5× magnification by two individuals blind to experimental group. The mean number of lung foci per animal was determined for both TFD PAIII immunized and control groups.

Histology

Primary challenge tumors (see Fig. 1), and TFD PAIII masses were fixed in 10% neutral buffered formalin for sectioning and staining with hematoxylin and eosin (H&E). Lungs were saved in Bouin's solution and switched to 70% ETOH ~17 h later.

Statistical analysis

Differences in challenge tumor weights and number of metastatic lung foci between groups were evaluated by unpaired, two-way, Student's t tests. Differences were considered significant at $P < 0.05$. The highest outlier (>2 standard deviations from the mean) of lung foci number from each group was removed for statistical comparisons.

Results

There were no differences in mean body weights between TFD PAIII vaccinated rats and control rats at the time of necropsy. TFD PAIII masses were microscopically characterized by extensive necrosis with central liquefaction. Microscopically, challenge PAIII tumors were moderate to poorly differentiated adenocarcinomas with abundant fibrous stroma. There was no apparent histological difference between the PAIII (challenge) tumors of vaccinated and control rats. TFD vaccinated animals exhibited significantly fewer metastatic lung foci ($P = 0.016$), averaging 12 foci per animal, compared to unvaccinated controls, which averaged 32 foci (Fig. 2a). The mean weight of challenge tumors in vaccinated rats was significantly smaller (18 g; $P < 0.003$) than the mean weight of tumors (24 g) in unvaccinated controls (Fig. 2b).

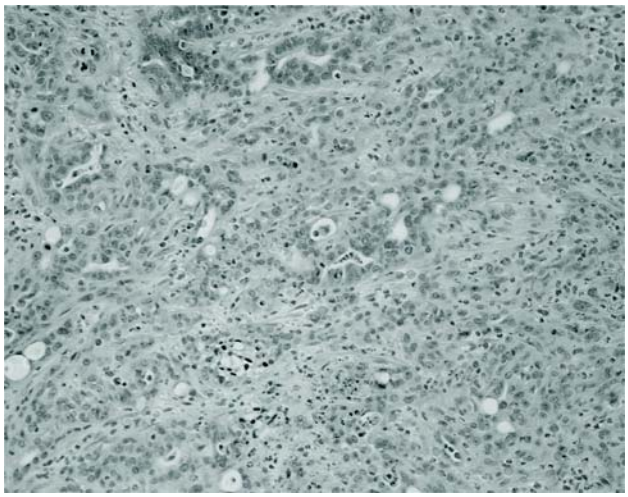

Fig. 1 Typical section of PAIII tumor mass showing scattered poorly organized acinar structures with many mitiotic figures and within abundant connective tissue stroma. Section stained with H&E, magnified ×400

Summary/discussion

The attenuated tumor cell vaccine described here proliferates in vivo, recruits a stromal microenvironment and subsequently undergoes necrosis, likely confronting its host with a larger, more sustained dose of tumor associated antigens than that provided by typical cancer immunotherapy. The demonstration that the TFD PAIII cell immunization confers protection against the growth of transplanted wt PAIII cells and their subsequent metastases offers the intriguing possibility that the strategy demonstrated by TFD PAIII cell vaccine may be an effective way to treat and/or prevent PC.

There are several reasons why the TFD PAIII vaccine may ultimately prove superior to traditional cancer vaccine methodologies. First, malignant cells are notoriously prone to genetic/epigenetic transformation and thus often permanently lose immunogenic

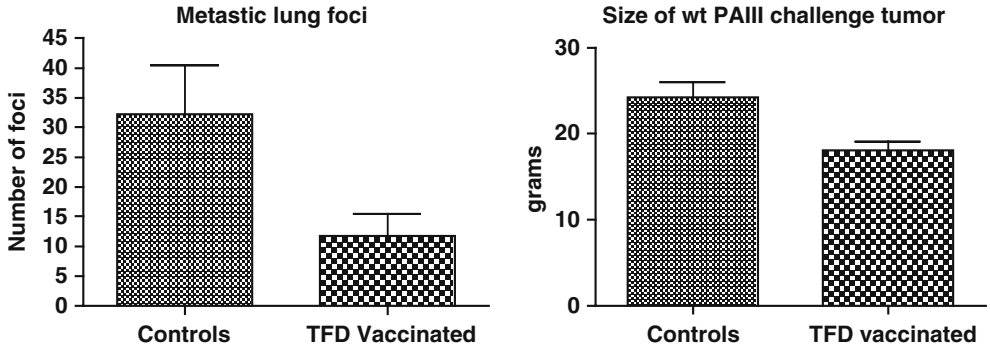

Fig. 2 **a** Average number of metastatic lung foci in control L–W rats (*left*) administered subcutaneous wt PAIII ($n = 9$) compared to TFD vaccinated animals ($n = 23$, *right*) given the same wt PA-III inoculation. **b** Average size (in grams) of wt PAIII challenge tumor excised from control L–W rats ($n = 10$) compared with wt PAIII challenge tumor from TFD vaccinated rats ($n = 24$). All samples were taken 43 days after wt PAIII administration

epitopes. In fact, there is considerable evidence suggesting that neoplastic cells mutate to avoid immune recognition, rendering vaccines designed against single, static targets in the immune system insufficient (reviewed in [19–21]). The most effective targets may instead be the tumor-expressed antigens that are most likely to persist despite the inevitable evolution of tumor cells: those related to the growth, reproduction and support of the tumor cells. While *whole* tumor cells inherently express a more comprehensive antigenic profile, they must be inactivated to prevent uncontrolled tumor cell proliferation within the host. Non-proliferating whole tumor cell vaccines maintain the ability to secrete cytokines for a short period of time, yet are also more rapidly processed [22, 23], and elicit a weaker immunogenic response than their live counterparts [22, 24–28]. This is significant in light of the recent use of "suicide" gene therapy to regulate the onset of an apoptotic death *after* vaccine administration. While these live, eventually apoptotic, cells exhibit improved efficacy over ex vivo killed vaccines, cancer cells that die a necrotic death are better able to invoke a systemic antitumor response [25, 29–31]. Thus, TFD PAIII, may serve as an inherently safe, replicating tumor cell vaccine, that can present a comprehensive variety of immunogens including exposure to generational genetic/epigenetic changes in cancer cells, as well as the process of tumor necrosis.

A second advantage of the TFD vaccine is that, unlike traditional anti-tumor vaccines, it exploits the antigenic complexity associated with a *growing* tumor. For more than a week the TFD PAIII cells replicate and recruit a tumor microenvironment within the host. Studies have shown that when reciprocal interactions between vaccine cancer cells and normal neighboring cells are permitted, subsequent immune targeting specifically of tumor stroma can result [32–35]. Importantly, anti-stromal responses are not necessarily based on a single histiotype, and immune evasion mechanisms are less applicable since stromal cells are more genetically stable than neoplastic cells [34, 36–38]. Thus, as the TFD PAIII line exposes the immune system to cancer cells, in addition to associated connective tissue, it may provide a source of antigen exposure and cytokine production not available from vaccines composed merely of nonreplicating malignant epithelial cells.

Finally, these results provide evidence of immunoprotection against PC cells *without* the assistance of immunostimulatory adjuvants. Adjuvants may be effective in counteracting immunosuppressive factors produced by developing tumors, yet ideally, clinical vaccines would be sufficient without the addition of artificial immune stimulation [39]. Notably, the single difference between metastatic wt PAIII cells, and the TFD PAIII vaccine is expression of a protein that has been implicated in immunosuppressive signaling itself. Over-expression of tissue factor at the cell surface has been tightly correlated with malignancy and metastasis [10, 11, 13–15]. It has been hypothesized that tissue factor may "cloak" neoplastic cells allowing them to escape immunosurvellience during pulmonary metastasis [40]. Because tissue factor expression has been implicated in immunosuppressive signaling, tumors cells deficient in tissue factor may allow for an improved immune response, perhaps negating the need for adjuvants.

Obviously, protection from metastatic PC cell challenge provides only a snapshot of the immune response to the development and spread of PC. Here we provide proof of principle that a live tumor cell vaccine may provide a safe and effective alternative to irradiated, or otherwise 'reduced', cancer vaccines. Further studies are needed to perfect the timing, administration, and

stability of the TFD PAIII vaccine, to bring this technology to clinical application. Fortunately, our animal model system of PC, the L–W rat, recapitulates many aspects of the disease in man. This model will assist us in testing the therapeutic value of the TFD vaccine against autochthonous prostate tumors as well as the prophylactic merits of the vaccine in an immune system not already compromised by PC. Ultimately reduction in tissue factor expression, or other means of administering live self-regressing whole tumor cells, may serve as a general immunotherapeutic approach to protect against a variety of cancers.

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