Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer

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Abstract

Purpose: Prostate tumors express antigens that are recognized by the immune system in a significant proportion of patients; however, little is known about the effect of standard treatments on tumor-specific immunity. Radiation therapy induces expression of inflammatory and immune-stimulatory molecules, and neoadjuvant hormone therapy causes prominent T-cell infiltration of prostate tumors. We therefore hypothesized that radiation therapy and hormone therapy may initiate tumor-specific immune responses.

Experimental Design: Pretreatment and posttreatment serum samples from 73 men with nonmetastatic prostate cancer and 50 cancer-free controls were evaluated by Western blotting and SEREX (serological identification of antigens by recombinant cDNA expression cloning) antigen arrays to examine whether autoantibody responses to tumor proteins arose during the course of standard treatment.

Results: Western blotting revealed the development of treatment-associated autoantibody responses in patients undergoing neoadjuvant hormone therapy (7 of 24, 29.2%), external beam radiation therapy (4 of 29, 13.8%), and brachytherapy (5 of 20, 25%), compared with 0 of 14 patients undergoing radical prostatectomy and 2 of 36 (5.6%) controls. Responses were seen within 4 to 9 months of initiation of treatment and were equally prevalent across different disease risk groups. Similarly, in the murine Shionogi tumor model, hormone therapy induced tumor-associated autoantibody responses in 5 of 10 animals. In four patients, SEREX immunoscreening of a prostate cancer cDNA expression library identified several antigens recognized by treatment-associated autoantibodies, including PARP1, ZNF707 + PTMA, CEP78, SDCCAG1, and ODF2.

Conclusion: We show for the first time that standard treatments induce antigen-specific immune responses in prostate cancer patients. Thus, immunologic mechanisms may contribute to clinical outcomes after hormone and radiation therapy, an effect that could potentially be exploited as a practical, personalized form of immunotherapy.

Prostate tumors are recognized by the immune system in a significant proportion of patients, as evidenced by serum antibody responses to prostate-specific antigen (PSA), prostatic acid phosphatase, p53, and HER2/neu (1). In addition, many prostate tumors contain significant numbers of tumor-infiltrating lymphocytes, which are associated with favorable outcomes in prostate cancer and other cancers (2–10). In an attempt to enhance the immune response to prostate cancer, clinical vaccine trials have been conducted targeting such antigens as PSA (11, 12), prostate-specific membrane antigen (13), and prostatic acid phosphatase (14). For example, Gulley et al. (15) conducted a phase II clinical trial combining a poxvirus vaccine encoding PSA with external beam radiation therapy (EBRT) and showed significant increases in PSA-specific T cells in the majority of patients, along with emergent responses to antigens not present in the vaccine, providing evidence of antigen spreading. A second example is Provenge (APC8015), an autologous dendritic cell vaccine loaded with human prostatic acid phosphatase–granulocyte macrophage colony-stimulating factor fusion protein (Dendreon Corporation, Seattle, WA; ref. 16). In a phase III trial, Provenge induced prostatic acid phosphatase–specific T-cell responses and significantly increased 3-year overall survival in patients randomized to APC8015 compared with those randomized to placebo (25.9 versus 21.4 months, \( P = 0.01 \)), representing the first survival advantage attributed to an immunotherapy product in prostate cancer (17, 18).

Despite this progress with immune-based therapies for prostate cancer, there is little information available on whether...
standard treatments, such as hormone therapy and radiation therapy, induce tumor-specific immune responses (19). One might expect the apoptotic cellular material resulting from cytotoxic therapies to be processed by macrophages and dendritic cells and presented to the immune system (20). Furthermore, radiation therapy creates an inflammatory milieu by inducing the expression of inflammatory cytokines, MHC molecules, B7 and other costimulatory molecules, adhesion molecules, death receptors, and heat shock proteins in tumor cells, stroma, and vascular endothelium, which can contribute to effective antigen cross-presentation, leading to a CD8+ cytolytic response (21–23). For its part, hormone therapy can induce prominent T-cell infiltration of human prostate tumors (2) and cause the release of tumor antigens for processing by dendritic cells and presentation to both cytotoxic and helper T cells (24). Finally, in normal tumor-free mice, androgen deprivation transiently increases levels of peripheral T cells, and T cells proliferate more vigorously after antigen stimulation (25). Despite these intriguing findings, there is little information available concerning the effect of standard treatments on tumor immunity in prostate cancer patients and the potential influence this has on clinical outcomes.

To investigate whether standard treatments induce tumor-specific immune responses, we studied a cohort of 73 prostate cancer patients undergoing various forms of standard treatment, including hormone therapy, EBRT, brachytherapy and radical prostatectomy, alone or in combination, as well as nine patients who chose watchful waiting instead of treatment. Pretreatment and posttreatment serum samples were evaluated by Western blot and SEREX (serological identification of antigens by recombinant cDNA expression cloning) antigen arrays for the appearance of treatment-associated autoantibody responses. In parallel, an androgen-dependent mouse model was assessed for treatment-induced serologic changes. We show for the first time that standard treatments induce antigen-specific autoantibody responses in a significant proportion of prostate cancer patients. In the future, it may be possible to enhance the immunologic effects of standard treatments as a practical, personalized form of immunotherapy.

**Materials and Methods**

**Subjects.** All blood samples were collected with informed consent and approval from the Research Ethics Board of the BC Cancer Agency. Seventy-three patients with nonmetastatic prostate cancer were recruited at the BC Cancer Agency in Victoria, BC, Canada. Typically, pretreatment serum was collected on the first patient visit and subsequent samples were collected approximately every 3 months during treatment, every 6 months for the first year after the completion of treatment, and yearly thereafter to coincide with regularly scheduled PSA tests. If more than one treatment was initiated, samples were collected during and after each treatment. Of the 73 patients, 9 chose watchful waiting instead of treatment and served as an untreated control group. A second control group consisted of 50 men over the age of 50 years with no personal history of cancer. For 27 of these controls, two to three serial blood draws were collected at 2- to 6-month intervals; for the remainder, only a single blood sample was collected. Serum was aliquoted and stored at −80°C.

**Western blot assay.** LNCaP or PC3 cells were lysed at $-1 \times 10^6$ cells/ml in radioimmunoprecipitation assay buffer [50 mmol/L Tris-HCl (pH 7.5); 150 mmol/L NaCl; 1% NP40; 0.5% sodium deoxycholate; 0.1% SDS] on ice for 30 min. After centrifugation, 400 μg of protein were separated using NuPAGE Novex 4% to 12% Bis-Tris gels (Invitrogen, Burlington, ON, Canada) and transferred to nitrocellulose using the XCell SureLock Mini-Cell (Invitrogen). Sera were diluted 1/500 in Blotto (5% dry milk powder; 0.1% Tween 20; 50 mmol/L Tris; 150 mmol/L NaCl) and incubated with nitrocellulose membranes for 1 h at room temperature using a multichannel immunoblotting device (Mini Protein II Multiscreen, Bio-Rad, Mississauga, ON, Canada). The membrane was then incubated for 1 h at room temperature with horseradish peroxidase–conjugated goat anti-human IgG (H+L; Jackson ImmunoResearch, West Grove, PA) diluted 1/10,000 in Blotto and visualized by enhanced chemiluminescence.

**SEREX screening and antigen arrays.** A prostate cancer phage cDNA expression library was constructed with a total of 5.0 μg mRNA from three prostate cancer cell lines (LNCaP, PC3, and DU-145) using the ZAP cDNA library construction kit (Stratagene, La Jolla, CA). The LNCaP and PC3 cell lines were purchased from American Type Culture Collection (Manassas, VA), and the DU-145 cell line was a kind gift from Dr. Ralph deVere White (University of California, Davis Medical Centre, Davis, CA). The library contained 6.8 × 10^8 cloning with a 98.6% recombination frequency. SEREX screening, antigen arrays, and plasmid conversions were done as previously described (26) with the following exception: an additional blocking step was done using TBS [50 mmol/L Tris/150 mmol/L NaCl (pH 8.0)] + 1% bovine serum albumin before secondary antibody was added. SEREX screening of the prostate cancer library was done using serum from a total of 15 patients. A previously described testis library (26) was also screened in an attempt to obtain antigens of the cancer-testis class (27). To make screening more efficient, sera from two to five patients were pooled, each represented at a 1/200 dilution.

**Shionogi mouse model.** Ten male DD/S mice were inoculated s.c. with 5 × 10^6 Shionogi tumor cells in one flank. When the tumor reached a diameter of 8 mm in size (approximately day 14), the mice were castrated to simulate androgen deprivation–type hormone therapy, causing the tumor to regress. Blood was collected from each mouse before castration, 2 weeks postcastration, and at euthanization. Cytoplasmic protein lysates were made from intact Shionogi tumor using an NP40 lysis buffer [50 mmol/L Tris (pH 7.5); 150 mmol/L NaCl; 1% NP40]. Western blotting using precastation and postcastration serum samples was done as described above.

**Results**

**Description of the treatment and control groups.** To assess the effect of standard treatments on the immune response to prostate cancer, we collected serum samples from patients with various stages of disease before, during, and after standard treatments, which included hormone therapy, EBRT, brachytherapy, radical prostatectomy, or combinations thereof. Patient characteristics are shown in Table 1. Control groups included nine prostate cancer patients who underwent watchful waiting in lieu of treatment, as well as 50 age-matched men with no personal history of cancer. The median ages of the patient group, the watchful waiting control group, and the cancer-free control group were 69 years (range 51-81 years), 70 years (range 55-80 years), and 71 years (range 53-85 years), respectively.

**Serologic changes associated with hormone therapy.** To broadly assess whether standard treatments for prostate cancer induce immune responses to tumor antigens, patient and control sera were first immunoblotted against lysates from the prostate cancer cell line LNCaP. All positive and most negative results were confirmed by at least two independent immunoblots.
We evaluated 24 patients undergoing hormone therapy. All but one of these patients was subsequently treated with EBRT ($n = 21$) or radical prostatectomy ($n = 2$); however, analysis of their serum at the completion of hormone therapy and before EBRT or radical prostatectomy allowed assessment of the effects of hormone therapy alone. These 24 patients represented a range of disease risk levels for localized disease, including 4 low-risk (all of PSA $\leq 10$, Gleason $\leq 6$, and stage T1/T2a), 5 intermediate-risk (any of PSA $11-19$, Gleason $7$, or stage $> T2b$), and 15 high-risk (any of PSA $>20$, Gleason $\geq 8$ or stage $\geq T3a$) patients. By Western blot, 7 of 24 patients (29.2%) showed the emergence of new seroreactivities during hormone therapy, as indicated by the appearance of one or more immunoreactive bands by Western blot (Fig. 1A). Treatment-associated changes arose 4 to 9 months after the initiation of hormone therapy and, with one exception described below, persisted for the duration of study, even in patients who went on to receive a second treatment modality.

We next examined whether the emergence of hormone therapy–associated serologic responses correlated in some way to the extent of tumor regression, as measured by changes in PSA values. All 24 patients experienced a decline in their PSA value, with the median value dropping from 11.0 ng/mL (range 2.7-42 ng/mL) to 0.15 ng/mL (range 0.02-20 ng/mL), and for most patients, the PSA nadir occurred within 3 months of the initiation of hormone therapy. Intriguingly, patients exhibiting a hormone therapy–associated immune response achieved a lower median PSA value (0.14 ng/mL) at 3 months after hormone therapy than those who did not (median 0.26 ng/mL). Moreover, the median time to PSA nadir was less in those patients showing an immune response (7 months, range 6-16 months) than those who did not (9 months, range 2-16 months). Although these differences did not reach statistical significance, possibly owing to small sample sizes, they suggest that tumors undergoing more rapid and extensive regression may be the most likely to trigger a serologic response.

### Table 1. Patient characteristics and treatment

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<th>BT</th>
<th>HT + BT</th>
<th>RP</th>
<th>HT + RP</th>
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<td>10</td>
<td>12</td>
<td>2</td>
<td>9</td>
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<td>69.5 (55-75)</td>
<td>68.5 (58-76)</td>
<td>62.5 (53-71)</td>
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<td>70 (55-80)</td>
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<td>6</td>
<td>6.5</td>
<td>6</td>
<td>6*</td>
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<td>6.5*</td>
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<td>Median (range)</td>
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<td>5.7</td>
<td>6.65 (1.2-12.7)</td>
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<td>6</td>
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**Abbreviations:** HT, hormone therapy; BT, brachytherapy; RP, radical prostatectomy; WW, watchful waiting.

*One patient’s Gleason score and stage were unknown.

*Two patients’ PSA values were unknown.

Fig. 1. Standard treatments induce autoantibody responses to tumor proteins in prostate cancer patients. Western blot analysis of serum from five patients probed against LNCaP cell lysates. A, patient PC036. Hormone therapy (HT) is associated with the appearance of a ~ 55 kDa protein (arrow). B, patient PC051, demonstrating a hormone therapy–associated antibody response to one antigen, which intensifies after EBRT (top arrow) and an EBRT-associated response (bottom arrow). C, patient PC051, showing an EBRT-associated antibody response (arrow) of ~ 100 kDa. D, patient PC026, showing a brachytherapy (BT)-associated antibody response (arrow).
timing of serologic responses with respect to changes in PSA, in three of seven patients (PC009, PC015, and PC060), the hormone therapy–associated autoantibody response occurred ≥3 months after the PSA nadir (Fig. 2). In one of seven patients (PC036), the immune response occurred during (or possibly before) the drop in PSA. In the remaining three of seven patients, the available time points did not allow us to determine whether the immune responses occurred during or after the drop in PSA.

We considered other factors that might influence the development of hormone therapy–associated immune responses. In general, all patients began hormone therapy with a combination of flutamide and leuprolide acetate or flutamide and goserelin. We found no obvious differences in the hormone therapy drug regimen between those patients who developed a serologic response and those who did not. Similarly, there was no obvious correlation with disease severity, as autoantibody responses were seen in 1 of 4 low-risk, 1 of 5 intermediate-risk, and 5 of 15 high-risk patients.

Although the above results concerned the emergence of new seroreactivities during hormone therapy, we also observed one example (PC001) in which a seroreactive band of ~55 kDa disappeared within 7 months of the initiation of hormone therapy (data not shown). At the same time, a seroreactive band (~62 kDa) appeared, indicating that hormone therapy may both induce and inhibit autoantibody responses to tumor-associated antigens.

Serologic changes associated with radiation therapy. Twenty-nine patients were evaluated by Western blot for the effect of EBRT on the autoantibody profile. The majority (28 of 29) had received prior hormone therapy; therefore, we first considered whether EBRT enhanced or inhibited autoantibody responses that arose during hormone therapy. Five of the patients described above who showed a hormone therapy–associated response went on to receive EBRT. With only one exception, hormone therapy–associated autoantibody responses persisted after EBRT, with follow-up ranging from 7 to 14 months. The one exception was patient PC001, in which a ~62 kDa seroreactive band that appeared during hormone therapy diminished within 3 months of starting EBRT. We do not know whether this change was due to EBRT or would have occurred irrespective of further treatment. One patient (PC015) had a serologic response induced by hormone therapy that intensified after EBRT, as well as a second seroreactivity that emerged after EBRT (Fig. 1B). Thus, in most cases, EBRT is associated with retention or even enhancement of hormone therapy–induced autoantibody responses.

We next considered the emergence of new autoantibody responses associated with EBRT. Four of 29 patients (13.8%) showed new seroreactivities after EBRT (Fig. 1C), none of whom had shown a hormone therapy–associated immune response. One of the four patients underwent EBRT without prior hormone therapy, meaning that the immune response was attributable to EBRT alone. In the other patients that had neoadjuvant hormone therapy, it is not possible to determine whether the response was due to the effects of the EBRT, a combination of the hormone therapy and EBRT, or a delayed response to hormone therapy.

As an alternative to EBRT, 20 patients received brachytherapy, either alone (n = 10) or after hormone therapy (n = 10). Three of 10 hormone therapy + brachytherapy patients showed the emergence of new seroreactivities after brachytherapy that were not seen during hormone therapy. Of the 10 patients receiving brachytherapy without prior hormone therapy, two (20%) showed new immunoreactivities at 4 to 5 months after brachytherapy (Fig. 1D).

The presence or absence of a serologic response during radiation therapy was not attributable to radiation dose, as all patients who underwent EBRT received the same dose of radiation (a total of 7,400 cGy in 37 fractions over 7.5 weeks).

![Fig. 2: PSA values over time for the seven patients who showed a hormone therapy–associated seroreactive change by Western blot. PSA values were plotted with respect to the initiation of treatment (t = 0). Shaded bars, timeframe in which the hormone therapy–associated seroreactivity emerged according to available blood draws.](image-url)
Similarly, all patients who underwent brachytherapy received a peripheral dose of 144 Gy delivered by 120 to 130 $^{125}$I seeds (0.334 mCi). Therefore, it appears that patient-specific factors unrelated to radiation dose influenced whether an autoantibody response emerged during treatment.

Serologic analysis of surgical cases and control participants. Fourteen patients in the study had surgery, two of whom received neoadjuvant hormone therapy. No serologic changes were seen by Western blot in any of these patients with a follow-up ranging from 5 to 20 months after radical prostatectomy. Similarly, the nine patients who underwent watchful waiting instead of treatment showed no serologic changes over a 5- to 30-month interval.

We also evaluated 27 cancer-free control subjects from whom two to three serial blood draws were available. Control sera showed a number of immunoreactive bands that varied from individual to individual but did not change over time in 25 of 27 controls. However, 2 of the 27 controls did show a serologic change over a 5- to 10-month interval. One of these individuals displayed a seroreactive band of $\sim$125 kDa at their third time point that was not present at the first two time points. The second individual showed a seroreactive band of $\sim$42 kDa at the second and third time points, as well as a seroreactive band of $\sim$62 kDa at the third time point (data not shown). Thus, the serologic changes seen by Western blot are not exclusive to prostate cancer patients. Nevertheless, the frequency of responses in patients treated with hormone therapy and/or radiation therapy (28.8%; 15 of 52) is significantly higher than that seen in cancer-free controls (2 of 27, 7.4%; $P = 0.028$, $\chi^2$ test) and prostate cancer patients treated surgically (0 of 14, 0%; $P = 0.022$, $\chi^2$ test), and approaches significance in patients undergoing watchful waiting (0 of 9, 0%; $P = 0.064$, $\chi^2$ test), suggesting the majority of responses are indeed induced by the hormone or radiation treatments.

**Androgen withdrawal induces autoantibody responses in a murine tumor model.** Although the above studies using patient...
sera show a correlation between hormone therapy/radiation therapy and the emergence of serologic responses, they do not establish a causative link. Therefore, to experimentally test whether hormone therapy can induce humoral immunity to tumor antigens, we used the murine Shionogi mammary carcinoma model (SC-115), which has been used extensively to study the conversion from androgen-dependent to -independent neoplasia (28–30). Shionogi tumors are initially androgen dependent and hence are grown in male mice. Castration precipitates apoptosis and tumor regression similar to that seen after androgen withdrawal in prostate cancer patients (31, 32). To evaluate whether tumor regression induces an autoantibody response, Shionogi tumor cells were injected into the flank of 10 male DD/S mice. When tumors reached ~8 × 8 mm, mice were castrated, inducing tumor regression in all cases. Precastration and postcastration sera were subjected to immunoblotting against Shionogi tumor protein lysates. Intriguingly, 5 of 10 mice showed the emergence of an autoantibody response to an ~40 kDa tumor protein within 3 weeks of castration (Fig. 3). Thus, androgen withdrawal can indeed induce autoantibody responses to tumor antigens. However, even in the context of a well-controlled tumor model, there is some degree of variability between individuals, suggesting that other factors influence the initiation of these responses.

Cloning prostate cancer antigens by SEREX. SEREX immunoscreening has been used successfully to identify a large number of antigens from various human cancers, including prostate cancer (33–37). Therefore, to clone the tumor antigens underlying the Western blot results from the above-mentioned prostate cancer patients, a prostate tumor cDNA expression library was constructed using pooled mRNA from the prostate cancer cell lines LNCaP, PC3, and DU-145. Immunoscreening was done using serum from 15 prostate cancer patients, 10 of whom showed a treatment-associated serologic change by Western blot analysis. Each of the 15 patient sera was screened against 1 × 10⁵ to 5 × 10⁵ plaque-forming units of the prostate library, and many were also screened against a previously described testis library (26) in an attempt to identify antigens of the cancer-testis class (27). In total, 21 unique immunoreactive clones were identified (Table 2). The majority of clones contained single cDNA inserts; however, two clones, SWAP-70 + CHYS1 and ZNF707 + PTMA, contained two contiguous cDNA segments, likely representing a double cDNA insertion event during library construction.

To evaluate the frequency of autoantibody responses to these antigens among prostate cancer patients and cancer-free controls, we constructed a SEREX antigen array containing the 21 antigens, as well as three antigens from our previous ovarian cancer study (26). Each array also contained a positive IgG control and a negative control consisting of a nonrecombinant clone. All phage were spotted in duplicate in distinct locations on the array. Replicate arrays were probed with pooled time point sera from 55 patients and 50 cancer-free controls (Table 3). Based on this analysis, antigens could be grouped into three classes. Nine antigens were reactive in approximately equal numbers of patient and control sera, suggesting they represent common autoantigens. Indeed, this group included RBPSUH and SDCCAG1, both of which are known autoantigens (38, 39). By contrast, nine antigens were reactive with serum from cancer patients only. This included two known tumor antigens (MBD2 and p53), one known disease-associated autoantigen (PARP1), and six novel autoantigens (C14orf35, USP11, WDR36, ODF-2, ZNF707+PTMA, and CEP78). A third group encompassed antigens that were recognized by autoantibodies from prostate cancer patients at least twice as frequently as cancer-free controls. This included two known cancer-associated autoantigens (PECI and SEB4), two known cancer- and disease-associated autoantigens (DLD and KTN-1), and two novel autoantigens (RALBP1 and SON).

We evaluated whether autoantibody responses to tumor antigens were more prevalent in the patient versus control groups. For this analysis, we excluded the 15 patients who were used to screen the SEREX libraries, because this would introduce a bias in favor of the patient group. In addition, we excluded the nine antigens that were equivalently reactive with patient and control sera, because these likely represent common autoantigens that are unrelated to prostate cancer. Using these criteria, 13 of 40 (32.5%) of patients were seroreactive to at least one antigen on the array, compared with 7 of 50 (14%) of controls (P = 0.036, χ² test). It is perhaps not unexpected that some control sera would be seropositive, as the prevalence of autoantibody responses increases with age (40–42) as does the risk of prostate cancer (43). The six antigens that were seroreactive in the 14% of control subjects included PECI, SEB4, DLD, KTN-1, RALBP1, and SON. Of these, only DLD has been previously reported to be immunoreactive in a healthy control subject (44), and PECI, SEB4, DLD, and KTN-1 have all been deemed to be cancer-

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<th>Known common autoantigens</th>
<th>Prostate cancer sera (n = 55)</th>
<th>Cancer-free control sera (n = 50)</th>
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<tr>
<td>ZNF707 + PTMA</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>CEP78</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>
-associated autoantigens in other studies (45–47). In the current study, all six were more frequently immunoreactive in prostate cancer patients than controls. Thus, although not definitive, these results suggest that some or all of these controls may have undiagnosed prostate cancer or another cancer.

An equivalent proportion of patients with high-risk versus low/intermediate-risk disease were seroreactive to one or more antigens on the array [51.7% (15 of 29) versus 54.5% (6 of 11)]. Likewise, 50% (16 of 32) of patients with stage ≤T2b disease were seroreactive versus 62.5% (5 of 8) of patients with stage ≥T2c disease. Thus, similar to the Western blot results, there was no obvious association between disease severity and seroreactivity to antigens on the array.

Treatment-associated immune responses to SEREX-defined antigens. Serial serum samples were analyzed to determine whether autoantibody responses to SEREX-defined antigens developed in association with treatment. In 4 of the 34 patients analyzed, we observed antigen-specific autoantibody responses that were present in posttreatment but not pretreatment sera. In one patient, PC036, autoantibody responses to two antigens (ZNF707+PTMA and CEP78) arose after 7 months of hormone therapy (Fig. 4A). In a second patient, PC011, an autoantibody response to SDCCAG1 arose after EBRT. In a third patient (PC001), three different serologic changes were seen. Specifically, hormone therapy was associated with the disappearance of autoantibodies to two different antigens (KTN1 and RALBP1), whereas EBRT was followed by a significant increase in autoantibodies to a third antigen (ODF2). Finally, patient PC015 had a hormone therapy–associated response to PARP1 that appeared within 4 months of hormone therapy and intensified after EBRT (Fig. 4B). Interestingly, this patient had a ~120 kDa treatment-induced band appear on Western blot after 4 months of hormone therapy (Fig. 1B), making it likely that this band is in fact PARP1, a 113 kDa protein.

For those control subjects who showed seroreactivity to the treatment-associated antigens on the array, serial time point serum samples were analyzed individually. No changes in seroreactivity were seen for any control over a >6-month interval.

One patient, PC011, showed a treatment-associated seroreactivity by antigen array that was not detected by Western blot. Therefore, when the results for the Western blot assay and SEREX antigen array were combined, hormone therapy was associated with a serologic response in 29.2% of patients, EBRT in 17.2% of patients, and brachytherapy in 25.0% of patients.

Discussion

The combined use of adjuvant hormone therapy and radiation therapy in high-risk prostate cancer has led to significantly improved outcomes, with 76% of patients remaining disease-free, without PSA failure at 5 years (48). Our prior results have shown that neoadjuvant hormone therapy given before radiation therapy for >8 months provided prolonged PSA disease-free survival in patients with less well-differentiated tumors (49). Although these clinical results are encouraging, we do not fully understand the basis of synergy between hormone therapy and radiation therapy, which limits our ability to improve upon current treatments. Here, we show for the first time that hormone therapy and radiation therapy induce antigen-specific immune responses in ~29% of prostate cancer patients, as evidenced by the development of autoantibody responses to tumor-associated antigens. Responses typically appeared within a few months of the start of treatment and in most cases persisted for the duration of the study (up to 30 months). A diverse repertoire of antigens was recognized, several of which were successfully cloned by SEREX. In the murine Shionogi tumor model, androgen withdrawal by castration also induced an autoantibody response to the tumor in ~50% of animals. Our findings raise the possibility that the host immune response may influence clinical outcomes after standard treatments. If so, enhancing the frequency, magnitude, and character of these immune responses with immunomodulatory agents could potentially improve outcomes further.

The ~29% prevalence of treatment-associated immune responses shown here may be an underestimate given the limitations of the assays used. First, *Escherichia coli*–based expression systems do not recapitulate the full range of posttranslational modifications characteristic of mammalian proteins, which precludes the expression of many epitopes. We mitigated this in part by performing Western blots of tumor cell lysates, which allows detection of glycosylated or other posttranslationally modified epitopes; however, antigens cannot
be cloned using this method. In the future, use of a yeast cell surface display library (50) may allow cloning of additional posttranslationally modified antigens. Second, we focused exclusively on IgG autoantibodies and therefore would have missed IgM responses, which predominate early in the primary immune response (51). Third, by focusing on autoantibody responses, we may have overlooked treatment-induced responses involving T cells or innate immune effectors such as natural killer or NKT cells. Nevertheless, serologic screening is a useful starting point as it provides recombinant antigens that can then be used to assess antigen-specific T-cell responses, as has been done with SEREX-defined antigens such as NY-ESO-1 (52). In a similar manner, we are currently evaluating whether treatment-associated serologic responses are associated with the emergence of CD4+ and/or CD8+ T-cell responses to the SEREX-defined antigens described here.

Many of the cancer-associated antigens we found have intriguing properties that suggest an involvement in tumor development or progression, including proteins involved in cell cycle regulation (SC65 and CEP78) and DNA damage and repair (USP11 and PARP1). Other antigens are involved in signaling (RALBP1, SWAP-70) or show homology to other oncoproteins (SON). Interestingly, none of the proteins we identified have been previously implicated in prostate cancer (34–37, 53–55). Furthermore, despite extensive screening of both a prostate cancer and testis library, we did not isolate previously reported prostate cancer antigens such as NY-ESO-1, LAGE-1, and XAGE-1 (52, 54). The lack of overlap between different studies suggests that prostate cancer triggers responses to a broad repertoire of autoantigens.

How might tumor antigens become immunogenic during standard treatments? In theory, some may be newly expressed by stressed or dying tumor cells. Others might become immunogenic due to loss of tolerance. Indeed, hormone therapy induces apoptosis of hormone-dependent tumor cells (56), increases tissue levels of dendritic cells and costimulatory molecules, and triggers vigorous T cell–mediated inflammation in the prostate (2). Similarly, radiation therapy induces the apoptosis and necrosis of tumor cells (21) and increases expression of inflammatory and immune mediators, including cytokines, MHC molecules, costimulatory molecules, adhesion molecules, death receptors, and heat shock proteins (57). Although apoptosis was historically considered a nonimmunogenic process, this no longer appears to be the case. In autoimmune disease and cancer, the surface blebs of apoptotic cells contain high concentrations of intracellular antigens that trigger autoantibody responses (58, 59). This process can be enhanced when antigens undergo structural modifications such as cleavage by apoptosis-specific proteases, phosphorylation, or complex formation (58). Dendritic cells can take up apoptotic material and cross-present antigens to CD8+ cells (20), thereby breaking tolerance. In the present study, three of the five treatment-associated antigens we discovered (ZNFP707+PTMA, CEP78, and ODF-2) have not been described previously; therefore, we currently have little insight into their mechanism of immunogenicity. On the other hand, PARP1 is a well-known target for cleavage by caspase-3 during apoptosis and necrosis (60) and has been identified as an autoantigen in colorectal cancer (39) and autoimmune conditions such as systemic lupus erythematosus (61). Thus, PARP1 likely becomes immunogenic by virtue of caspase-3–mediated cleavage in apoptotic tumor cells. Intriguingly, anti-PARP1 autoantibodies from patients with systemic lupus erythematosus have been shown to inhibit PARP1 function, even in intact cells (62), which raises the possibility that autoantibodies to antigens such as PARP1 might affect tumor cell biology.

It is difficult to predict whether treatment-induced autoantibody responses will have a positive or negative effect on clinical outcomes. This issue has been most extensively studied with autoantibodies to p53, which are associated with favorable outcomes in lung cancer (63, 64) and poor outcomes in breast, colon, oral, and gastric cancers (65). It remains to be determined whether these disparate results reflect immunologic differences between tumor sites, or an inconsistent biological effect overall. In various autoimmune disorders, autoantibodies are associated with present or future tissue destruction, and may act via complement reactions, antibody-dependent cytotoxicity, immune complex formation, or direct triggering of cell death pathways (66, 67). As mentioned above, autoantibodies can also penetrate target cells and alter the function of their cognate antigen (62). In addition to any potential direct effects on target cells, autoantibodies are usually indicators of an underlying T-cell response, which, in turn, can mediate tissue destruction. For example, coincident autoantibody and CD8+ T-cell responses to antigens such as p53 and NY-ESO-1 have been documented in cancer patients (68, 69). On the other hand, the Th1/Th2 paradigm would suggest that autoantibodies and cytolytic T-cell responses may be mutually exclusive in some cases, as they are promoted by Th2 and Th1 T helper responses, respectively (70).

Given the diversity of autoantigens recognized by cancer patients in the present study and many previous SEREX studies (33), it seems likely that investigation of a single antigen such as p53 is unlikely to fully reveal any relationship between tumor immunity and outcomes. Furthermore, the vast majority of studies to date have measured autoantibody responses at the time of diagnosis rather than posttreatment. As shown here, autoantibody profiles can change significantly during the course of treatment, and one would expect outcomes to be more influenced by the immune status of patients after treatment than before. Indeed, a recent study involving patients undergoing IFN therapy for melanoma showed that the emergence of autoantibody responses to a panel of antigens during treatment correlated with increased relapse-free survival and overall survival (71). Importantly, this latter study focused on common autoimmune indicators such as antinuclear and anti-DNA antibodies. This suggests that even common autoantigens, such as many of the antigens identified in the present study, can potentially serve as clinically relevant targets in cancer, or at least represent surrogate markers for effective antitumor responses.

Although much progress has been made with cancer immunotherapy, the clinical effect and feasibility of many immunotherapeutic approaches remain a challenge. For example, peptide-loaded dendritic cells are considered among the most potent of vaccines, yet require specialized expertise and considerable expense to administer (72). By contrast, our data show that standard treatments for cancer may represent a form of in situ vaccination, at least at the level of serologic responses. Ongoing studies will address whether standard treatments also induce CD4+ and CD8+ T-cell responses that may contribute
to an antitumor response. The prevalence, magnitude, and nature of such responses could potentially be optimized by administration of immune-stimulatory agents during standard treatments. For example, Flt3 ligand has been shown in preclinical studies to enhance the immune-stimulatory effects of radiation therapy (73). A phase I clinical trial combining conformal radiotherapy and intratumoral injection of dendritic cells was shown to induce tumor-specific and innate immunity (74). Finally, a phase II trial combining chemotherapy and s.c. injection of interleukin 2 and granulocyte macrophage colony-stimulating factor showed strong immunologic and antitumor activity in colorectal cancer patients (75). As shown here, the Shionogi mouse model provides an experimental system to test whether or not other immunomodulatory strategies can enhance the immunologic effects of hormone therapy and radiation therapy to therapeutic levels. The concept of using immunomodulatory agents to maximize the immunologic effects of standard cancer treatments might ultimately provide a cost-effective, clinically feasible alternative to personalized cancer vaccines.

References


Standard Treatments Induce Antigen-Specific Immune Responses in Prostate Cancer

Nancy J. Nesslinger, Robert A. Sahota, Brad Stone, et al.


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