Dendritic Cell-based Immunotherapy of Prostate Cancer: Immune Monitoring of a Phase II Clinical Trial

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Abstract

We assessed both non- and peptide-specific immune responses in prostate cancer patients before and after immunotherapy with dendritic cells exogenously pulsed with the prostate-specific membrane antigen-derived peptides, PSM-P1 and PSM-P2. For all subjects, we observed that clinical responses were strongly associated with two indicators of immunocompetence: skin test responses to recall antigens and cytokine secretion by T cells after nonspecific stimulation. In a subset of responders, we observed cytokine secretion or cytotoxicity against the immunizing peptides or an immunodominant epitope from an influenza recall antigen. The clinical results support the use of monitoring for overall immunocompetence to help determine why a patient has or has not responded to therapy. Moreover, it could be useful as an inclusion criterion to select those more likely to benefit from treatment.

Introduction

The concept of the immune system being responsible for policing the body for aberrant cells was initially put forth years ago by F. MacFarlane Burnet, who named it immune surveillance (1). With the recent explosion in the identification of tumor-specific and tumor-associated antigens (reviewed in Refs. 2 and 3), there is renewed interest in the theory of immune surveillance. Multiple strategies for using immune cells as adjuvant therapy to eradicate residual cancer cells after primary treatment have recently been, or are currently, in clinical trials (4–9). In addition to assessing the clinical outcome in these trials, investigators have also begun developing methods to monitor the immune response during, as well as after, therapy (10, 11). Evaluation of the immune response for patients involved in immunotherapy is important for several reasons. It should help determine the underlying mechanism(s) responsible for the clinical response. Once the mechanism is understood, the treatment can be refined and improved. Immunological monitoring assays aim to provide a surrogate marker for clinical response, allowing for earlier evaluation of the efficacy of the treatment. Finally, patients may be prescreened to determine which are viable candidates for immunotherapy. We recently completed a Phase II clinical trial treating prostate cancer patients with autologous dendritic cells pulsed with derived peptides (PSM-P1 and PSM-P2) and designated PSM-P1 (peptide sequence, LLHETDSAV) and PSM-P2 (peptide sequence, ALFDIESKV). As many as 30% of the patients achieved clinical benefit, based on PSA changes and other established criteria. Participants were evaluated for both nonspecific and vaccine-specific reactivity. Most importantly, the realization of clinical responders allowed us to assess whether any immune parameters correlated with treatment outcome. Although we detected occasional and anecdotial immune reactivity to PSM-P1 and PSM-P2, it did not show relevance to the patient’s clinical situation. Overall immunocompetence prior to the initial infusions, as assessed by: (a) skin test; and (b) cytokine secretion after nonspecific T cell stimulation, displayed an association with clinical status. Our data confirm well-established conceptions about a link between immune status and the ability to mount an effective antitumor response. However, this is the first demonstration of such a link in actual clinical responders after DC-based vaccination against prostate cancer.

Materials and Methods

Peptides and Cytokines. The source of peptides and cytokines used in these experiments has been delineated in prior studies (14). Briefly, PSMA peptides with HLA-A0201-specific motif (PSM-P1, MLHETDSSAV; PSM-P2, ALFDIESKV) were synthesized and purified (>95%) by Peninsula Laboratories, Inc. (Belmont, CA). The influenza matrix peptide M15 8–66 (GILGFVFTL), derived from the influenza matrix protein M1, was synthesized by Multiple Peptide Systems (San Diego, CA). It is also presented in the context of HLA-A0201 (15). Granulocyte-macrophage colony-stimulating factor was provided by Immunex Corp. (Seattle, WA). IL-4 was purchased from Peprotech, Inc. (Rocky Hill, NJ).

DC Generation and Infusion. The generation of DCs from PBMCs has been described previously (14). Briefly, PBMCs were isolated using Lymphoprep (Life Technologies, Inc., Gaithersburg, MD) or Histopaque 1077 (Sigma Chemical Co., St. Louis, MO). After a 1-h, 37°C incubation step in tissue culture flasks, adherent PBMCs were cultured with granulocyte-macrophage colony-stimulating factor (500 units/ml) and IL-4 (500 units/ml) for 7 days. The treatment regimen has been detailed in previous work (12, 13, 16, 17). All patients had confirmed locally recurrent prostate cancer or hormone-refractory, advanced disease. Immediately prior to treatment, DCs were exogenously pulsed with peptide via incubation with 10 μg/ml PSM-P1 and PSM-P2 for 2 h. After washing, the DC suspension was infused i.v. over 30 min in a total volume of 10 ml of 0.9% saline. Patients received six infusions of peptide-pulsed autologous DCs at 6-week intervals. Patients were followed before, during, and after therapy with periodic PSA (Tandem-E PSA kit; Hybritech, Inc., San Diego, CA), chest X-rays, bone scans, and ProstateScint scans (18). Clinical responses were determined by modified criteria of the National Prostatic Cancer Project (19) as well as PSA determinations.

Measurement of DTH. DTH was measured prior to the first infusion and after the final treatment with a Multitest CMI (Pasteur Merieux Connaught, Swiftwater, PA). The recall antigens included in the test were: tetanus, streptococcus, tuberculin, glycerin, Candida, trichophyton, and protein. Antigen (0.1 ml) was injected intradermally. The skin test was read 48–72 h after administration. The widest diameter of distinctly palpable induration was recorded in millimeters. A 5-mm or greater induration was scored as a positive test.

Measurement of Nonspecific T-Cell Response. Blood was drawn from patients prior to the first infusion and at time points after every infusion. PBMCs were isolated at each time point and stored in liquid nitrogen. PBMCs from all time points were evaluated in the same assay for any one patient. To stimulate with anti-CD3, 100 μl of monoclonal antibody (Biosource International, Camarillo, CA) at three concentrations (0.5, 0.25, and 0.1 μg/ml) was

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2 The abbreviations used are: PSMA, prostate-specific membrane antigen; PS, prostate-specific antigen; HLA, human leukocyte antigen; IL, interleukin; DC, dendritic cell; PBMC, peripheral blood mononuclear cell; DTH, delayed-type hypersensitivity; Th1, T helper cell type 1.
added in triplicate in a 96-well, round-bottomed plate. After incubation overnight at 4°C, antibody was removed, and 2 × 10^6 PBMCs were added in 0.2 ml of AIM V (Life Technologies, Inc., Gaithersburg, MD). After a 48-h incubation, supernatant was removed. A sandwich ELISA was used to determine the amount of IFN-γ in the supernatant. ELISA plates (Greiner, Palatine, IL) were coated with 100 µl of capture antibody (Endogen, Inc., Woburn, MA) diluted in PBS at 1 µg/ml. After overnight incubation at 4°C, antibody was removed, and the plate was blocked with 100 µl of PBS/BSA for 1 h at room temperature. Plates were then washed four times. Sample or IFN-γ standard and detection antibody were added in a volume of 100 µl, and the plate was incubated for 1.5 h at room temperature. Plates were washed four times with PBS/0.05% Tween 20 using a Microplate Autowasher (Bio-Tek Instruments). Streptavidin peroxidase (Zymed, South San Francisco, CA), diluted 1:10,000 in PBS/Tween, was added in a volume of 100 µl and incubated for 30 min at room temperature. Plates were washed four times, and then 100 µl of OPD substrate (Zymed) was added to each well. The reaction was incubated for 30 min at room temperature and then stopped with 100 µl of 2N H₂SO₄. The plates were read at 490 nm with a 630-nm reference using an EL 340 Microplate reader (Bio-Tek Instruments).

**Microwell Cytokine Assay.** Our procedure was a modification of the semiquantitative assay described recently by Romero et al. (Ref. 11; Fig. 1). PBMCs from prostate cancer patients enrolled in the clinical trial were used. IFN-γ secretion was measured using PBMCs collected before the onset of therapy (pretreatment) and during or after the completion of the regimen (posttreatment). They were added to 96-well plates at a concentration of 1 × 10⁶/ml in a volume of 100 µl. They were cocultured with autologous DCs exogenously pulsed for 2 h with 25 µg of peptide(s) consisting of either: (a) PSM-P1 and PSM-P2; or (b) M1. An E/T ratio of 10:1 was used. DMEM (Biofluids, Inc., Gaithersburg, MD) supplemented with 10% human serum (Sigma Chemical Co., St. Louis, MO), antibiotics, and gentamicin was used for all cultures. Cells were cultured in a total volume of 200 µl. Three days after initial stimulation, 300 IU/ml IL-2 were added to each 96-well culture. Cultures were restimulated with peptide-pulsed DCs at day 10 of culture. On day 11, 1 day after restimulation, 300 IU/ml IL-2 were added to each microwell.

Each 96-well plate contained two rows (24 wells) of the following: (a) preimmune PBMCs stimulated in vitro with PSM-P1 and PSM-P2 peptides; (b) preimmune PBMCs stimulated in vitro with M1 peptide; (c) postimmune PBMCs stimulated in vitro with PSM-P1 and PSM-P2; and (d) postimmune PBMCs stimulated in vitro with M1. The aforementioned microwells were set up in duplicate; one set of plates was dedicated to ELISA, and one set was dedicated to cytotoxicity analysis.

On day 17, all microwells were gently resuspended. One hundred µl of each effector T-cell suspension were added to 100 µl of T2 cells (5 × 10⁵/ml) that had previously been exogenously pulsed with whichever peptide(s) (PSM-P1 and PSM-P2, or M1) were used for the stimulations in vitro. The remaining 100µl of each effector T-cell suspension was added to 100 µl of T2 cells (5 × 10⁵/ml) alone (unpulsed). Twenty-four h later, the supernatants were harvested and measured for IFN-γ and IL-10 production in an ELISA using paired antibodies from the manufacturer (Endogen, Inc., Woburn, MA). All experimental assay points were in singlicate. Specific cytokine secretion was determined by subtracting the amount of IFN-γ and IL-10 from a particular microwell in response to T2 alone from that secreted in response to T2 plus peptide(s). Standards were performed in duplicate.

**Microwell Cytotoxicity Assay.** The procedure was identical to that of the microwell cytokine analysis, with the exception that the T2 cells were labeled. Target cells (1 × 10⁶) were labeled with In (Nycomed Amersham, Buckinghamshire, United Kingdom) in 0.5 ml of PBS or AIM-V (Lymphoprep; Life Technologies, Inc.) for 10 min at 37°C, then washed three times before the addition of T cells. Incubation of target and effector cells was carried out for 4 h at 37°C. Supernatants were collected and counted on a gamma counter. The percentage of specific lysis was calculated as: (sample counts – spontaneous counts/maximum counts – spontaneous counts) × 100%. Spontaneous release was determined from targets incubated with AIM-V. Maximum release was determined from targets incubated with 2% SDS. All experimental assay points were in singlicate. Spontaneous and maximum releases were determined in triplicate. Spontaneous counts did not exceed 30% of maximum. The percentage of specific cytotoxicity was determined by subtracting the percentage of cytotoxicity from a particular microwell in response to T2 alone from the lytic activity in response to T2 plus peptide(s). Only the percentage of specific cytotoxicity >10% was scored as an antigen-specific microwell.

**Results**

A total of 107 patients with either local recurrence of prostate cancer after primary treatment failures or with hormone-refractory metastatic prostate cancer were treated in this Phase II clinical trial (13). Patients received six infusions of autologous DCs pulsed with two HLA-A2 binding, PSMA-derived peptides every 6 weeks. The treatment was well tolerated by all study participants. Approximately 30% of the trial participants had clinical responses defined by modified National Prostate Cancer Project criteria including a 50% reduction in PSA.

A cohort of patients was clinically evaluable (i.e., 46 for DTH testing), and it is those patients who were assessed for this report. The immune status of the patient was evaluated using two different assays: (a) DTH; and (b) cytokine secretion. The first assay was a skin test measuring DTH to six different recall antigens: tetanus, diphtheria, streptococcus, tuberculin, glycerin, Candida, trychophyton, and protex. Patients were tested prior to infusion and after completion of all six infusions. As shown in Table 1, patients that responded clinically to DC-based immunotherapy were more likely to have a DTH response than those patients who did not. Moreover, there was an association between response to treatment and the number of patients with three or more positive skin tests. Because of the small number of complete responders and the number of patients in each group overall, both Kruskal-Wallis analysis and P test did not indicate that the differences between groups were statistically significant. However, the tendency was for partial and complete responders to have a greater
percentage of patients with three or more positive reactions, and this greater reactivity remained constant throughout treatment.

The second assay used to assess the immune response of the patients was cytokine secretion by T cells after nonspecific stimulation. PBMCs, from the pretreatment time point, were stimulated with anti-CD3 monoclonal antibody, and IFN-γ production was measured 48 h later by ELISA. As shown in Table 2, production of low amounts of IFN-γ (<2000 pg/ml) correlated with poor clinical response, whereas production of greater amounts (>300 pg/ml) indicated a probability of a positive clinical outcome. Although two patients with progressive disease demonstrated high cytokine secretion from pre-immune PBMCs, it is important to point out that this reactivity was not durable. That is, when using PBMCs from posttreatment time points, only low IFN-γ secretion was observed. This is in contrast with PBMCs from responders, whose high IFN-γ secretion was maintained throughout treatment. In total, our cytokine secretion data suggest that DC-based immunotherapy is more effective in patients with a functional immune system.

To assess specific immunity induced by the therapy, DTH responses against vaccine components were measured. Patients were given autologous DCs alone and autologous DCs pulsed with both PSMA-derived peptides. Six partial responders demonstrated enhanced DTH responses to both DCs plus peptides as well as DCs alone (data not shown). Therefore, such reactivity could not be classified as peptide-restricted in nature. In fact, only one partial responder demonstrated an enhanced DTH response solely to DCs plus peptides after treatment when compared with the pretreatment response.

We performed a semiquantitative analysis (Fig. 1) for PSMA- and M1-restricted T-cell reactivity by using a modified microwell ELISA (Fig. 2) and cytotoxicity assay (Fig. 3). Instead of a bulk method, usually using 24-well culture dishes, we established singlicate microcultures of PBMCs in 96-well plates. For patients in our clinical trial, usually using 24-well culture dishes, we established singlicate microcultures (Fig. 2) and cytotoxicity assay (Fig. 3). Instead of a bulk method, using a modified microwell ELISA (Fig. 1 and as described in “Materials and Methods.” Briefly, each of effector T-cell stimulators was added in duplicate to 96-well plates in a volume of 100 μl. Effector T cells (5 × 10^5) and 5 × 10^5/ml DC stimulators were added in duplicate to 96-well plates in a volume of 100 μl each. Twenty-four h later, the supernatants were harvested and measured for IFN-γ production in an ELISA using standard, matched antibody pairs. The ELISA was set up as shown in Fig. 1 and as described in “Materials and Methods.” Briefly, each of effector T-cell stimulators was added in duplicate to 96-well plates in a volume of 100 μl each. Twenty-four h later, the supernatants were harvested and measured for IFN-γ production in an ELISA using standard, matched antibody pairs. The ELISA was set up as shown in Fig. 1 and as described in “Materials and Methods.”

We have demonstrated that clinical responses to DC-based immunotherapy strongly correlated with two measurements of immunocompetence: DTH responses to recall antigens, and secretion of cytokine by T cells after nonspecific stimulation. Using skin testing, we showed that human subjects achieving treatment benefit displayed strong, durable DTH responses against a panel of recall antigens, as compared with subjects treated similarly whose disease was not impacted. Using cytokine release by T cells after nonspecific stimu-
We defined the percentage of specific cytotoxicity as described previously: % specific CTL from responders but not progressors demonstrate specific lytic activity of T2 alone). We defined the percentage of specific cytotoxicity as the percentage of lysis (T2/PSM − T2 alone). Therefore, we defined the percentage of specific cytotoxicity as described previously: % specific cytotoxicity = % lysis (T2/M1 − T2 alone). PBL, peripheral blood lymphocytes; PRE, pretreatment; POST, posttreatment.

Importantly, we demonstrated the importance of strong, durable immunocompetence in a human system. These correlations suggest that despite our inability to demonstrate peptide-specific immune reactivity in a significant percentage of responders, the mechanism is immune based. It suggests that a patient with a comparatively healthy immune system is more likely to benefit from immunotherapy. Although the need for preexisting and stable immunocompetence is not a novel concept, we were able to demonstrate its validity in actual complete and partial clinical responders obtained using a DC-based immunotherapy of cancer patients, not simply an animal system using a model antigen.

Lastly, for those whose cancer is of an earlier stage, monitoring a patient’s immune reactivity will be an important adjunct to imaging studies such as bone scans for the clinician to assess whether a patient is responding to immunotherapy.

It is noteworthy that the durable complete responder in our protocol demonstrated a Th1-type of immune response. This patient demonstrated specific cytokine secretion in when stimulated in vitro and subsequently tested against either the immunizing peptides or the recall antigen M1. The number of complete responders sampled is far too small to draw any firm conclusions. But it must be recognized that our results are interesting in light of the difficulty in producing any partial or complete responders in patients with hormone-refractory, metastatic prostate cancer.

Finally, in a finite cohort of partial responders, we demonstrated an association between clinical outcome and either cytokine secretion or lytic activity against the immunizing PSM-P1/P2 peptides or against the recall epitope M1. Pre-existing immunity seemed to be most strongly associated with clinical outcome for those microwell analyses that demonstrated antigen-specific cytokine secretion or cytotoxicity. But it must be mentioned that this observation, although not anecdotal, was not observed in a vast majority of patients. That is, we did not observe any repeatable, reliable correlation between peptide-specific immunoreactivity and clinical outcome. Therefore, it is highly questionable whether the time, effort, and cost of setting up microwell analyses of this sort are justified, and any observed correlation must be considered far from conclusive.

The paucity of immunological data concerning PSMA could be attributable to several reasons. The antigen may be poorly immunogenic. It is not inconsequential to note that there is not a single report in the literature of T-cell reactivity against PSMA-derived peptides. Alternatively, the assays used may not have been sensitive enough to detect PSMA-specific responses. T-cell receptor-ligand interactions are relatively weak compared with antigen-antibody interactions. In addition, there is great variation in the sensitivity of immune monitoring methods, depending upon the approach used (11), and if the precursor T-cell frequency was below 1/10^6, it would likely be below the threshold of detection of the methods we used. Perhaps PSMA-specific cells are sequestered at tumor sites and not found in the peripheral blood. Finally, the mechanism responsible for the clinical response may have not been addressed. For example, DCs have been reported recently to stimulate natural killer cells (20): natural killer activity would not have been detected by any of the assays used in our evaluation.

There has been a great deal of work concerning immune monitoring (reviewed in Ref. 10). Five methods commonly used to detect B- or T-cell responses in PBMCs are: (a) antibody titer determinations; (b) cytotoxicity; (c) cytokine release (via ELISA, ELISPOT, or intracellular staining); (d) molecular analysis of T-cell receptor usage; and (e) direct quantification of antigen-specific cells using tetramers (11). A complimentary tactic is to look for defects in a patient’s immune cells (21–23), or prostate tumor escape mechanisms (24, 25), as reasons for a subject to fail treatment. There is currently no consensus on which methods to use or how a monitoring scheme should be designed. This is appropriate, considering that the field of cancer vaccines directed against defined antigenic targets is in its infancy. One fundamental, inherent problem with true immunological monitoring is that a sufficient number of clinical responses must be achieved before statistical correlations between in vitro and in vivo parameters are declared; for experimental immunotherapy of advanced cancers, this is not trivial. For tumor types other than melanoma, there exists another dilemma:

the glaring lack of basic knowledge concerning human tumor-associated antigens. It is a problem that must be addressed if monitoring and the immune-based therapies they are based upon are to fulfill their early promise.

References

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