Tumor Prevention and Antitumor Immunity with Heat Shock Protein 70 Induced by 15-Deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ in Transgenic Adenocarcinoma of Mouse Prostate Cells

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Abstract

The biological modifier $\Delta^{12}$-prostaglandin J$_2$ and related prostaglandins have been reported to have significant growth-inhibitory activity with induction of heat shock proteins (Hsps). Tumor-derived Hsps have been shown previously to elicit specific immunity to tumors from which they are isolated. In this study, 15-deoxy-$\Delta^{12,14}$-prostaglandin J$_2$ (15d-PGJ$_2$)-induced Hsp70 was purified from transgenic adenocarcinoma mouse prostate cells (TRAMP-C2). It was then tested for its ability to activate specific CTLs and induce protective immunity against prostate cancer in C57BL/6 mice. Treatment of cells with 8.0 $\mu$m 15d-PGJ$_2$ for 24 h caused significant induction of Hsp70 expression. The yield of Hsp70 purified from 15d-PGJ$_2$-treated cells was 4–5-fold higher when compared with untreated TRAMP-C2 cells. Vaccination of mice with Hsps isolated from TRAMP-C2 cells elicited tumor-specific CTLs and prevented the growth of TRAMP-C2 tumors. These results indicate that the induced heat shock proteins may have promising applications for antitumor, T-cell immunotherapy. In particular, these findings have important implications for the development of novel antitumor therapies aimed at promoting an immune response to prostate tumors.

Introduction

Prostate cancer in its early stages is amenable to surgery, radiation treatment, and hormone therapy. The major concern is that the prognosis for late-stage metastatic prostate cancer is poor. Manipulation of the immune system appears to be a promising means that may allow elimination of metastatic cells. However, most human cancer cells are not sufficiently immunogenic to trigger an immune response in vivo (1–3). One possible reason for this lack of tumor cell immunogenicity is that most tumor antigens are either masked or inaccessible. One of the most effective ways to stimulate antitumor immunity has been to promote cross-priming of CTLs by host professional antigen-presenting cells (4, 5). Cross presentation is thought to occur when antigen-presenting cells take up Hsps (6) and may represent a mechanism for inducing immunotherapy for prostate cancer.

The antiproliferative activity of 15d-PGJ$_2$ causes nonapoptotic cell death in prostate tumor cells (7). The growth-inhibitory effect of 15d-PGJ$_2$ on tumor cells involves the induction of Hsp70 synthesis (8). Hsp overexpression leads to an increased chaperoning of antigenic peptides into a particular subset of macrophages or other antigen-presenting cells, leading to their efficient presentation via class I or class II pathways (9, 10). Heat shock proteins activate the resting antigen-presenting cells to take up and process the tumor antigens and up-regulate the expression of costimulatory molecules necessary for T-cell activation (6). There is now comprehensive experimental evidence that the antigenicity of tumor-derived Hsp70 and gp96 preparations results from diverse arrays of endogenous peptide antigens complexed with the Hsps (11). Therefore, Hsps isolated from a patient’s tumor represent a customized, patient-specific, pan-valent vaccine. This is because the Hsps chaperone an entire array of antigenic peptides generated by a tumor, instead of one or a few selected antigenic epitopes (12).

Several animal tumor models have examined the role of Hsps in antitumor responses. Vaccination of mice with Hsp preparations derived from autologous tumor cells have been shown to cause resistance to a subsequent challenge with live cancer cells in Zajdel ascitic hepatoma, in Meth A fibrosarcoma, and in B16 melanoma cells (13–15). This phenomenon has been shown with three major Hsps, gp96, Hsp90, and Hsp70. When the relative immunogenicities of the Hsps are compared in the Meth A sarcoma, the immunogenicity of Hsp90 was ~10% that of gp96 or Hsp70 (16). In the poorly immunogenic UV-induced mouse carcinomas, vaccination with gp96 preparation has been shown to elicit CTL and memory T-cell responses in addition to tumor prevention (17). These results illustrate that different Hsps may have different antigenic responses in various tumors.

Recent studies in the Dunning prostate cancer rat model showed tumor preventive response by vaccination with gp96, with delay in the development of tumor (18). However, previous work has not investigated the role of Hsps in the ability to activate tumor-specific CTLs in antiprostate tumor therapy or the role of Hsp70 in prostate cancer immunotherapy. Expression of Hsps after 15d-PGJ$_2$ treatment may provide a functional signal to the immune system that could contribute to the breaking of tolerance to tumor antigens that would otherwise have remained immunologically hidden. Therefore, we studied whether 15d-PGJ$_2$-induced Hsp expression may have an effect on recognition of prostate tumor cells by the immune system.

In our study, TRAMP-C2 cells (transgenic adenocarcinoma mouse prostate cancer C2 cells) from the TRAMP model were used to evaluate the antitumor effect of Hsps induced by 15d-PGJ$_2$ in a prostate cancer model. Previously, Greenberg et al. (19) described a spontaneous autochthonous transgenic mouse model for prostate cancer. In this mouse model, TRAMP mice, which are transgenic for the SV40 large T antigen (Tag) under the control of the rat probasin regulatory elements, express Tag at puberty (6 weeks of age; Ref. 20). The probasin regulatory element is androgen regulated and prostate specific in transgenic mice (21). The development and progression of prostate cancer in the TRAMP model closely mimics the human disease. Three cell lines, TRAMP-C1, TRAMP-C2, and TRAMP-C3 were obtained from a 32-week-old TRAMP mouse prostate adenocarcinoma. C1 and C2 are transplantable in syngeneic C57BL/6 mice. It has been shown that Tag is not expressed in C1 and C2 cells in vitro.
or in vivo (22). We used TRAMP-C2 cells to investigate whether 15d-PGJ2-induced tumor-derived Hsp70 can be used as a vaccine to generate a specific antitumor immune response to prostate cancer and to protect immune-competent C57BL/6 mice from prostate tumor growth.

Materials and Methods

Mice. Male C57BL/6 mice, 6–8 weeks of age were obtained from Jackson Laboratories (Bar Harbor, ME). Animals were housed in the Mayo Animal Resources Facilities under controlled temperature, humidity, and a 12-h light and dark cycle with food and water ad libitum in the virus-free mouse facility. Animals were allowed to acclimate 5 days prior to the experiment.

Tumor Cell Lines. TRAMP-C2 cells were cultured in DMEM supplemented with 5% Nu-serum IV, 5% FBS, 100 units/ml penicillin, 100 units/ml streptomycin, and 5 μg/ml insulin. Murine EL4 lymphoma cells were maintained in Iscove’s modified medium with 5% FCS, 4 μM β-mercaptoethanol, and 10 μg/ml gentamicin.

Treatment of Cell Lines with 15d-PGJ2. 15d-PGJ2 was obtained from Cayman Chemical Company as a solution in methyl acetate. The solvent is changed to ethanol by evaporation of methyl acetate under a gentle stream of nitrogen. TRAMP-C2 and EL4 lymphoma cells were seeded in 10-cm culture dishes at 1 × 10^6 cells in DMEM and Iscove’s media. The medium was changed to 5% charcoal stripped serum medium before 24 h of treatment. The cells were treated with varying concentrations of 15d-PGJ2 for 24 h, washed once with PBS, harvested, and centrifuged at 1000 rpm for 5 min. Whole-cell lysates were prepared according to the protocol provided by Santa Cruz Biotechnology, Inc. with minor modification. Cells were washed with cold 1× PBS once and lysed in RIPA buffer [1× PBS, 1% NP40 (Amresco), 0.5% sodium deoxycholate, 0.1% SDS] (23). Protein concentration was determined by detergent compatible assay (Bio-Rad).

Antibodies. The anti-Hsp70 mouse monoclonal antibody was obtained from Dr. David Toft (Department of Biochemistry, Mayo). It recognizes both constitutive (Hsp73) and inducible (Hsp72) forms. Mouse anti-Hsp70 monoclonal antibodies SPA-810 and SPA-815 specific for inducible (Hsp72) and constitutive (Hsp73) forms, respectively, were obtained from StressGen Biotechnologies Corp. (Victoria, British Columbia, Canada). β-Tubulin mouse monoclonal antibody was obtained from Sigma Chemical Co. (St. Louis, MO).

Western Blot Analysis. The expression of Hsp70 in cells treated with 15d-PGJ2, 24 h was examined by Western blot analysis. Aliquots of the cell lysates (25 μg) or purified Hsp (1.0 μg) were resolved on 10% polyacrylamide gels and transferred to nitrocellulose membrane. The membranes were blocked in 5% nonfat dry milk in TBST [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20] at room temperature for 1 h. Blots were incubated with Hsp70 antibody (1,5000 dilution), inducible Hsp70 antibody (1:1000), constitutive Hsp70 antibody (1:1000), or β-tubulin antibody (1:10,000) for 1 h at room temperature. Blots were washed three times with TBST and then incubated for 1 h with 1:10,000 diluted horseradish peroxidase-conjugated antimoise IgG antibody (Amersham Life Science, Arlington Heights, IL), and the proteins were detected using an enhanced chemiluminescence Western blotting analysis system (Amersham Life Science). β-Tubulin was used as the control of protein loading and transfer efficiency.

Purification of Hsp70 PCs from TRAMP-C2 and EL4 Cell Lines. Cells were seeded in T-175 cm^2 culture flasks at 4 × 10^6 cells and were treated with 8 μM 15d-PGJ2 for 24 h. The cells were collected by centrifugation at 1000 rpm for 10 min. The pellet was washed with PBS and homogenized in hypotonic buffer (10 mM NaHCO3, 0.5 mM pefabloc, pH 7.1). The Hsp70 PC was isolated according to the method of Peng et al. (24), using ADP agarose column (Sigma) and further purified by FPLC system (Mono Q; Pharmacia Biotech). We used pefabloc SC instead of phenylmethylsulfonyl fluoride as protease inhibitor. Proteins were quantified by Bradford assay, and BSA was used as standard (Sigma).

Prophylactic Assay. Male mice, 6–8 weeks of age, were divided randomly into three groups of six animals each. Group I received 200 μl of PBS, group II received Hsp70 PCs purified from TRAMP-C2 cells, and group III received Hsp70 PCs purified from EL4 cancer cells. Hsps were injected s.c. under the nape of the neck, in 200 μl of PBS, twice a week for 2 weeks. The animals were challenged with 3 × 10^6 live cancer cells (isolated on the same day) s.c. on the right flank, 10 days after the final immunization, and the kinetics of tumor growth was monitored.

Generation of CTL Effector Population. Mice were immunized as described in prophylactic assay. Ten days after the final immunization, the mice were sacrificed, spleens from mice in the same treatment group (four mice/group) were harvested, and the unfractionated splenocytes were restimulated in vitro with corresponding TRAMP-C2 and EL4 Hsps. The cells were cultured in 24-well plates for 6 days at a concentration of 5 × 10^6 cells in 1.0 ml of Iscove’s medium with the addition of recombinant mouse IL-2 (50 units/ml) after 24 h of culture. Cytolytic activity was assayed after 6 days of incubation.

CTL Assay. Target cells TRAMP-C2 and EL4 were cultured in flasks with 100 units/ml IFN-γ before 72 h of CTL assay. The cells were collected by trypsinization and labeled with 300 μCi of chromium chloride (51Cr) for 90 min. Effector cells were plated in 96-well plates at various E/T cell ratios in triplicates. The total reaction volume was kept at 200 μl/well. After 4 h of incubation of effector and target cells at 37°C/5% CO2, 30 μl of cell free supernatant were collected from each well and counted in the Top Count NXT (Packard) counter. The amount of 51Cr spontaneously released was obtained by incubating target cells in medium alone. The total amount of 51Cr incorporated was determined by adding 2% Triton X-100 in PBS to the target cells, and the percentage of specific lysis was calculated as follows: % lysis = [(sample cpm – spontaneous cpm)/(total cpm – spontaneous cpm)] × 100.

Results

Induction of Hsps. To investigate the effect of 15d-PGJ2 on the expression of Hsps, TRAMP-C2 cells were incubated with various concentrations of 15d-PGJ 2 (2.5–15 μM) for 24 h. The cells were harvested, and whole-cell extracts were run on a gel and then immunoblotted with Hsp70 antibody. Fig. 1 shows dose-dependent induction of Hsp70 protein with 15d-PGJ2 concentrations ranging from 2.5 to 10 μM. Using Hsp70 antibody which recognizes both inducible and constitutive forms, maximum induction of Hsp70 was seen with 10 μM 15d-PG-J2. Hsp70 in EL4 cells was also induced by 8.0 μM 15d-PG-J2 when compared with the untreated group (Fig. 1B).

Purification of Hsp70 PCs. To isolate the Hsp70 PCs, the 15d-PGJ2-treated cell lysates were purified using an ADP-agarose column, followed by ion-exchange chromatography (Mono Q FPLC system). Next, the fractions were resolved by SDS polyacrylamide gels. Whole-cell lysates and the purified fractions from the mono Q column were immunoblotted with Hsp70 antibodies specific for the inducible and constitutive forms (Fig. 1C). Densitometry of the purified fractions revealed that 92% of the Hsp70 from TRAMP-C2 cells is the inducible form and 8% is the constitutive form. Purified fractions from both TRAMP-C2 and EL4 cells were stained with silver nitrate. The staining shows a single band of Hsp70 on the gel from both EL4 and TRAMP-C2 cells (Fig. 1D). This reveals homogeneity of preparation with little or no contamination from other proteins. We also purified Hsps from TRAMP-C2 cells without 15d-PGJ2 treatment (data not shown). The amount of the purified product of Hsp70 with 15d-PGJ2 treatment was 4–5-fold higher as compared with untreated cells.

Generation of Tumor-specific CTL Response by Vaccination with Hsp PCs. We evaluated the ability of 15d-PGJ2-induced Hsps to elicit a CTL response against TRAMP-C2 cells. As expected, mice treated with PBS had a low level of CTL activity against either TRAMP-C2 or EL4 targets (Fig. 2). Mice immunized with Hsps isolated from TRAMP-C2 cells developed high levels of CTL activity against TRAMP-C2 targets but not EL4 targets. Mice vaccinated with EL4 Hsps developed relatively low CTL activity against TRAMP-C2 cells or the lymphoma cells. These results indicate that the vaccination of mice with syngeneic C2 tumor-derived Hsps elicited a tumor-specific CTL response against TRAMP-C2 prostate tumor cells.

Antitumor Protection with Hsp70 PCs. To determine whether Hsp70 vaccination protects against a lethal challenge of TRAMP-C2
tumor cells, the mice were injected with Hsps or PBS, and then the C2 cells were observed for tumor growth over a 9-week period. Mice pretreated with PBS and EL4 Hsps developed palpable tumors by 4 weeks after tumor challenge. The tumors grew rapidly, leading to the death of the animals within 8 weeks (Fig. 3, A and B). In contrast, mice preimmunized with Hsps isolated from 15d-PGJ2-treated TRAMP-C2 cells showed resistance to tumor challenge, and only two of the six mice developed tumors (Fig. 3C). The tumors in these two mice exhibited delayed kinetics and were quite small, with the average diameter of 5.5 mm around 7 weeks after tumor challenge. Interestingly, the tumor in one of the two mice eventually disappeared (Table 1) by the eighth week. In the second mouse, the tumor grew slowly until the end of the experiment at 9 weeks.

Discussion

We used TRAMP-C2 tumors in syngeneic C57BL/6 mice to evaluate the efficacy of Hsp70 vaccination in a prostate cancer model. The disease progression in the original TRAMP mice closely resembles the progression of human prostate cancer. Therefore, it is a useful model for evaluating preventive and therapeutic approaches for prostate cancer. This model should provide better correlation between animal and human antitumor results than previous models (25, 26).

We found that 15d-PGJ2 caused nonapoptotic cell death (data not shown) with induction of Hsp70 synthesis in TRAMP-C2 cells. We have also observed 15d-PGJ2-induced Hsp70 overexpression in other prostate cells such as TRAMP C1, LNCaP, PC3, and DU145 cells (data not shown), indicating a broad Hsp70 induction with 15d-PGJ2. Maximum induction of Hsp70 was seen around 8–10 μM 15d-PGJ2. The use of an ADP-affinity column allowed the isolation of immunogenic peptides associated with Hsp70, and these preparations mostly contained the inducible (Hsp72) and constitutive form (Hsp73). Inducible fractions of the Hsp70 from the TRAMP-C2 cells were resolved by SDA-PAGE and stained with silver nitrate. Panel 1 is the low molecular weight marker, and panels 2 and 3 are purified Hsp70 from TRAMP-C2 and EL4 cells, respectively.

Fig. 1. A. 15d-PGJ2-induced expression of Hsp70 in TRAMP-C2 cells. TRAMP-C2 cells were treated with various concentrations of 15d-PGJ2 for 24 h and immunoblot analysis with Hsp70 monoclonal antibody recognizing both the constitutive and inducible forms. B. induction of Hsp70 expression in EL-4 cells with 8 μM 15d-PGJ2. C. TRAMP-C2 cells treated with 10 μM 15d-PGJ2, the whole-cell lysate, and the purified fraction of Hsp70 from the mono Q column were immunoblotted with mouse Hsp70 antibodies specific for the inducible form (Hsp72) and constitutive form (Hsp73). D. purified fractions of the Hsp70 from the TRAMP-C2 cells were resolved by SDA-PAGE and stained with silver nitrate. Panel 1 is the low molecular weight marker, and panels 2 and 3 are purified Hsp70 from TRAMP-C2 and EL4 cells, respectively.

Fig. 2. Induction of CTL activity after immunization with TRAMP-C2 Hsps, EL4 Hsps, and PBS for control animals. Spleens from each group were collected 10 days after the final immunization. Pooled splenocytes from each group were restimulated in vitro with Hsp PCs isolated from TRAMP-C2 and EL4 cells and tested for cytolytic activity after 7 days of culture. Targets consisted of TRAMP-C2 (A) and EL4 (B) cells.
preparations were obtained (12). Immunization of mice with Hsps elicits a specific cellular immune response not against the Hsps per se but against antigenic peptides chaperoned by them. Results from our study demonstrate that tumor-derived TRAMP-C2 Hsp vaccination as opposed to EL4 Hsps induces a specific CTL response against the C2 cells. The appearance of CTLs specific for TRAMP-C2 cells and capable of lysing C2 cells in vitro correlated with the development of protective immunity against TRAMP-C2 tumors in vivo. Similar results were obtained when the experiment was repeated. The possible reasons for low EL4 CTL activity with EL4 cell-derived Hsps might be because the conditions for vaccinations were not optimal (e.g., frequency and time interval of vaccination). Alternatively, the amount of Hsp70 PC used might be insufficient to develop a significant immune response.

A well-characterized major peptide binding Hsp70 was shown to elicit immunity to the tumors from which it was isolated but not to antigenically distinct tumors (28). The induction of Hsp72 in B16 melanoma cells significantly enhanced the immune recognition of tumor cells by increasing the levels of MHC class I antigens on their surface (29). A role of Hsp72 in the trafficking of antigenic peptides has been suggested by Srivastava et al. (30). They have shown that exogenous peptides administered as complexes with Hsp72 are efficiently shunted into the MHC class I presentation pathway. It was shown that Hsps localized in distinct intracellular compartments are associated with different sets of precursors for MHC class I binding, tumor antigenic peptides. The patterns of association of peptides were distinct and specific for each Hsp. In a mouse leukemia model, Hsp90 was found associated with an 8-mer epitope as well as two other precursor peptides, whereas Hsp70 was associated with only the 8-mer epitope and gp96 was associated with the 8-mer epitope and one of the 10-mer precursor peptides. The antigenic peptides associated with Hsp70 in the prostate cancer model are not yet known. However, vaccination with autologous tumor-derived Hsp PCs uses the entire antigenic repertoire of the cell, which circumvents the need to identify a large number of CTL epitopes.

Our study shows that the 15d-PGJ 2-induced Hsp72 can be used to generate a specific CTL response. The immune response elicited by Hsp72 expression was also able to protect against tumor challenge. The advantage of using Hsp PCs for vaccination is that the antitumor immune responses will be generated for the entire antigenic repertoire of the cancer cells. The observations reported in this study provide some of the important elements needed for development of Hsp peptides as the basis of a new generation of vaccines against prostate cancer.

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References

HSP VACCINATION TO PREVENT PROSTATE CANCER

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