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PRINCIPAL INVESTIGATOR: Charles Y. Young, Ph.D.

CONTRACTING ORGANIZATION: Mayo Clinic Rochester
Rochester, MN 55905

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7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) Mayo Clinic Rochester Rochester, MN 55905 E-Mail: youngc@mayo.edu		8. PERFORMING ORGANIZATION REPORT NUMBER	
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13. ABSTRACT (Maximum 200 Words) Prostate cancer is a heterogeneous disease for which the only effective treatments for organ-confined cancer at this time include radical surgery, radiation or androgen ablation/hormonal therapy. But advanced prostate cancer is almost no cure. Effective ways to prevent and/or treat advanced prostate cancer are needed. Our recent work has shown that a terminal metabolite, 15-deoxy- Δ 12,14-prostaglandin J ₂ (15d-PGJ ₂), of some essential polyunsaturated fatty acids can induce cell death of human and mouse prostate cancer cells and overexpression of heat shock protein 70 (Hsp70). Our preliminary results also demonstrated that Hsp70 purified from 15d-PGJ ₂ treated prostate cancer cells can induce prostate cancer specific immunity which can highly effectively prevent cancer growth. Tumor-associated peptides in purified Hsp70 from cancer cells are critical for induction of cancer-specific immunity. We hypothesize that heat shock protein from 15d-PGJ ₂ treated prostate cancer cells can generate higher anti-tumor immunity than that from untreated cancer cells and that 15d-PGJ ₂ may be a useful chemotherapeutic and immunotherapeutic agent for prostate cancer. Because a full immune competent host is required to evaluate and validate this proposed study before it can be tested in humans, TRAMP C2 cells and syngeneic male mice will be used for this proposal. This proposal will establish whether 15d-PGJ ₂ treatment will exhibit both chemotherapeutic and immunotherapeutic benefits for combating prostate cancer in animal models. If these goals can be accomplished, initial clinical trials would be appropriate to pursue in the near future.			
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Introduction:

Prostate cancer is a heterogeneous disease for which the only effective treatments for organ-confined cancer at this time include radical surgery, radiation or androgen ablation/hormonal therapy (1-4). But advanced prostate cancer is almost no cure. Effective ways to prevent and/or treat advanced prostate cancer are needed. Our recent work (5) has shown that a terminal metabolite, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J_2 (15d-PGJ₂), of some essential polyunsaturated fatty acids can induce cell death of human and mouse prostate cancer cells and overexpression of heat shock protein 70 (Hsp70) (6). It has been proposed that Hsp70 purified from cancer cells can induce cancer specific immunity which can highly effectively prevent cancer growth (7-12). Tumor-associated peptides in purified Hsp70 from cancer cells are critical for induction of cancer-specific immunity. We hypothesize that 15d-PGJ₂ can inhibit *in vivo* tumor growth and heat shock protein from 15d-PGJ₂ treated prostate cancer cells can generate high anti-tumor immunity. Therefore, 15d-PGJ₂ might be an excellent chemotherapeutic and immunotherapeutic agent for prostate cancer.

Body:

Statement of Work

1. Study whether 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ₂) can inhibit the growth of TRAMP C2 tumor and produce anti-tumor immunity. (months 1-15)

a. Evaluate the growth response of prostate tumor to 15d-PGJ₂ treatment.

i. Determination of tumor growth response to 15-d PGJ₂.

ii. Measure whether the expression of Hsp70 is increased by 15d-PGJ₂ treatment.

iii. Determine whether infiltration of immune cells can occur in tumor.

iv. Measure *in vitro* anti-tumor immune response by cytotoxic T cell (CTL) assay.

b. Determine *In vivo* anti-prostate cancer immune response.

2. Determine whether heat shock protein 70 (Hsp70)- peptide complex induced by 15-deoxy $\Delta^{12,14}$ prostaglandin J_2 (15d-PGJ₂) has higher potency for producing antitumor immunity when compared to hsp70 from untreated cells. (months 5-16)

a. Isolation of Hsp70-peptide complexes:

b. Prophylactic assay

c. Measuring anti-tumor immune response by cytotoxic T cell (TCL) assay

3. Determine whether the Hsp70 - peptide complex can prevent the development and progression of as well as treat prostate cancer in transgenic adenocarcinoma of the mouse prostate (TRAMP) model. (months 13-24)

a. Can anti-prostate cancer immunity be generated by 15-d PGJ₂ induced Hsp 70 for preventing and/or treating the cancer ?

b. Can 15-d PGJ₂ induce anti-cancer immunity for preventing and/or treating the cancer ?

Until now we have finished most of work outlined in specific aims 1 and 2 (see the above Statement of Work).

Since our work as proposed in Specific aim 1 showed that, although 15-d PGJ₂ is highly effective for inducing cell death of human prostate cell lines *in vitro*, it did not show any significant effects in *in vivo* studies as presented below. Because of this result, we have to modify our strategy of ongoing studies as described below.

To address the *in vivo* efficacy of 15-d PGJ₂ on tumor growth, mouse prostate cancer cell line, TRAMP C2, was used for this study. Adult male mice were used to inject s.c. TRAMP C2 cells (5×10^6 cells/mouse). After one month, the tumor becomes palpable, two concentrations of 15-d PGJ₂ in ethanol was mixed with phosphate buffered saline (PBS), or PBS were used for direct injection into tumor (6 mice per group) every two days for four times. Tumor size was measured three times a week thereafter for three weeks.

However, no differences in tumor size among the groups were noted. We did not observe any increase in Hsp70 protein expression in tumor tissues after 15-d PGJ₂ administration. To increase the efficiency of 15-d PGJ₂, we did another experiment by using lipiodol as a solvent vehicle and taxol as a positive control. Lipiodol is a lipid containing solvent that can enhance drug delivery to cancer cells. Again after implanted tumor became palpable, four group of tumor bearing mice (6 mice/group) received injection of vehicle (100 ul lipiodol), or vehicle plus the following 15-d PGJ₂ (3 mg), 15-d PGJ₂ (3 mg) + taxol (0.04 mg), or taxol (0.04 mg), respectively. This injection was repeated every other day for six times. Tumor sizes were measured as shown in Figure 1, which shows that the treatment of 15-d PGJ₂ had very little benefit to prevent tumor growth. Although taxol showed good inhibitory effects, surprisingly, 15-d PGJ₂ seemed to offset the effects of taxol. The reason for that is not clear. Moreover, why the *in vivo* effect of 5-d PGJ₂ is quite different from its effects *in vitro* is not understood at present time. Perhaps it is very labile and easily inactivated in *in vivo* conditions.

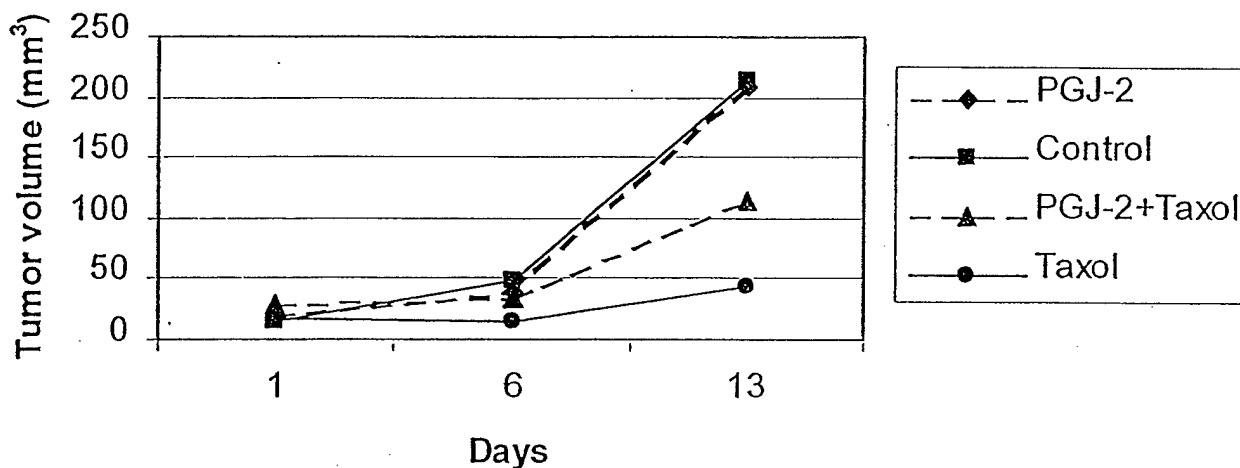


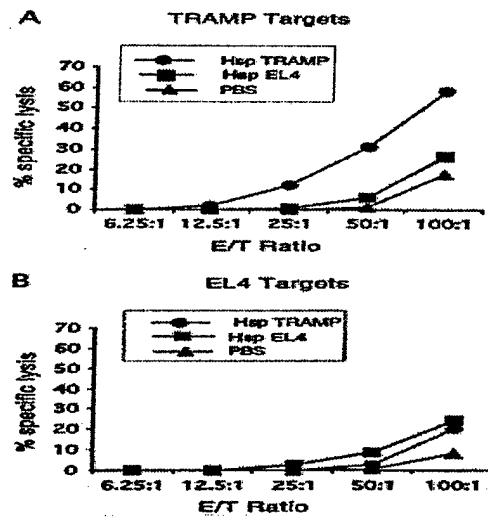
Figure 1. The *in vivo* effects of 15-deoxy- Δ 12,14-prostaglandin J₂ (15d-PGJ₂) on TRAMP C2 tumor growth.

These four groups of mice were further challenged with TRAMP cells (5×10^6 cells/mouse) to test whether there is anticancer immunity that can be induced. Unfortunately, no significance of tumor growth among groups could be observed. While we were starting to do the above studies, we finished most of work as proposed in Specific Aim 2 as summarized in Fig. 2 and table 1 (please also see the appendix, part of the study was published in *Cancer Research* 60, 4714-4718, 2000). The following shows some representative data of the results in this manuscript

Generation of a tumor-specific cytotoxic T lymphocyte (CTL) response by vaccination with

Hsp70-peptide complexes (HspPCs): The ability of HspPCs to elicit a CTL response against TRAMP cells was evaluated. Mice immunized with Hsp70PCs isolated from 15-d PGJ₂ treated TRAMP cells developed high levels of CTL activity against TRAMP targets but not EL4 targets. Mice vaccinated with EL-4 HspPCs developed relatively low CTL activity against TRAMP cells or EL4 thymoma cells. These results (Fig. 2) indicate that the vaccination of mice with autologous tumor derived Hsps elicit a tumor specific CTL response.

Fig 2 Induction of CTL activity following immunization with TRAMP-C2 Hsps, EL4 Hsps and PBS. Splens from each group were collected 10 days after the final immunization. Pooled splenocytes as effector cells (E) from each group were restimulated *in vitro* with HspPCs isolated from TRAMP-C2 and EL-4 cells and tested for cytolytic activity after 7 days of culture. Target cells (T) consisted of (A) ⁵¹Cr labeled TRAMP-C2 and (B) ⁵¹Cr labeled EL4 cells. Various ratios of effector and target cell mixtures were incubated at 37°C for 4 hours. Target cell lysis by effectors was measured. Note that only Hsp from TRAMP C2 generates highly specific anti-tumor T cells.



Anti-tumor protection with Hsp70 peptide complexes : Mice pretreated with PBS and EL 4 Hsps developed palpable tumors by four weeks of 3×10^6 live TRAMP C2 tumor cell challenge. The tumors grew rapidly leading to the death of the animals within 8 weeks (not shown). In contrast mice pre-immunized with Hsps isolated from TRAMP cells showed resistance to tumor challenge and only two of the six mice developed tumors with delayed kinetics. The tumors were quite small with the average diameter of about 5.5 mm around 7 weeks after tumor challenge. Interestingly, the tumor in one of the two mice eventually disappeared (Table 1) at the eighth week. In the second mouse the tumor grew slowly. The tumor growth was monitored up to nine weeks. This study showed at least 83 % of mice free of tumor at the end of the experiment.

Table 1.

No .weeks after challenge	4	5	6	7	8	9
Control (PBS)	4/6	6/6	6/6	6/6	6/6	NA ^a
Hsp70 PCs (EL-4)	5/6	6/6	6/6	6/6	6/6	NA ^a
Hsp70 PCs TRAMP-C2	0/6	0/6	0/6	2/6	1/6	1/6

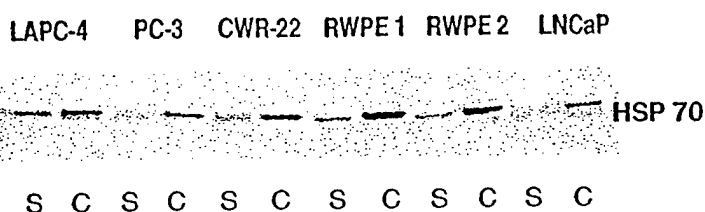
^aNA, not available

In addition, a modified and approved plan from Phase I proposal of this grant was accomplished. Since this work is not directly related to immunotherapy. It will not be described in this section (but see the publication in the appendix).

From our above studies with *in vivo* 15-d PGJ₂ treatment did not show any significant effects. Therefore, we wanted to change the course originally proposed in the specific aim 3 and concentrated on developing Hsp70 to a useful vaccine for prostate cancer. Also, this modification was partly based on our novel finding that endogenous Hsp can secrete into extracellular surrounding (Fig. 3a). Hsp has been recognized as an intracellular protein. We further demonstrated that ectopic overexpression of mouse Hsp70 can enhance the secretion of Hsp70 (Fig3b). Therefore we propose that ectopic overexpression of Hsp70 in cancer cells can enhance secretion of Hsp70 which in turn may have potential to stimulate anti-cancer immunity. Gene transfer of Hsp70 expression DNA vector into prostate tumor may be a new mechanism for tumor vaccine. To prove our theory, we performed the following experiments. After injection of transient transfected TRAMP C2 cells with mouse Hsp70 expression vector, we showed that tumor growth in mice was significantly reduced by Hsp70 expression in comparison with empty vector (Fig 4). We are also

making Hsp70 or empty vector stably transfected cells to determine whether higher levels of Hsp expression can improve antitumor activities. The goal of this study is to develop a gene transfer technology for immuno-neoadjuvant therapy to prostate cancer by injecting Hsp expression DNA vector into diseased prostate prior to surgical operation. We requested the consideration of and obtained the approval of this modification and continue this work in the no cost extension term (extends to 02/28/04). This work actually replaced the original specific aim 3. Futher we have finished this work which was published in British J. Cancer (please see it in Appendix).

a.



b.

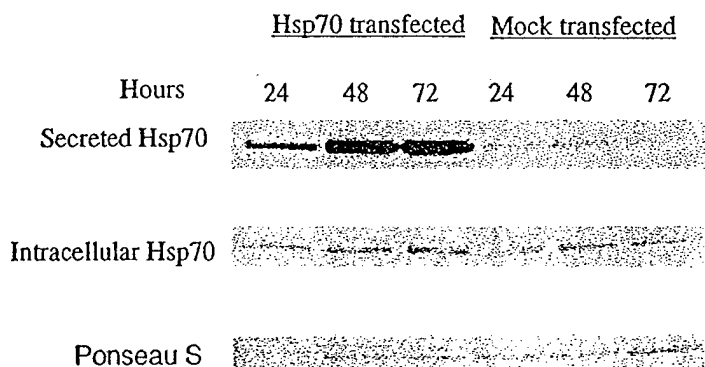


Figure 3. Detection of Hsp70 in secretion of prostate cancer cells by western analysis. (a) Concentrated spent media and cell extract from six human prostate cell lines were obtained after 48 hours of growth and used for western analysis of Hsp70. (b) Concentrated spent media and cell extract from mouse TRAMP C2 cells transfected with Hsp70 plasmid or mock transfection were used for Hsp70 analysis. Ponceau S protein staining was used for protein normalization.

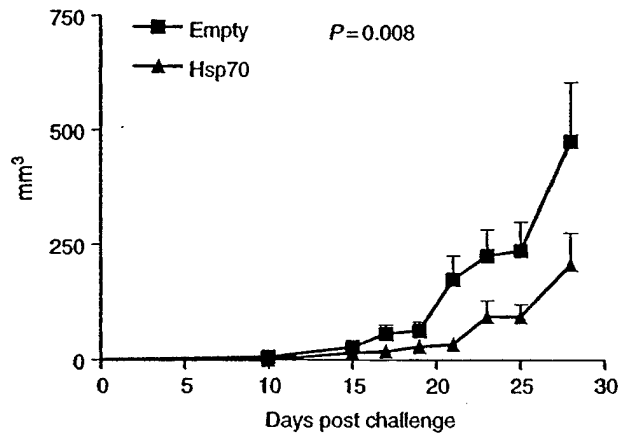


Figure 4 Effects of overexpression of Hsp70 on *in vivo* growth of TRAMP C2. C57 BL/6 male mice were injected s.c. three times with C2 cells transfected with Hsp70 vector or empty vector in ten days. Ten days after the last injection, mice were challenged with 3×10^6 C2 cells on the opposite flank. Tumor size was measured every other day. Tumor volumes were measured by the formula: Volume = Length x width x depth x (3.14/6). $P=0.008$

Key research accomplishments

1. Although we found that 15-d PGJ_2 is not effective in inhibiting growth of prostate tumor *in vivo*, we did show that Hsp70 induced by 15-d PGJ_2 *in vitro* has strong efficacy in producing anti-prostate tumor immunity and prevent tumor growth *in vivo*.

2. Our findings that endogenous Hsp70 can be secreted and ectopic overexpression of Hsp70 can enhance its secretion lead to the hypothesis that gene transfer of Hsp70 into cancerous prostate may stimulate the development of anti-tumor immunity. Our data seemed to support this notion as published in this year.

Reportable outcomes

We showed that 15-d PGJ_2 related lipid chemicals may have anti-prostate cancer activities *in vitro*. Chung BH, Mitchell SH, Zhang JS, Young CY. (2001) Effects of docosahexaenoic acid and eicosapentaenoic acid on androgen-mediated cell growth and gene expression in LNCaP prostate cancer cells. *Carcinogenesis* 22(8):1201-1206.

The above study led to a USA patent application

USA patent application pending No. 09/957,006. Methods and compositions for inhibiting the proliferation of prostate cancer cells. 2002.

We reported that Hsp70 isolated from prostate cancer cells treated with 15-d PGJ_2 can induce anti-cancer immunity and prevent prostate tumor growth *in vivo*.

Donkena K V, Grossmann, ME, Celis, E and. Young CYF. (2000) Tumor prevention and anti-tumor immunity with heat shock protein 70 induced by 15-deoxy- $\Delta^{12,14}$ prostaglandin J_2 in transgenic adenocarcinoma of the mouse prostate cells. *Cancer Research* 60: 4714-4718.

4. We reported that overexpression of Hsp70 can enhance its secretion into extracellular environment and potentially increased anti-tumor activity of the host.

Wang MH, Grossmann ME, Young CY. Forced expression of heat-shock protein 70 increases the secretion of Hsp70 and provides protection against tumour growth. Br J Cancer. 2004 Feb 23;90(4):926-31

Conclusions:

From our *in vivo* 15-d PGJ₂ study, we conclude that 15-d PGJ₂ was not effective for inhibiting tumor growth. However, we found that Hsp70 isolated from 15-d PGJ₂ treated prostate cancer cells can produce anti-prostate tumor immunity and effectively inhibit *in vivo* tumor growth. Additionally, we discover that gene transfer of Hsp70 into prostate cancer cells can produce secreted Hsp70 protein that may enhance anti-tumor activities *in vivo*.

Personnel

Charles YF Young, Ph.D.
K Vanaja Donkena, Ph. D.
Mike E. Grossmann Ph. D
Esteban Celis, M.D, Ph. D.
Ming H. Wang, M.D.

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Appendices

Three manuscripts

Forced expression of heat-shock protein 70 increases the secretion of Hsp70 and provides protection against tumour growth

M-H Wang¹, ME Grossmann¹ and CYF Young^{*,1}

¹Department of Urology and Biochemistry/Molecular Biology, Mayo Graduate School, Mayo Clinic/Foundation, Rochester, MN 55905, USA

Although heat-shock protein 70 (Hsp70) has been considered an intracellular protein, we report that Hsp70 is secreted under normal cell culture conditions by human prostate cell lines, LAPC-4, PC-3, CWR-22, RWPE-1 and -2, LNCaP, and TRAMP (transgenic adenocarcinoma mouse prostate)-C2. We found that the secretion can be enhanced by transfection with cDNA encoding for Hsp70. To verify that the Hsp70 detected in the supernatant was not secondary to cell leakage, C2 cells were cotransfected with cytoplasmic *Renilla luciferase* as a reporter. High levels of activities were noted in the cell extracts, while no enzyme activities were detected in the supernatants. To verify that forced oversecretion of Hsp70 could protect against tumour growth, mice were injected with C2 cells transfected with an Hsp70 DNA construct and challenged with live tumour cells. Mice injected with cells transfected with the Hsp70 DNA construct demonstrated a significantly decreased rate of tumour growth compared to those injected with empty vector. In addition, a difference in survival rate as defined by a surrogate end point was noted between the two groups. In a second experiment, we developed a cell line that stably overexpressed Hsp70. Mice injected with these cells also demonstrated a significant decrease in tumour growth and significantly increased survival.

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Keywords: heat-shock protein 70; secretion; prostate cancer; transgenic adenocarcinoma mouse prostate; gene therapy

Prostate cancer is the second leading cause of cancer death in men in the United States, exceeded only by lung cancer. The American Cancer Society estimates that 28 900 men in the United States will die of prostate cancer in the year 2003. Currently, for patients with early stage, organ confined disease, there are well-defined treatment options, including radical prostatectomy, radiation therapy, or watchful waiting. However, no definitive treatments are available for advanced or recurrent disease. It is known that tumour regression can be achieved with androgen blockade; however, disease usually recurs within 1–2 years, leading to significant morbidity and mortality (Mahler and Denis, 1992).

The idea of gene transfer to enable the use of host immune system against tumours has generated new treatment options for patients with prostate cancer. This therapy is based on the assumption that it is possible to break tolerance to tumour antigens by increased expression of immunomodulators and chemokines (Houghton, 1994). Specifically in prostate cancer studies, various gene transfer strategies using human or murine granulocyte/macrophage colony-stimulating factor (Sanda *et al*, 1994), interleukin-2 (Fearon *et al*, 1990), and interferon gamma (Vieweg *et al*, 1994) have been shown to elicit antitumour responses.

The antitumour property of heat-shock proteins (hsp's) was recognised in the 1980s when purified hsp's from tumour cells were shown to elicit immunity (Srivastava *et al*, 1998). Subsequent

studies have contributed to the understanding of the mechanism by which purified hsp's interact with the immune system. In their role as housekeeping proteins and chaperones, hsp's can bind to multiple intracellular peptides including tumour peptides (Gething and Sambrook, 1992; Parsell and Lindquist, 1993). These hsp-peptide complexes have the unique ability to promote crosspriming of cytotoxic T lymphocytes (CTLs), one of the most effective ways to stimulate antitumour immunity (Cavallo *et al*, 1993; Suto and Srivastava, 1995; Cayeux *et al*, 1997). Once released from tumour cells, these hsp complexes bind to CD91 receptors on host antigen-presenting cells (APCs) (Binder *et al*, 2000). The binding of hsp-peptide complex with CD91 leads to the internalisation of the complex and presentation of tumour peptides with MHC class I with the activation of CD8+ cells. Studies also suggest that a small proportion of the hsp-peptide complex is loaded onto MHC class II, leading to the stimulation of CD4+ cells (Matsutake and Srivastava, 1999). Other receptors, such as CD36 and CD40, were identified recently on APCs that can also interact with hsp's (Panjwani *et al*, 2000; Wang, 2001). These receptors, once activated, cause the secretion of nonspecific inflammatory cytokines such as tumour necrosis factors and interleukins (Ishii *et al*, 1999).

Recent studies indicated that hsp's interact with natural killer (NK) cells. Studies have demonstrated a correlation between tumour cell hsp's expression and increased NK cell-mediated cell lysis (Ponomarev *et al*, 2000). This observation is further supported by the identification of the extracellular C-terminal epitopes on Hsp70, 504–617, which are important for NK cells' killing activities (Botzler *et al*, 1998).

*Correspondence: Dr CYF Young; E-Mail: youngc@mayo.edu
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Previously in our laboratory, we found that purified Hsp70 from transgenic adenocarcinoma mouse prostate (TRAMP)-C2 cells (Foster *et al*, 1997) can induce an antitumour response (Vanaja *et al*, 2000). TRAMP-C2 is a murine prostate cancer cell line derived from TRAMP mice (transgenic adenocarcinoma of mouse prostate) that spontaneously develop prostate cancer (Greenberg *et al*, 1995). In the process of extending our previous studies on hsp's, we noted the presence of Hsp70 in the routine cell culture media of prostate cancer cells. This led us to investigate Hsp70 secretion and its significance in antitumour therapy. The advantage of using hsp's gene transfer is that it bypasses the need to purify large quantities of hsp's, in addition to allowing for systemic delivery.

MATERIALS AND METHODS

Cell lines

TRAMP C2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO, CA, USA) supplemented with 5% fetal calf serum (FCS), and 1% penicillin/streptomycin. Cells were maintained in T162 cm flasks at 37°C, 5% CO₂, and passaged weekly. Cells used for animal injections were collected by trypsinisation and washed with DMEM three times prior to injections. Specified number of viable cells (100 µl of DMEM per mouse) was determined by trypan blue exclusion, and used for injection.

LNCaP, PC-3, CWR-22, and LAPC-4 are human prostate adenocarcinoma cell lines. Each cell line was cultured in RPMI 1640 media (GIBCO, CA, USA) containing 5% FCS and 1% penicillin/streptomycin. Cells were maintained at 37°C, 5% CO₂. RWPE-1 and RWPE-2 cells derived from normal human prostate cells immortalised with human papilloma virus 18 were maintained in keratinocyte media (GIBCO, CA, USA), 37°C, and 5% CO₂ (Bello *et al*, 1997).

Hsp70-expressing cells

The full-length cDNA coding for inducible mouse Hsp70 was inserted into a mammalian expression vector pcDNA3.1 (+) (Invitrogen, CA, USA), and transiently transfected into TRAMP-C2 cells as per the protocol (Superfect, Qiagen, CA, USA). Empty vector was transfected as a control. Supernatants and cell extracts were collected at 24, 48, and 72 h. Whole-cell extracts were prepared as per Santa Cruz Biotechnology research applications. Spent media were spun down at 1000 r.p.m. for 5 min; supernatant was collected and concentrated with Vivaspin column concentrator, 10 000 MWCO (Vivascience, CA, USA). Protein levels were quantified with DC protein assay or Bradford (Bio-Rad, CA, USA). Success of the transfections was verified by Western analysis for Hsp70. To generate stable clones, cells were transfected with pcDNA3.1 + Hsp70 or empty vector as above and selected with gentamicin. Positive clones were selected and verified by Western analysis for Hsp70.

Secretion study

TRAMP-C2 cells were cotransfected with pcDNA3.1 + Hsp70, and *Renilla luciferase* vector (Promega, WI, USA). Supernatants and cell extracts were collected at 24, 48, and 72 h. Proteins were collected and analysed by Western analysis for Hsp70 as described below and luciferase activity was measured as per the manufacturer's instructions (Promega, WI, USA). Western blots of Hsp70 protein were quantified by densitometry and luciferase activity was measured by luminescence. All experiments were performed in triplicate.

LNCaP cells were cultured in RPMI 1640 media (GIBCO, CA, USA) containing 5% FCS, 1% penicillin/streptomycin, and 1 nm mibolerone, a synthetic androgen. Various concentrations of

brefeldin A (BFA, Sigma, MO, USA) dissolved in RPMI 1640 were added to each plate. At 16 h after the BFA treatment, both supernatants and cells were collected and prepared as above for Western analysis.

Western blot analysis

Whole-cell protein extracts and supernatants were prepared and quantified using DC assay or Bradford assay (BioRad, CA, USA). Equivalent protein samples were loaded into a precast 4–12% NuPage gel (SDS-PAGE), followed by electrophoresis and subsequent transfer onto a nitrocellulose membrane. Ponceau S staining was performed for total protein staining. The membrane was blocked overnight at 4°C with 5% milk in PBST (phosphate buffer solution with 1% Tween 20) and washed five times, 5 min each with PBST. This was followed by incubating the membrane at room temperature with either inducible Hsp70 primary antibody (StressGen, Canada) at 1:5000 dilution in PBST or prostate-specific antigen (PSA, Dako, CA, USA) at 1:2000 dilution. After an hour of incubation with the indicated antibodies, the membrane was washed as above, followed by a second anti-rabbit/mouse horseradish peroxidase antibody (1:100 000) incubation for an additional hour. Protein detection was performed with SuperDura chemiluminescence reagent as per the manufacturer's instructions (Pierce, IL, USA) and visualised with a digital camera. For quantitation, the various bands were analysed with AlphaEaseFC Software version 3.1 (Alpha Innotech Corporation, San Leandro, CA, USA).

Animal studies

All studies were approved by The Mayo Foundation Institutional Animal Care and Use Committee. Male C57BL/6 mice, 5–6 weeks of age were obtained from Jackson Laboratory and 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 a virus-free environment. Eight mice per group were used for each study. TRAMP-C2 cells were transfected with either pcDNA3.1 + Hsp70 or empty vector and collected 24 h post-transfection as described, and irradiated (10 000 rads) and injected subcutaneously. Three separate injections were performed 3 days apart. Each mouse received 1×10^6 cells per injection. At 10 days after the last injection, mice were challenged with 3×10^6 wild-type TRAMP-C2 cells on the opposite flank. In the second study, stably transfected TRAMP-C2 cells were collected, irradiated (10 000 rads), and injected into mice as above. At 10 days after the last injection, mice were challenged with 3×10^6 wild-type TRAMP-C2 cells on the opposite flank. Animals were examined and tumours were measured in three dimensions every other day using a caliper. Tumour volume was calculated, $V = (\text{length})(\text{width})(\text{depth})$. Animals were removed from the study when tumour diameter was greater than 1 cm.

Statistics

Data from the animal studies were analysed by log-rank test or Wilcoxon's signed-rank test as described in the figures. *P*-values <0.05 were considered to be statistically significant.

RESULTS

Forced overexpression of Hsp70 can increase Hsp70 secretion

During our studies on hsp's, we observed that Hsp70 is present in the routine culture media of TRAMP-C2 cells (data not shown). To test if the presence of Hsp70 in the spent media is a dynamic process and if overexpression can increase its secretion, we

transiently transfected TRAMP-C2 cells with a vector coding for murine Hsp70 (pcDNA3.1 + Hsp70). As shown in Figure 1, Hsp70 is detected in both the spent media and the cytosol of transfected and mock-transfected cells. However, increased levels of Hsp70 were noted only in the spent media of transfected cells, while intracellular Hsp70 remained relatively constant over time (Figure 1a and b). The relative amounts of Hsp70 in the supernatants and cell extracts were calculated, and greater percentages of Hsp70 were noted in the supernatants of the transfected samples (24, 48, and 72 h) when compared to the mock-transfected samples (Figure 1c).

Hsp70 in supernatant is not due to cell leakage

To eliminate the possibility of nonspecific cell leakage by physical damage, TRAMP-C2 cells from the above experiment were cotransfected with pcDNA3.1 + Hsp70 and a vector containing

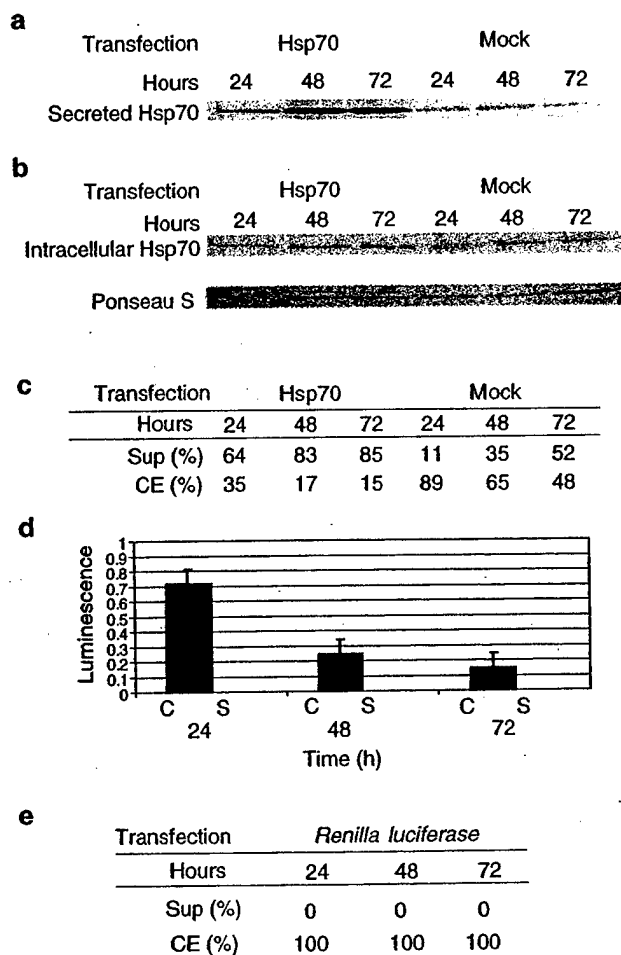


Figure 1 (A) Western analysis for Hsp70 in supernatants of pcDNA3.1 + Hsp70 and *Renilla luciferase* or mock-transfected TRAMP-C2 cells. Spent media were collected at 24, 48, and 72 h and concentrated as in Materials and methods. (B) Western analysis for Hsp70 in cell extracts of Hsp70, *Renilla luciferase*, and mock-transfected TRAMP-C2 cells. Ponceau S was used for normalisation. (C) Percent of Hsp70 in supernatants (Sup) and cell extracts (CE) of Hsp70 and mock-transfected TRAMP-C2 cells as determined by densitometry. (D) Comparison of luciferase activity in cell extracts (C) vs supernatants (S) in C2 cells transfected with pcDNA3.1 + Hsp70 and *Renilla luciferase* at various time points. Spent media and whole-cell protein extracts were prepared as above. (E) Percentage of renilla protein was determined by luminescence.

the cytoplasmic *Renilla luciferase* as a reporter. Luminescence was used to quantitate the relative amount of renilla protein in the supernatants and cell extracts. No luciferase enzyme activity was detected in the supernatants at any time points (Figure 1d). In addition, results were adjusted to account for concentrated supernatants and represented in percentages (Figure 1e).

Hsp70 secretion can be found in other human prostate cell lines

Our findings in TRAMP-C2 cells raised the question as to whether Hsp70 secretion is occurring in other prostate cell lines. We examined the spent media of various spontaneous prostate adenocarcinoma cell lines, including LAPC-4, PC-3, CWR-22, and LNCaP cells, and two additional transformed human prostate cell lines, RWPE-1 and RWPE-2. Note that RWPE-1 is not tumorigenic in athymic mice. RWPE-2, derived from RWPE-1, further transformed by Ki-Ras oncogene, is tumorigenic. Western analysis of these human prostate cell lines incubated under routine cell culture conditions was positive for Hsp70 in the supernatants and cell extracts, strongly suggesting that Hsp70 is secreted (Figure 2).

Hsp70 secretion is not blocked by a secretion inhibitor, BFA

To determine if the observed secreted Hsp70 was through the classical secretory pathway, BFA was used to study the secretion of Hsp70 and PSA in LNCaP cells. At 16h after treatment, supernatants and cell extracts were collected as above. Western analysis for Hsp70 was performed with PSA as a positive control. LNCaP cells provided a useful model because they are an androgen-responsive human prostate adenocarcinoma cell line that expresses androgen-inducible genes such as PSA (Murtha *et al*, 1993). Prostate-specific antigen is a classical secretory protein that has been well studied, and its secretion and production have been shown to be inhibited by BFA (Gau *et al*, 1997; Konno *et al*, 1998). As shown in Figure 3, Hsp70 was detected in both supernatants and cell extracts and is not decreased with the addition of BFA, while a decrease in PSA secretion was noted at a concentration of 0.5 $\mu\text{g ml}^{-1}$ BFA or higher. The presence of tubulin in the cell extracts but to only a small extent in the supernatants indicates that the Hsp70 detected in the supernatants is not due to cell death and lysis.

Forced overexpression of Hsp70 from TRAMP-C2 cells delays tumour growth and extends survival of C57BL/6 male mice

Transient overexpression experiment To test whether forced oversecretion of Hsp70 from prostate cancer cells can provide protection from tumour growth *in vivo*, TRAMP-C2 cells were transiently transfected with pcDNA3.1 + Hsp70 and injected subcutaneously into syngeneic C57BL/6 male mice.

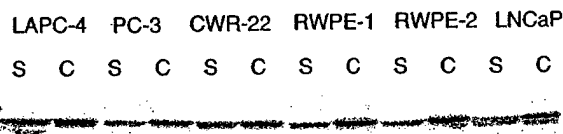


Figure 2 Western analysis for Hsp70 in supernatants (S) and cell extracts (C) of various prostate cell lines. Whole-cell protein extracts and spent media were collected at 48 h after being plated and subjected to Western analysis. Proteins were quantified with Bradford assay and equivalent amounts of proteins were loaded onto SDS-PAGE gel.

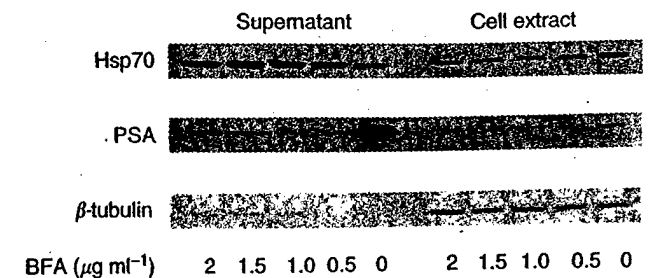


Figure 3 Western analysis for Hsp70, PSA and β -tubulin in supernatant and cell extract of LNCaP cells. Whole-cell protein extracts and spent media were prepared 16 h after treatment with various concentrations of BFA ($\mu\text{g ml}^{-1}$). Protein concentrations were quantified and equal amounts of proteins were loaded onto a single gel.

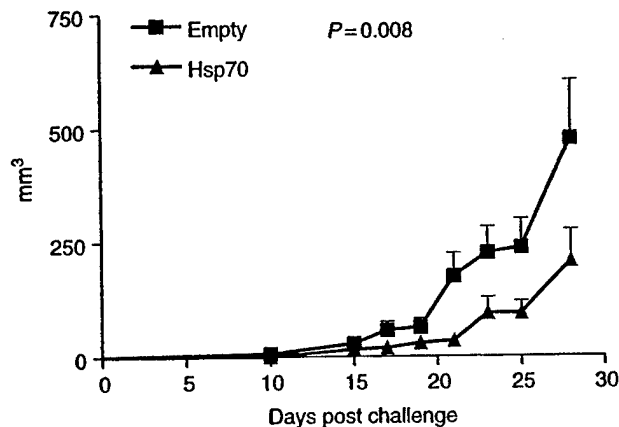


Figure 4 TRAMP-C2 tumour growth rate in C57BL/6 male mice after injecting with transiently transfected TRAMP-C2 cells with Hsp70 or empty vector. Tumours were measured every other day. *P*-values of <0.05 were considered statistically significant.

Cells transfected with empty vector were used as a control. At 10 days after last injection, mice were challenged with nontransfected TRAMP-C2 cells on the opposite flank. As shown in Figure 4, there is a delay in TRAMP-C2 cell growth in mice previously inoculated with Hsp70-expressing C2 cells. Statistical significance was observed at $P=0.008$ as analysed by Wilcoxon's signed-rank test between the groups (Figure 4). We also examined survival as defined by the time until the diameter of the tumour was greater than 1 cm. We found that there was a difference in the survival rates between the two groups that was not statistically significant (Figure 5, $P=0.11$). This experiment suggests a protective effect offered by inoculation with cells forced to overexpress Hsp70.

Stable overexpression experiment

Furthermore, stable TRAMP-C2 transfectants with pcDNA3.1 + Hsp70 or empty vector as a control were used to reproduce the above experiment. Western analysis of these clones verified an increase in intra- and extracellular Hsp70. Injections with stable clones and subsequent live, nontransfected TRAMP-C2 challenge were performed as above. As shown in Figure 6, there was a statistically significant delay in TRAMP-C2 tumour growth in mice previously injected with Hsp70-expressing stable clones ($P=0.001$). In addition, in this experiment there was a significant difference in survival between the two study groups (Figure 7, $P=0.02$).

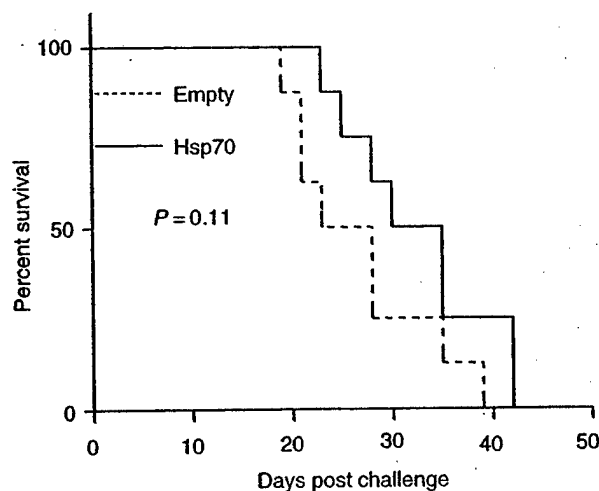


Figure 5 Percent survival of mice with transiently transfected Hsp70 TRAMP-C2 cells or empty vector.

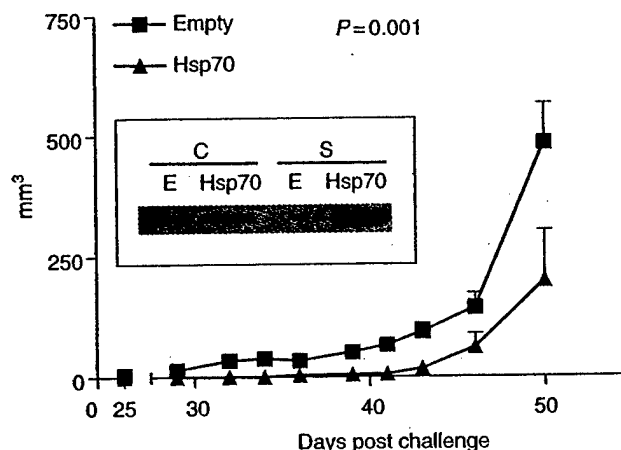


Figure 6 TRAMP-C2 tumour growth rate in C57BL/6 male mice after injecting with stably transfected Hsp70 TRAMP-C2 cells. Tumours were measured every other day. *P*-values of <0.05 were considered statistically significant. Inlet: Western analysis of Hsp70 in pcDNA3.1 + murine Hsp70 (Hsp70)- and pcDNA3.1 (E)-transfected stable clones. Cell extracts (C) and supernatants (S).

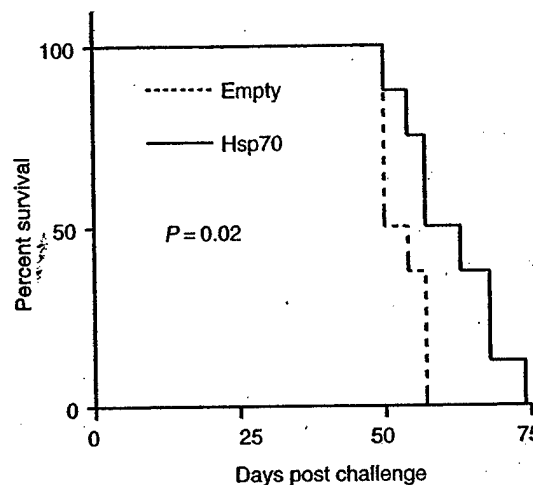


Figure 7 Percent survival of mice with stably transfected Hsp70 TRAMP-C2 cells or empty vector.

DISCUSSION

In the course of extending our previous studies on hsp's in prostate cancer, we found that Hsp70 is secreted into the spent media by some prostate cell lines under routine cell culture conditions, although a rigorous examination of all the cell lines remains to be completed. The significance of *in vivo* secretion of Hsp70 remains to be further elucidated. Previous studies have shown the release of hsp's into the cultured media by rat and chick embryo cells, squid glial cells, and yeast following heat shock (Hightower and Guidon, 1989; Russo *et al*, 1992; Guzhova *et al*, 2001). It is believed that these hsp's are important in cell proliferation during embryo morphogenesis, in addition to acting as protective factors for the surrounding cells in the presence of environmental stress (Hightower and Guidon, 1989; Russo *et al*, 1992; Guzhova *et al*, 2001). Our extensive literature search indicates that this is the first time that the Hsp70 secretion is documented in mouse and human prostate cancer cells. In addition, Hsp70 secretion can be increased with overexpression. This raised an interesting implication in that this oversecretion might have the potential to be utilised in generating antitumour immunity.

Hsp studies have attempted to elucidate the means by which intracellular hsp's can interact with extracellular immune cells. One of the possibilities is that hsp's are released during tumour cell necrosis, leading to the induction of immune response. Studies by Melcher *et al*, utilising the suicide gene transfer system, herpes simplex virus thymidine kinase/gancyclovir (HSVtk/GCV), noted different patterns of cell death in various tumour cells. Herpes simplex virus thymidine kinase/gancyclovir utilises the strategy in which a gene coding for a prodrug-converting enzyme is delivered into tumour cells, followed by the administration of the prodrug. Thus, the enzyme converts the prodrug into a toxic compound that kills the cells (Vile *et al*, 1997). In this particular study, cells that became necrotic with HSVtk/GCV treatments were found to express higher levels of hsp's mRNA when compared to cells that were apoptotic. Follow-up *in vivo* studies showed a decrease in tumorigenicity of hsp-transfected cells (Melcher *et al*, 1998). Studies subjecting tumour cells to rapid freeze-thaw cycles to mimic necrosis also noted an increase in hsp's in the cell lysates and supernatants, with corresponding decrease in tumorigenicity. Studies also supported cell surface expression as an avenue by which hsp's can interact with extracellular immune cells. Studies utilising membrane-bound hsp constructs noted an increase in immunogenicity of transfected cells (Wu *et al*, 1999; Chen *et al*, 2002). Recently, a study demonstrated a decrease in tumorigenicity of hsp110-overexpressing colon cancer cells (Wang *et al*, 2002). Further studies should clarify the role each of these mechanisms has on immunogenicity.

Although Hsp70 has been regarded as an intracellular protein, we found its presence in the extracellular media despite the addition of BFA, a reversible inhibitor that blocks protein translocation at the level of the endoplasmic reticulum-Golgi juncture and the trans-Golgi network (Schatz and Dobberstein, 1996; Cleves, 1997). Our findings indicate that these prostate cancer cells secrete Hsp70 via a mechanism other than the well-studied classic vesicular secretory pathway. This 'nonclassical' secretion of proteins that lack a typical N-terminal signal peptide has been observed in several other proteins such as fibroblast growth factors 1 and 2, interleukin-1, and viral proteins (herpes simplex tegument proteins) (Cleves, 1997). The fact that Hsp70 is released into the culture media by prostate cells without known stressors, coupled with its anticancer activity raised some interesting questions: first, whether hsp's are secreted *in vivo* by prostate cancer cells, second and more importantly, how does this phenomena fit into the evolution of host tolerance to cancer cells.

In order to test our hypothesis that Hsp70 oversecretion from prostate cancer cells can potentially be utilised as an anticancer agent, murine Hsp70 was overexpressed in TRAMP-C2 cells and tested *in vivo*. TRAMP-C2 cells, a transplantable murine epithelial prostate cancer cell line, provide a useful model for the study of prostate cancer therapies (Greenberg *et al*, 1995). Our study showed that there is a significant decrease in the tumorigenicity of TRAMP-C2 cells in mice injected with TRAMP-C2 cells over-secreting Hsp70, in addition to a significant difference in survival between mice injected with Hsp70 oversecreting cells and control.

We can speculate from our *in vitro* results and previous studies that an undefined level of Hsp70 extracellularly might be involved in cancer cell protection. This is in concordance with correlation studies that suggest hsp's as unfavourable prognostic factors for progression in some types of cancer (Nylandsted *et al*, 2000a,b; Lebret *et al*, 2003). Moreover, studies in our laboratory and others have shown that increased hsp's induces antitumour activities. A study by Podack and co-workers who constructed a gp96-Ig fusion protein noted an increase in tumour immunogenicity in cells transfected with this construct, suggesting that increased hsp secretion can act as a stimulatory signal for the breaking of host immune tolerance (Yamazaki *et al*, 1999). Additional studies of hsp secretion will likely offer insights and help us to answer fundamental immunologic questions with respect to the development of tolerance and immunity.

ACKNOWLEDGEMENTS

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Effects of docosahexaenoic acid and eicosapentaenoic acid on androgen-mediated cell growth and gene expression in LNCaP prostate cancer cells

Byung Ha Chung^{1,2,3}, Susan H. Mitchell¹, Jin-San Zhang and Charles Y.F. Young^{1,2,4}

¹Department of Urology and ²Department of Biochemistry and Molecular Biology, Mayo Foundation, Guggenheim Building 1742B, 200 First Street SW, Rochester, MN 55905, USA

³Present address: Department of Urology, Yonsei University College of Medicine, CPO Box 8044, Seoul, Korea

⁴To whom correspondence should be addressed

There is some epidemiological support for a protective influence of ω -3 fatty acids against prostate cancer. We wanted to explore whether ω -3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can affect androgen receptor function in prostate cancer cells. Our study showed that both DHA and EPA inhibit androgen-stimulated cell growth. Androgenic induction of prostate-specific antigen (PSA) protein was repressed by DHA and EPA in a dose-dependent manner. The mRNA levels of five androgen up-regulated genes, PSA, ornithine decarboxylase, NKX 3.1, immunophilin fkbp 51 and Drg-1, were decreased with DHA treatment in the presence of androgens. Transfection experiments using a DNA vector containing androgen-responsive elements demonstrated that both DHA and EPA could interfere with transactivation activities of the androgen receptor (AR). However, western blot analysis of AR protein showed that DHA and EPA treatments did not change AR expression levels. Interestingly, the proto-oncoprotein c-jun was increased by DHA treatment. A transient transfection found that forced expression of c-jun inhibited AR transactivation activity. Thus, this study found that the inhibitory effects of ω -3 polyunsaturated fatty acids on AR-mediated actions are due, at least in part, to an increase in c-jun protein.

Introduction

Prostate cancer is the most commonly diagnosed cancer in the USA after non-melanoma skin cancer and it is the second leading cause of cancer death in American men. Although prostate cancer is just as common in Japan as in the USA, death rates from prostate cancer are significantly lower in Japan (1). It is unlikely that these differences are all genetic, because Japanese men who migrate to the USA die of prostate cancer with increasing frequency as a function of the number of years they reside in the USA (2). It is possible a portion of this paradox could be explained by dietary factors.

Dietary intake of essential fatty acids, including both ω -3 and ω -6 fatty acids, is crucial for many important cellular processes, including cell proliferation and differentiation (3).

Abbreviations: AR, androgen receptor; ARE, androgen-responsive element; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; β -gal, β -galactosidase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Mib, mibolerone; ODC, ornithine decarboxylase; PSA, prostate-specific antigen.

Epidemiological studies demonstrated a correlation between high consumption of animal fat and death from prostate cancer (4). Controlled case studies added support to a positive association between dietary fat, particularly saturated animal fat, and prostate cancer (5). Note that animal fat contains high amounts of ω -6 fatty acids. A large prospective study of American men showed a positive association between α -linoleic acid (an ω -6 fatty acid) in the diet and prostate cancer (6). *In vitro* studies (7,8) showed that the growth of PC-3 human prostate cancer cells is stimulated in the presence of linoleic acid. Diets high in ω -6 polyunsaturated fatty acids can stimulate prostate cancer development (9,10). On the other hand, a nested case-control study on plasma lipid levels and the development of prostate cancer suggested that low plasma levels of α -linoleic acid might be associated with a reduced risk of prostate cancer (11). Wang *et al.* showed that lowering the proportion of fat in the diet decreased the growth rate of human prostate adenocarcinoma cells in nude mice (12).

Conversely, long-chain ω -3 polyunsaturated fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) can inhibit *in vitro* and *in vivo* cell growth of prostate cancer cells (13-16). There is epidemiological support for a protective influence of ω -3 fatty acid against prostate cancer (17-19). Japanese and Alaskan Eskimo men who eat large quantities of fish have a low risk for prostate cancer. Fish traditionally provide the major source of animal proteins and fat for these people. Fish oil is rich in ω -3 fatty acids. It has been suggested that external factors such as fish oil may have a role in repressing the development and growth of prostate cancer (19). Moreover, Mishina *et al.* reported that Japanese men that consumed low quantities of seafood were associated with increased prostate cancer risk (18). Therefore, long-chain ω -3 polyunsaturated essential fatty acids found in fish oil and other dietary factors may be beneficial for prostate cancer chemoprevention.

Androgens play an important role in proliferation, differentiation, maintenance and function of the prostate (20). Evidence shows that androgens are also involved in the development and progression of prostate cancer (21). The androgen receptor (AR) is a ligand-dependent transcription factor belonging to the nuclear steroid hormone receptor superfamily (22) and is the essential mediator for androgen action. In addition to its physiological functions, the AR plays a critical role in the development of prostate cancer. The LNCaP cell line is a well-established, androgen-responsive prostate cancer cell line obtained from a lymph node metastasis of a prostate cancer patient (23). LNCaP cells express the AR and a number of androgen-inducible genes, such as prostate-specific antigen (PSA) and hK2 (23,24). This study explored whether DHA and EPA can inhibit the function of the AR in LNCaP prostate cancer cells.

Materials and methods

Cell culture

Human prostate cancer cell line LNCaP (American Type Culture Collection) was grown in RPMI 1640 medium supplemented with 5% fetal bovine serum

(FBS) at 37°C and 5% CO₂ until reaching ~50–70% confluence. The medium was changed to serum-free RPMI 1640 to deplete undesired steroids for 24 h prior to experiments. Cells were then treated with RPMI 1640 containing 5% charcoal-stripped FBS and *cis*-4,7,10,13,16,19-docosahexaenoic acid (DHA) or *cis*-5,8,11,14,17-eicosapentaenoic acid (EPA) (Sigma, St Louis, MO) dissolved in ethanol at designated concentrations with or without mibolerone (Mib) (NEN, St Louis, MO), a non-metabolizable synthetic androgen, dissolved in ethanol. Equivalent amounts of solvent were added to control cells.

Cell proliferation and PSA quantification assays

LNCaP cells were seeded at 2×10^4 cells/well in 24-well plates. After 72 h they were treated with serum-free RPMI 1640 for 24 h and then incubated with varying amounts of DHA or EPA with or without 1 nM Mib in medium containing 5% charcoal-stripped serum. Six days after treatment 400 μ l of spent medium was collected for total PSA protein measurement by the Tandem-E PSA kit (Hybritech). An MTS assay was performed (Promega, Madison, WI) to measuring cell viability. Four wells per treatment were used for the above assays and they were repeated twice. PSA levels in spent medium were normalized to cell number.

Transient transfections

LNCaP or PC-3 cells were plated in 60 mm dishes. Cells were transiently transfected as previously described (25) with either three copies of the hK2 ARE in a pGL3 promoter plasmid, CMV β -gal, a human AR expression vector or c-jun expression vector as indicated. The AR and c-jun constructs are driven by the SV40 promoter (26). LNCaP cells were transfected with liposomes containing dimethyldioctadecyl ammonium bromide (Sigma) and L- α -lecithin (Sigma) (4:10 w/w) and PC-3 cells were transfected with lipofectamine (Gibco BRL, Grand Island, NY). After 24 h LNCaP cells were treated with 150 μ M DHA or EPA with or without 3.2 nM Mib. Whole cell extracts were prepared for luciferase assay according to the manufacturer's instructions (Promega). A CMV β -galactosidase (β -gal) expression vector and a parental vector (pGL3) were included as controls in the above transfections. β -Gal activity and total protein were assayed using the Bradford assay (Bio-Rad, Hercules, CA) for normalization purposes. Each transfection was done three times and standard deviations were calculated.

Northern blots

After steroid depletion as mentioned above, LNCaP cells were treated with varying amounts of DHA and 1 nM Mib as indicated and RNA was collected by the guanidine isothiocyanate method (27). A denaturing RNA gel was run and transferred to a nylon membrane (Bio-Rad) according to the GeneScreen protocol of New England Nuclear. Fifteen micrograms of total RNA were loaded in each lane. cDNAs for PSA, ornithine decarboxylase (ODC) (28), NKX 3.1 (29), fkbp 51, Drg-1 (30) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) were labeled with [³²P]dCTP by random priming and used as probes. Hybridization was performed according to Clontech protocols with ExpressHyb hybridization solution (Clontech, Palo Alto, CA). The films were autoradiographed at -70°C.

Western blot analysis

LNCaP cells were plated in 10 cm dishes at 9×10^5 cells/dish in RPMI 1640 (Mediatech, Herndon, VA) and 5% FBS (Biofluids, Rockville, MD). After steroid depletion as mentioned above the cells were treated with 1 nM Mib and varying concentrations of DHA and EPA. Cells were collected at designated times for whole cell protein preparation according to the Santa Cruz Biotechnology protocol. Protein levels were measured by a DC protein assay (Bio-Rad). Fifteen micrograms of protein were loaded into precast 4–20% NuPage gels (Novex, San Diego, CA), run with MOPS buffer and transferred according to the manufacturer's instructions to a nitrocellulose membrane (Bio-Rad). Ponceau S was added to the membranes and they were visualized with a digital camera. The membranes were blocked overnight at 37°C in TBST (20 mM Tris-HCl, pH 8.0, 137 mM NaCl and 0.1% Tween 20) and 5% dry milk. The membranes were washed three times for 10 min each with TBST. Primary antibody for the AR (Pharmingen, San Diego, CA) at a 1:1000 dilution or c-jun (Calbiochem, La Jolla, CA) at a 1:500 dilution were incubated at room temperature for 1 h. The membranes were washed three times for 10 min each with TBST. The appropriate horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ) at a 1:10 000 dilution was also incubated for 1 h at room temperature. The membranes were washed again and Renaissance chemiluminescence (NEN, Boston, MA) was used according to the manufacturer's instructions. β -Tubulin detected by specific β -tubulin antibody (1:1000) (Sigma) was also used as a control for protein loading and transfer efficiency.

Statistics

Results were analyzed by Student's *t*-test. $P < 0.05$ was accepted as the level of significance.

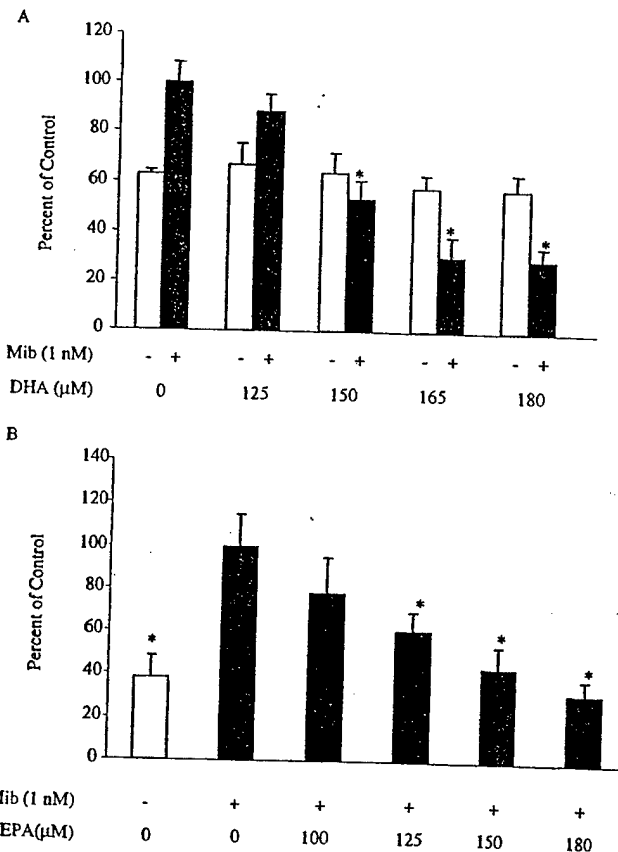


Fig. 1. Effects of DHA and EPA on androgen-stimulated growth responses in LNCaP cells. LNCaP cells were incubated with DHA (A) or EPA (B) as indicated for 6 days with or without Mib followed by a MTS assay performed in quadruplicate (bars indicate standard deviation). *, Significant inhibition compared with the no treatment control in (A) and with Mib treatment in (B).

Results

We examined the effects of DHA and EPA on androgen-stimulated growth in LNCaP cells. DHA-treated cells in the presence Mib showed a dose-dependent decrease in cell growth with a statistically significant P value of <0.05 . However, DHA-treated cells without androgen stimulation showed little response. This suggests that DHA inhibits the androgen-mediated growth response. Interestingly, androgens seemed to further potentiate the inhibitory effect of DHA when compared with that without androgens (Figure 1A; 31). EPA treatment showed a similar effect, decreasing androgen-stimulated cell growth to the same level as cells with no androgen treatment (Figure 1B).

To ascertain that DHA and EPA indeed affect androgen action in LNCaP cells we then examined the effect of these lipids on androgen-regulated expression of a well-known androgen-regulated gene, PSA. The normalized data in Figure 2A showed a dramatic decrease in androgen-stimulated PSA secretion by DHA. Figure 2B, for EPA treatment, demonstrates a similar pattern to Figure 2A, except that EPA treatment required higher concentrations for significant inhibition. Similar results were seen for another androgen-regulated protein, hK2 (data not shown).

Northern analysis was performed to see whether different androgen-regulated genes are affected by DHA treatment. The androgen-responsive genes NKX 3.1, ODC, the immunophilin fkbp 51, Drg-1 and PSA were all tested to ascertain the effects

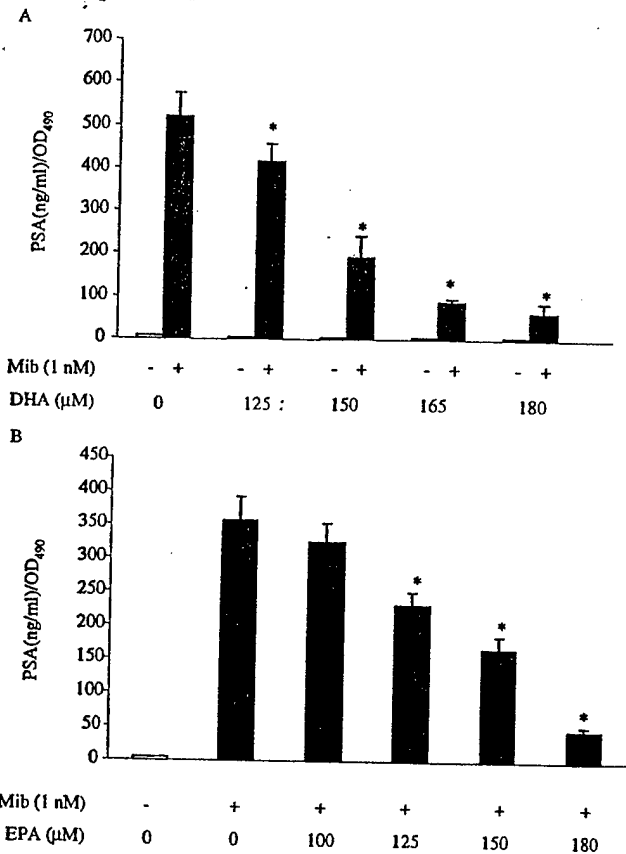


Fig. 2. Androgen-induced expression of PSA protein is affected by DHA and EPA in LNCaP Cells. Total PSA quantification was performed on the spent medium from cells treated with DHA (A) or EPA (B). These protein levels were normalized to the MTS measurements shown in Figure 1. *, Significant inhibition compared with the no treatment controls.

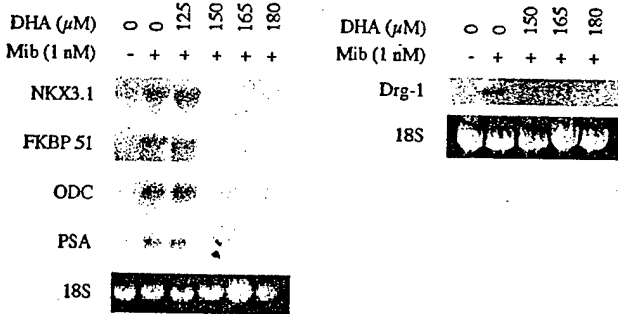


Fig. 3. Effects of DHA on androgen-induced expression of androgen-regulated genes. Northern blot analysis of PSA, ODC, NKX 3.1, fkbp 51, Drg-1 and GAPDH mRNAs in LNCaP cells treated with DHA and Mib for 4 h. 18S rRNA is shown as a loading control.

f the ω-3 fatty acids. Figure 3 shows that all mRNAs probed were up-regulated by androgens and that treatment with DHA at 150 μM or higher concentrations greatly inhibited the induced response.

Given that all the androgen-inducible genes tested were inhibited at the mRNA level, the next step was to determine if DHA and EPA had any effect on AR-mediated transactivation of androgen-regulated genes. Therefore, a construct containing three copies of an androgen-responsive element (ARE) in front of a luciferase reporter gene and an empty vector were transfected into LNCaP cells with or without Mib for 24 h to test whether DHA or EPA can directly affect AR-mediated

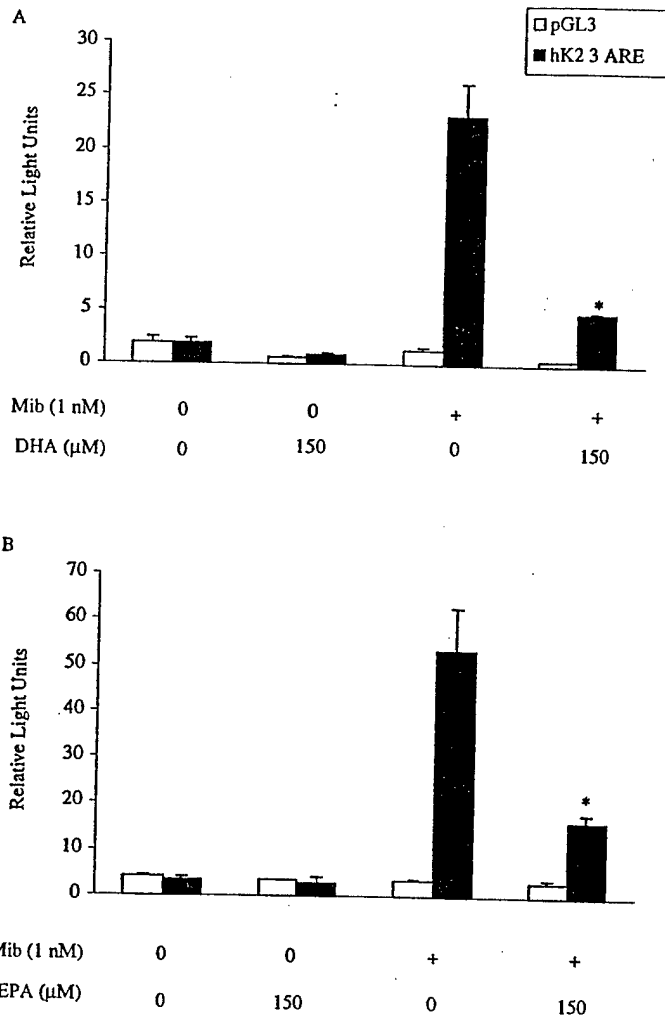


Fig. 4. Effects of DHA and EPA on AR-mediated transcription of a heterologous reporter gene. LNCaP cells were transiently transfected with PGL3 SV40 or pGL3 SV40-3 ARE. After 24 h cells were treated with DHA (A) or EPA (B) in the presence or absence of Mib. Cell extracts were prepared for luciferase and β-gal assays. Luciferase activities were normalized to β-gal activities and are presented as relative light units/mU β-gal. Transfection was repeated three times (bars indicate standard deviation). *, Significant inhibition compared with the no treatment controls.

transcriptional activity. Figure 4 shows the activity of cells treated with or without Mib. In Mib-treated cells the ARE gives a strong androgenic induction of luciferase activity. However, DHA and EPA treatments inhibited this androgenic response ($P < 0.05$). This assay strongly suggests that these lipids can affect transcriptional function of the AR.

Furthermore, western blot analysis was performed to determine whether the lipids have an effect on expression of AR protein. As shown in Figure 5, AR protein levels were increased by androgens, which could not be changed by DHA treatment for 24 or 36 h. Similarly, EPA did not alter AR expression.

In order to ascertain what is inhibiting AR function the levels of c-jun and c-fos were checked. Figure 6 shows that the level of c-jun increased with DHA treatment up to 3.5 times the control levels. The graph depicts the normalized data. This experiment was repeated twice and representative data are shown. However, the levels of c-fos were unaltered by DHA treatment (data not shown).

Previous studies by us and others showed that stimulated

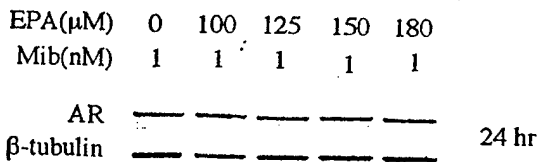
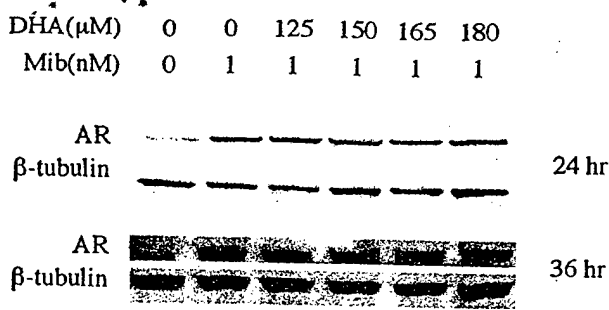


Fig. 5. Effects of DHA and EPA on expression of AR protein. A representative western blot analysis of the AR in LNCaP cells treated with varying amounts of DHA or EPA for the indicated times is shown. β-Tubulin was used as an internal control.

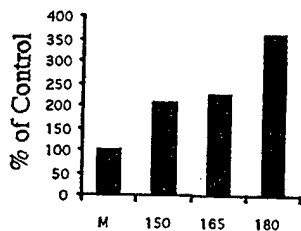
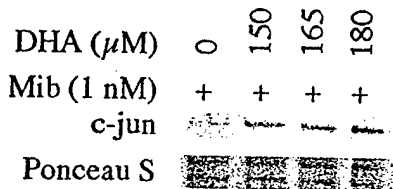


Fig. 6. Effect of DHA treatment on expression of c-jun protein. A representative western blot analysis showing c-jun protein levels in LNCaP cells treated with varying amounts of DHA and EPA for 24 h. Ponceau S staining is included as a loading and transfer control. The graph depicts protein levels normalized to Ponceau S staining as a percentage of the control.

overexpression of c-jun protein can inhibit function of the AR. Transient transfection was performed to determine the effects of c-jun expression on AR function. The hK2 3 ARE construct was transfected into PC-3 cells lacking the AR along with an AR expression vector and a c-jun expression vector (26,32-34). Figure 7 demonstrates that increasing amounts of c-jun cause decreases in androgen-induced activities of the ARE.

Discussion

Latent, non-infiltrating prostate cancer prevalence throughout the world varies little, however, mortality rates from prostate cancer differ greatly among industrialized countries (35). Asian men have a much lower mortality rate from prostate cancer than do American men. However, the rate increases when Asian men emigrate to the USA (36). The epidemiology suggests that dietary factors are the main cause of this increased risk of prostate cancer in the west (37). One aspect

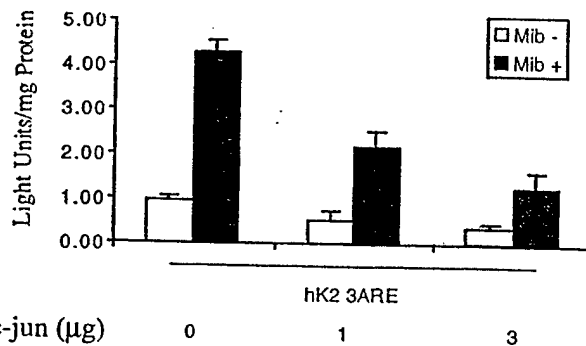


Fig. 7. Effects of c-jun on transcriptional activity of the AR. A transient transfection was performed using the hK2 3×ARE construct in PC-3 cells co-transfected with an AR and c-jun expression vector. The various concentrations of c-jun are indicated. The cells were treated with or without Mib for 24 h then cell extracts were prepared for luciferase assay and total protein was measured. The open bars represent no Mib treatment and the closed bars denote Mib treatment. The results are shown as light units/mg protein. The experiment was performed in triplicate (bars indicate standard deviation).

of the diet that differs greatly between Asia and the USA is the consumption of fish oil enriched in long-chain ω-3 fatty acids. Laboratory and epidemiological studies suggest that these ω-3 fatty acids may be useful for cancer prevention.

Previous studies have shown that ω-3 fatty acids inhibit prostate cancer cell growth *in vitro*, however, the mechanism of inhibition was not completely elucidated (7,8). Our data show for the first time that the ω-3 fatty acid DHA decreases androgen-stimulated LNCaP cell growth. Furthermore, androgenic induction of five androgen-regulated genes was significantly repressed by DHA at steady-state mRNA levels. Gene transfer experiments demonstrated that DHA repressed androgenic up-regulation of at least the PSA and hK2 genes at the transcriptional level. Similarly, EPA was able to reduce both the translational and transcriptional levels of these two androgen-regulated genes. The above results strongly suggest that EPA and especially DHA treatment inhibit androgen action, including the cell growth response.

One observation in our study requires further discussion. Northern blots showed that 125 μM DHA had no inhibitory effects on mRNA levels of the genes tested, including PSA, after 24 h treatment. One possibility as to why PSA and hK2 protein levels but not their mRNA levels were decreased by 125 μM DHA is that these cells were incubated for 6 days as opposed to 24 h. Therefore, it is possible that the extended treatment time is responsible for the concentration change. In addition, expression or secretion of both PSA and hK2 protein may potentially be more sensitive to DHA treatment than that of their mRNAs. Further study will be required to clarify the mechanism for the above concentration effect.

Our results certainly demonstrate that the ω-3 fatty acids exhibit repressive effects on androgenic induction of gene expression. Both PSA and ODC are well-known genes as direct targets of the AR (24,38). The lipids inhibit expression of the prostate-specific genes PSA and NKX 3.1 (29) and the ubiquitous genes ODC (28), fkbp 51 (39) and Drg-1 (30). Fkpb 51 has for the first time found to be up-regulated by androgens in this laboratory (submitted for publication). Together with the transient transfection study, the data suggest that the ω-3 fatty acids can impair the transactivation ability of the AR.

The inhibition of expression of ODC could explain, in part,

the decrease in cell growth. Polyamines are known to be involved in the processes of proliferation and differentiation of normal and neoplastic cells (38). ODC is the key enzyme in the biosynthetic pathway of polyamines (40). Forced over-expression of ODC can induce transformation of mammalian cells (41). It has been suggested that overexpression of ODC may be involved in the oncogenic process. Inhibitors of ODC have been used in cancer chemoprevention (42). Therefore, it is possible that repression of expression of ODC may be involved in the growth inhibitory effect of DHA in LNCaP cells.

The function of nuclear receptors like the AR can be affected by expression level (43), phosphorylation (44), dimerization (45), nuclear localization (46), ligand binding, interaction capability with various proteins such as heat shock proteins (e.g. hsp70 and hsp90), co-activators and other cross-talking factors (47,48). The ω -3 fatty acids do not seem to be able to reduce AR protein levels, although AR function is inhibited. Androgens can stabilize the AR and hence increase AR levels (49). Western blot analysis of the AR shows that the lipids do not interfere with the androgen-mediated stabilizing effect. In addition, we have examined the effects of the ω -3 fatty acids on the levels of hsp70 and hsp90 as well as the AR-specific co-activator ARA70 and did not find any inhibitory effects (data not shown).

Since AR protein levels were unaffected by DHA and EPA treatment and AR function was clearly affected, the levels of c-jun and c-fos were examined. The AP-1 transcription factors are composed of c-fos and/or c-jun nuclear proteins and can act as cross-talking factors for the AR. There is increased evidence that AR function could be affected by interaction with AP-1 proteins (26,32-34) in prostate cells. Previous studies have shown that c-jun alone could inhibit the formation of AR-ARE complexes (34). Our study found that c-jun expression is increased by DHA treatment and transcriptional activity of the AR is decreased with increasing c-jun expression. The gene transfer experiments performed in this study further confirm that inhibition of AR function is the result of increased levels of c-jun induced by DHA.

In this study we have shown a novel aspect of DHA and EPA in that they can attenuate AR-mediated action in androgen-responsive prostate cells. We show that inhibition of LNCaP cell growth is the result of a reduction in androgen action, possibly via impairment of AR function due to increased c-jun protein. Thus this study provides an additional mechanism for the anti-prostate cancer activities of the lipids. These lipids may have the potential to become chemopreventive and/or chemotherapeutic agents for prostate cancer.

Acknowledgements

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Tumor Prevention and Antitumor Immunity with Heat Shock Protein 70 Induced by 15-Deoxy- $\Delta^{12,14}$ -prostaglandin J₂ in Transgenic Adenocarcinoma of Mouse Prostate Cells¹

Donkena Krishna Vanaja, Michael E. Grossmann, Esteban Celis, and Charles Y. F. Young²

Departments of Urology [D. K. V., C. Y. F. Y.] and Immunology [M. E. G., E. C.], Mayo Clinic/Foundation, Rochester, Minnesota 55905

Abstract

The biological modifier Δ^{12} -prostaglandin J₂ 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 J₂ (15d-PGJ₂)-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 μ M 15d-PGJ₂ for 24 h caused significant induction of Hsp70 expression. The yield of Hsp70 purified from 15d-PGJ₂-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 anticancer 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 Hsps³ (6) and may represent a mechanism for inducing immunotherapy for prostate cancer.

The antiproliferative activity of 15d-PGJ₂ causes nonapoptotic cell death in prostate tumor cells (7). The growth-inhibitory effect of Δ^{12} -PGJ₂ 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 Zajdela 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 antiprostata tumor therapy or the role of Hsp70 in prostate cancer immunotherapy. Expression of Hsps after 15d-PGJ₂ 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-PGJ₂-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-PGJ₂ 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*

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²To whom requests for reprints should be addressed, at Departments of Urology and Biochemistry/Molecular Biology, Mayo Clinic/Foundation, Guggenheim 1742B, 200 First Street SW, Rochester, MN 55905. Phone: (507) 284-9247; Fax: (507) 284-2384; E-mail: youngc@mayo.edu.

³The abbreviations used are: Hsp, heat shock protein; 15d-PGJ₂, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J₂; PC, peptide complex; FPLC, fast protein liquid chromatography.

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 of light and dark cycle with food and water *ad libitum* in the virus-free mouse facility. The animals were allowed to acclimate 5 days prior to the experiment.

Tumor Cell Lines. TRAMP-C2 cell lines 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 cell line 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 was 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 flasks 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 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 $1 \times$ RBS once and lysed in RIPA buffer [$1 \times$ PBS, 1% NP40 (Amresco), 0.5% 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 for 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% SDS 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, and 0.1% Tween 20] at room temperature for 1 h. Blots were incubated with Hsp70 antibody (1:5000 dilution), inducible Hsp70 antibody (1000), constitutive Hsp70 antibody (1:1000), or β-tubulin antibody (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 anti-mouse 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 cm² culture flasks at 4×10^6 cells and were treated with 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 isotonic buffer (10 mM NaHCO₃, 0.5 mM pefabloc, pH 7.1), and the Hsp70 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 β-tubulin 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 cells. Hsps were injected s.c. under the skin 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 3×10^6 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-PGJ₂ (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. 1A 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-PGJ₂. Hsp70 in EL4 cells was also induced by 8.0 µM 15d-PGJ₂ 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 15-d 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

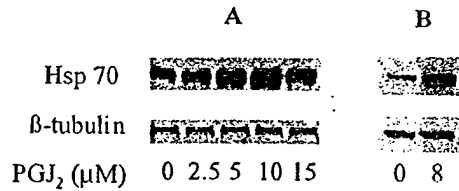
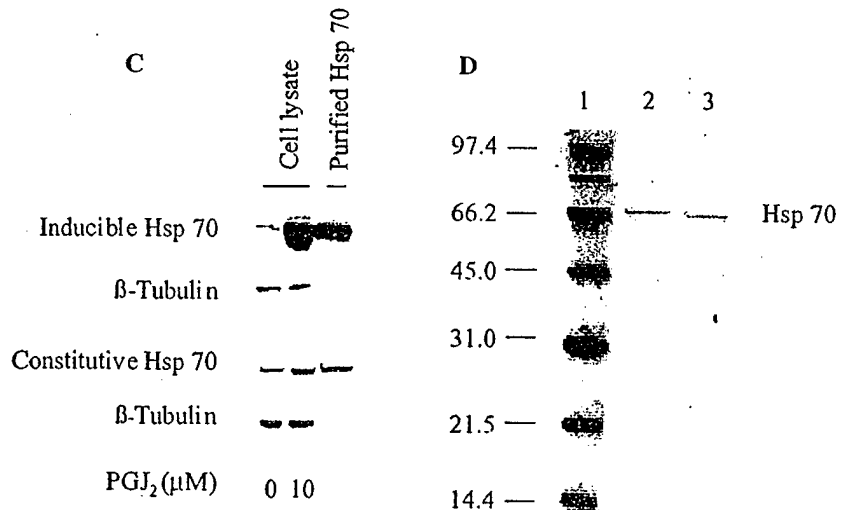


Fig. 1. A, 15d-PGJ₂-induced expression of Hsp70 in TRAMP-C2 cells. TRAMP-C2 cells were treated with various concentrations of 15d-PGJ₂ for 24 h and immunoblot analysis with Hsp70 monoclonal antibody recognizing both the constitutive and inducible forms. B, induction of Hsp70 expression in EL-4 cells 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.



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.

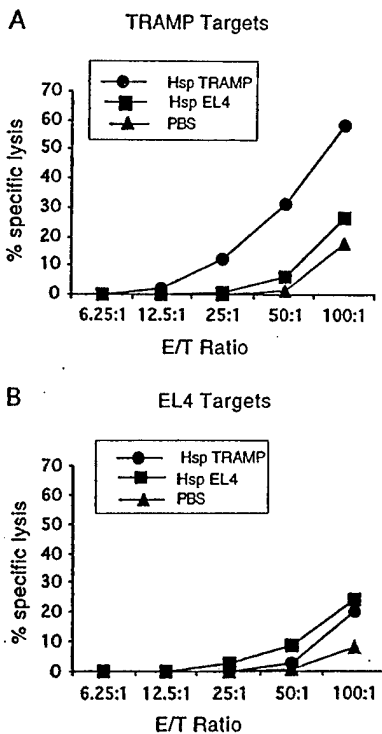


Fig. 2. Induction of CTL activity after immunization with TRAMP-C2 Hsps, EL4 Hsps, and PBS for control animals. Spleens from each group were collected 10 days after the final immunization. Pooled splenocytes from each group were restimulated *in vitro* with Hsp PCs isolated from TRAMP-C2 and EL4 cells and tested for cytolytic activity after 7 days of culture. Targets consisted of TRAMP-C2 (A) and EL4 (B) cells.

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) form. It was shown previously that Hsp PCs are important for generation of antitumor immunity. Vaccination with either Hsp70 alone or the peptides alone did not elicit tumor immunity in tumor rejection models (13, 27). The antigenic epitopes bound to Hsp70 may represent a broad range of unique, shared, and nonspecific normal cellular antigens.

It was demonstrated that Hsp preparations isolated from tumor cells could be used to immunize mice against the tumors from which the

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 by 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

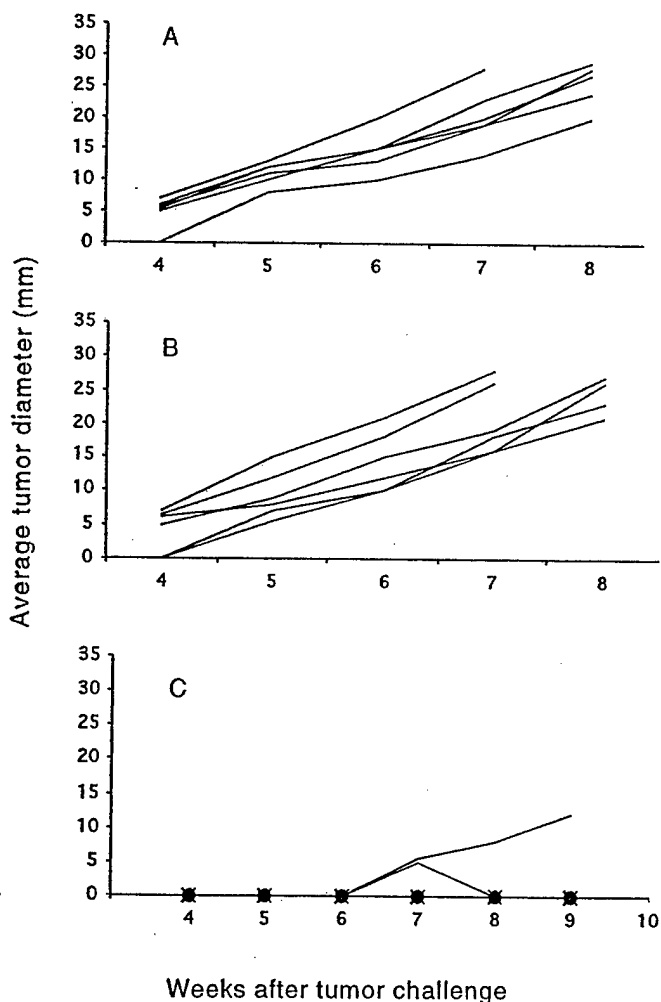


Fig. 3. Induction of antitumor protection by vaccination with Hsp70 PCs. Groups of six mice were injected with PBS for controls (A), Hsp PCs from EL4 cells (B), and Hsp PCs isolated from TRAMP-C2 cells (C). The animals were challenged 10 days later with s.c. injection of 3×10^6 TRAMP-C2 cells. Results depict tumor growth in individual animals over time.

Table 1 Immunization of Hsp70 PCs isolated from TRAMP-C2 and EL4 cells on the tumor incidence by challenge with TRAMP-C2 cells

The animals in the control group and EL4 Hsp group were sacrificed after 8 weeks. Tumor growth was monitored up to 9 weeks in animals that received TRAMP Hsp PCs.

	No. of weeks after challenge of 3×10^6 live TRAMP-C2 cells					
	4	5	6	7	8	9
Control (PBS)	4/6	6/6	6/6	6/6	6/6	NA ^a
Hsp70 PCs (EL 4)	5/6	6/6	6/6	6/6	6/6	NA
Hsp70 PCs (TRAMP-C2)	0/6	0/6	0/6	2/6	1/6	1/6

^aNA, not available.

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-PGJ₂-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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