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Radiation therapy induces circulating serum Hsp72 in patients with prostate cancer

Mark D. Hurwitz^a, Punit Kaur^b, Ganachari M. Nagaraja^b, Maria A. Bausero^{b,§}, Judith Manola^c, and Alexzander Asea^{b,*}

^a Department of Radiation Oncology, Dana-Farber/Brigham and Women's Cancer Center and Harvard Medical School, Boston, MA 02118

^b Department of Pathology, Scott & White Memorial Hospital and Clinic and The Texas A&M Health Science Center, College of Medicine, Temple, TX 76504

^c Department of Biostatistical Science, Dana-Farber/Brigham and Women's Cancer Center and Harvard Medical School, Boston, MA 02118

Abstract

Background and purpose—Hsp72 found in the extracellular milieu has been shown to play an important role in immune regulation. The impact of common cancer therapies on extracellular release of Hsp72 however, has been to date undefined.

Materials and methods—Serum from 13 patients undergoing radiation therapy (XRT) for prostate cancer with or without hormonal therapy (ADT) was measured for levels of circulating serum Hsp72 and pro-inflammatory cytokines (IL-6 and TNF- α) using the classical sandwich ELISA technique and the relative expression of CD8⁺ T lymphocytes and natural killer (NK) cells was measured using flow cytometry. Mouse orthotopic xenograft of human prostate cancer tumors (DU145 and PC3) were used to validate and further characterize the response noted in the clinical setting. The biological significance of tumor released Hsp72 was studied in human dendritic cells (DC) *in vitro*.

Results—Circulating serum Hsp72 levels increased an average of 3.5-fold (median per patient 4.8-fold) with XRT but not with ADT ($p = 0.0002$). Increases in IL-6 (3.3-fold), TNF- α (1.8-fold), CD8⁺ CTL (2.1-fold) and NK cells (3.2-fold) also occurred. Using PC3 and DU145 human prostate cancer xenograft models in mice, we confirmed that XRT induces Hsp72 release primarily from implanted tumors. *In vitro* studies using supernatant recovered from irradiated human prostate cancer cells point to exosomes containing Hsp72 as a possible stimulator of pro-inflammatory cytokine production and costimulatory molecules expression in human DC.

Conclusions—The current study confirms for the first time in an actual clinical setting elevation of circulating serum Hsp72 with XRT. The accompanying studies in mice and *in vitro* identify the released exosomes containing Hsp72 as playing a pivotal role in stimulating pro-inflammatory

*Corresponding author. Address: Division of Investigative Pathology, Scott & White Memorial Hospital and Clinic and the Texas A&M Health Science Center, College of Medicine, 1901 South First Street, Temple, Texas 76504 USA, aseaa@medicine.tamhsc.edu, aseaa@swmail.sw.org (A. Asea).

§Current address: Cell Biology Unit, Institut Pasteur de Montevideo, Calle Mataojo 2020 CP 11400 Montevideo, Uruguay

Disclosures

The authors have no conflict of interest.

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immune responses. These findings, if validated, may lead to new treatment paradigms for common human malignancies.

Keywords

androgen suppressive therapy; chaperokine; heat shock proteins; prostate cancer; radiation

Introduction

Heat shock proteins (HSP) have key roles in cellular stress response and immune modulation. Active areas of interest of study of HSP and malignancy include their role as prognostic factors, predictors of response to treatment, and their dual role in both tumor cell protection when expressed at high levels within tumors and conversely tumor cell destruction through antigen presentation and processing of tumor-derived HSP-peptide complexes.

A growing body of evidence supports the importance of HSP in human cancers in both the intracellular and extracellular environments. Intracellularly, HSP protect cells from proteotoxic stress by a variety of “holding and folding” pathways that prevent the formation of denatured proteins and the progression of lethal aggregation cascades by both necrotic and apoptotic pathways [1–6]. HSP also have a central role in modulation of the immune system, and Hsp72 can act as an immunological adjuvant [7–10]. Intracellular Hsp72 binds processed peptides derived from antigens and shuttles them to the cellular transporter associated with antigen processing (TAP). Hsp72 also appears to have additional effects on cytotoxic T-lymphocytes (CTL) that do not require the binding of tumor associated antigens to the HSP. Purified Hsp72 induces the activation of CD8⁺ CTL and the secretion of tumor necrosis factor- α (TNF- α) and IFN- γ in the absence of peptide loading [11]. The impact of HSP outside the cell has been further revealed in recent years. Hsp72, added exogenously to cells stimulates the production of pro-inflammatory cytokines TNF- α , interleukin-1 beta (IL-1 β) and IL-6 by antigen presenting cells (APC) [12–14]. Referred to as the chaperokine activity of Hsp72, Hsp72 appear to play a role in other aspects of non-specific immune responses: Hsp72, is found on the cell surface of some tumor cells and is a target of lymphoid activated killer (LAK) cells [15,16].

The majority of studies exploring the role of Hsp72 in cellular and immune regulation have been at the pre-clinical level. To date, studies of Hsp72 in human malignancies have largely focused on defining the expression of Hsp72 in extracted malignant tissue typically obtained at the time of diagnosis [17,18]. Relatively little is known about circulating serum levels of Hsp72 [19] and to date the impact of common cancer treatments on circulating Hsp72 has been undefined. As characterization of HSP profiles in clinically relevant settings may lead to development of specific new treatment strategies for cancer eradication, the present study was designed to assess the extracellular expression of Hsp72 and its potential effect on immune system response in patients undergoing treatment for prostate cancer. Subsequently, *in vivo* and *in vitro* studies were performed to further validate and characterize the clinical findings including the potential for tumor specific immune response and mechanisms for HSP release from intact irradiated tumors.

Materials and methods

Participants

Patients with clinically localized prostate cancer treated with external beam radiation therapy were eligible for this study. Institutional IRB approval was obtained prior to any patient enrollment. All patients were treated by a single radiation oncologist (MDH) and were enrolled in the study prior to the start of any treatment for prostate cancer. Samples suitable for analysis

of Hsp72, IL-6, TNF- α , CD8⁺ and NK cells prior to initiation of androgen suppression when administered, at the start of radiation therapy, and at the end of radiation therapy were obtained from 13 patients. Two additional patients from whom at least one serum sample was hemolyzed were excluded from analysis. Eight of the thirteen patients received neoadjuvant androgen suppressive therapy for 8 weeks prior to initiation of radiation. Blood (10 ml) was obtained per sample intravenously at the start and on the final day of radiation and for patients receiving 2 months of neoadjuvant AST prior to initiation of AST. Circulating serum levels of Hsp72, IL-6, TNF- α and plasma levels of CD8⁺ and NK cells were assessed at each of these time points in treatment.

Mice, tumor implantation and irradiation

Eight- to 10-week-old homozygous athymic male BALB/c *nude* mice were purchased from Taconic Farms (Germantown, NY) and housed in laminar flow isolation units in the Scott & White Clinic's vivarium under alternate dark and light cycles. The animals were housed 5 per cage in a pathogen free environment with air filter tops in filtered laminar flow hoods. Animals were maintained on food and water ad libitum. Animals were injected with 10⁶ tumors into the right hind leg. When tumors became palpable, tumor volume was measured every day. When tumors reach 100 mm³ mice were exposed to 0.5 Gy (sublethal) or 5 Gy (lethal) of γ -rays from a ⁶⁰Cobalt source at a rate of 2 Gy/minute. Blood was extracted at various times and immune parameters measured as described below. All animals were treated humanely and in accordance with the guidelines of the Committee on the Care and Use of Laboratory Animals of the Institute of Animal Resources, National Research Council and Scott & White Hospital and Clinic.

Cell lines, culture conditions and irradiation

PC-3, an androgen-negative, p53-negative prostate adenocarcinoma cell line was obtained from the American-Type Tissue Culture Collection (ATCC) and maintained at 37°C in Ham's F12 medium (F12K) supplemented with 2 mM L-glutamine, 1.5 g/L sodium bicarbonate and 10% fetal bovine serum. DU-145, a hormone insensitive prostatic carcinoma cell line obtained from the ATCC, was maintained at 37°C in minimum essential medium Eagle with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids and 1 mM sodium pyruvate, 10% FBS. All cells were incubated at 37°C with 5% CO₂ in air and passaged 2–3 times a week. For irradiation experiments, cells were plated in fresh medium and exposed to 0.5 Gy (sublethal) or 5 Gy (lethal) of γ -rays from a ⁶⁰Cobalt source at a rate of 2 Gy/minute. Cells were then incubated for an additional 96h at 37°C with 5% CO₂ in air.

Measurement of lactate dehydrogenase (LDH) release

LDH released into cell culture media by dead cells and total LDH contained in living cells was measured using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufactures instructions (Promega, Madison, WI) and as previously described [20].

Protein separation and Western blot analysis

Following various treatment protocols cells were washed once with complete medium, centrifuged and pellets lysed with 100 μ l of lysing buffer containing a cocktail of proteases inhibitors (antipain, bestain, chymostatin, E-64, pepstatin, phosphoramidon, pefabloc, EDTA, aprotinin; Complete Protease Inhibitor Cocktail Tablets®, Roche Diagnostics). Cells were then incubated for 30 min on ice and sonicated (Brandson 1510) for 15 min. The cells suspension was passed through a 26-gauge needle and protein quantification was performed using the Bradford method. Proteins were separated in a 10% SDS-PAGE by carefully placing 3 μ g of protein in each lane. Nitrocellulose membrane (GIBCO BRL) was used to transferring the

proteins and the membrane blocked with 5% skim milk (in TBS 1% pH 7.4 and 0.01% Tween 20) and incubated for 1 hour at room temperature with appropriate primary antibody; anti-Hsp72, and calnexin (StressGen Biotechnologies, BC, Canada), or anti-tubulin (Oncogene, San Diego, CA). Blots were incubated 50 min at room temperature with 0.5 µg of appropriate species matched anti-peroxidase and the reaction was detected using the Luminol reagent for chemiluminescence (Santa Cruz Biotechnology). The intensity of the bands were analyzed by densitometry with a video densitometer (ChemilmagerTM 5500; Alpha Innotech, San Leandro, CA) using the AAB software (American Applied Biology).

Hsp72 depletion assay

To determine the relative contribution of Hsp72, it was depleted from recovered supernatant by an affinity column chromatography as previously described [21]. Briefly, since Hsp72 binds to ATP-agarose, recovered supernatant was mixed with a buffer containing 0.5 M KC1 plus 10 mM EDTA and slowly passed over a 25-ml column of ATP-agarose. The column flow-through (50 ml) was attached to an Amicon filter and dialyzed overnight against two changes of Hepes buffer plus 100 mM KC1 and 5 mM dithiothreitol, and was then concentrated by Centricon C-10 ultrafiltration to one-half the original volume (0.5 ml). The Hsp72 retained by the ATP-agarose column was eluted with wash buffer containing 5 mM ATP and then concentrated and treated as above, and used as a positive control.

Enzyme-linked immunosorbent assay (ELISA)

After various treatment protocols cell culture medium was centrifuged to discard floating cells and cellular debris and the total protein content was determined by Bradford analysis using bovine serum albumin as a standard. The supernatant was aliquoted and treated with or without 1% Triton X-100 or 1% Lubrol WX or 0.5% Brij 98 for 10 min at 4°C with gentle rocking and Hsp72 content measured by standard sandwich ELISA as previously described with minor modifications [22]. The interassay variability of the Hsp72 immunoassays was <10%.

Sucrose density gradient centrifugation and acetylcholinesterase (AChE) activity

Exosomes were carefully layered onto a sucrose density gradient (ranging from 1.08–1.24 g sucrose/ml, and ultracentrifuged at 150,000g for 12 h at 4°C as previously described [23]. Gradient fractions were collected and analyzed by for the presence of Hsp72 by Western blot analysis. Acetylcholinesterase (AChE) activity of various fractions was measured using the protocol previously described [24]. Briefly, fractions (50 µl) were suspended in 1.25 mM AChE (supplemented with 0.1 mM 5,5-dithio-bis (2-nitrobenzoic acid)), and changes in absorption were monitored at 412 nm over a 10-minute period at 37°C.

Statistical analysis

Data from the clinical study was analyzed using a two-tailed Student's *t*-test after applying ANOVA to the data to assess for significant changes in Hsp72 and the correlative pro-inflammatory cytokines and components of the cellular immune response. A *p* value of < 0.05 was considered significant. Data obtained from western blot were analyzed according to the relationship between the signal intensity and the area and presented as optical density (OD). Data from flow cytometry is shown as histograms and fluorescent arbitrary units and the mean fluorescent intensity was obtained to compare between groups. Data are shown as percentages, mean and standard deviation (SD) comparison was done using a Student's *t*-test and *p* values <0.05 were considered statistically significant.

Results

Radiotherapy of human prostate cancer patients results in the release of bioactive mediators and an increase in the relative number of CD8⁺ T cells and NK cells

All patients enrolled had clinically localized prostate cancer (clinical T1c–T3b, N0, M0) treated with 8 weeks of 3D conformal radiation therapy. Patients with low-risk disease defined as clinical T1–T2a, Gleason ≤ 6 , and PSA < 10 ng/ml were treated with radiation alone while patients with adverse risk factors including bulky palpable disease, Gleason ≥ 7 , or PSA > 10 ng/ml typically received 8 weeks of neoadjuvant androgen suppressive therapy (AST) prior to initiation of radiation therapy (XRT) with AST continued through radiation therapy.

Serum Hsp72 taken at various time points after post-XRT was shown to be significantly elevated (Fig. 1A). Hsp72 levels significantly increased an average of 3.5x from 9.8 to 34.0pg/ml over 8 weeks of XRT ($p = 0.0002$) (Fig. 1A). Interestingly, no significant increase in serum Hsp72 levels was observed after 2 months of neoadjuvant AST. The average levels at baseline and 2 months into AST (day 1 of XRT) were 13.7 and 10.2pg/ml respectively ($p=0.95$).

Given the well established role that Hsp72 plays in modulation of the cellular immune response it was also hypothesized that an increase in serum Hsp72 would be associated with a concomitant increase in components of the cellular immune response. Important markers of enhanced immune function including effector cells like CD8⁺ cytotoxic T lymphocytes (Fig. 1B) and CD56⁺ NK cells (Fig. 1C) were upregulated post-XRT. There was a significant increase in CD8⁺ cell from 4.1 to 8.5% ($p = 0.005$). NK cell, known to play an important role in host defense against tumors was demonstrated to be significantly increased from 2.3 to 7.4% ($p = 0.001$). Similar to serum Hsp72, no change was noted for either CD8⁺ or NK cells over the course of neoadjuvant AST. Serum concentrations of pro-inflammatory cytokines interleukin-6 (IL-6) (Fig. 1D) and tumor necrosis factor-alpha (TNF- α) (Fig. 1E) were also increased in response to XRT but not to androgen suppression. An increase in serum levels of pro-inflammatory cytokines, expected with increased Hsp72 levels was observed over the course of XRT (Fig. 1D, E). Mean IL-6 levels increased from 9.8 to 32.3pg/ml ($p = 0.01$) and mean TNF- α levels increased from 10.0 to 17.9pg/ml ($p = 0.01$) from the start to the completion of 8 weeks of XRT. Consistent with Hsp72, no change was noted for either of these cytokines with 8 weeks of AST.

While an increase in Hsp72 over the course of radiation was noted for all patients, a wide range of baseline levels of Hsp72 was observed (0.3 – 56.3pg/ml). Given the variation in baseline levels of Hsp72 and the potential predictive/prognostic implications in such baseline heterogeneity, the degree of change in HSP and markers of immune response were assessed for patients with baseline Hsp72 < 10 pg/ml (6 pts) vs. > 10 pg/ml (7 pts). The absolute changes in Hsp72 and immune markers were relatively consistent regardless of baseline levels. Absolute and relative changes in Hsp72 for the low versus high groups were 26.7pg/ml/10.7x and 18.0pg/ml/2.3x. A similar pattern was found for markers of immune response implying a potentially greater impact for patients with low baseline Hsp72 levels.

Mouse orthotopic xenograft of human prostate tumors validates the clinical findings

To determine the mechanism by which radiotherapy mediates Hsp72 release and upregulates immune markers, we used a human xenograft model in mice. Two human prostate cancer cell lines the PC-3 (an androgen-negative) and DU-145 (a hormone insensitive) prostatic carcinoma cell lines were implanted into nude male mice. When tumors grew to a volume of ~ 100 mm³ animals were irradiated with either 0.5 or 5.0 Gy and serum Hsp72 concentrations measured. The concentration of serum Hsp72 in mice that were exposed to 5.0 Gy was significantly higher than mice that were exposed 0.5 Gy. The levels of serum Hsp72 were significantly higher in

both human prostate cancer xenografts 24 h post irradiation ($p < 0.05$) and levels decreased to baseline levels by 96 h post exposure (Fig. 2; top left and middle panels). To determine the source of Hsp72, non-tumor bearing mice were exposed to 0.5 and 5.0 Gy of irradiation. A similar significant increase in Hsp72 was observed ($p < 0.05$), albeit at a significantly lower concentration than in 4T1 tumor bearing animals (Fig. 2; top right panel). Interestingly, there was a slight but significant increase in serum Hsp72 concentrations even in mice not exposed to irradiation but simply placed in the same irradiation holding chamber as those exposed to 0.5 and 5.0 Gy irradiation (Fig. 2; top panels). To determine the cause of the increase serum Hsp72, we extracted serum from tumor bearing animals not placed into the irradiation holding chamber. This resulted in no significant increase in serum Hsp72 above baseline levels (data not shown). These results suggest that the slight increase in serum Hsp72 was due to psychological stress induced by confinement and/or being placed in the irradiation chamber.

To negate the possibility that the increased serum Hsp72 was released from other organ than the human xenograft, we utilized the fact that murine cells do not express Hsp27. We demonstrated that exposure of male BALB/c *nude* mice bearing the PC-3 human xenograft (Fig. 2; bottom left panel) and DU145 human xenograft (Fig. 2; bottom middle panel) to gamma irradiation induced the expression of the small heat shock protein, Hsp27, into the systemic circulation, but not the expression of serum Hsp27 in 4T1-tumor bearing mice (Fig. 2; bottom right panel). The 4T1 cells are a highly metastatic breast cancer cell line syngeneic in BALB/c mice and express Hsp25 but not Hsp27 [25,26].

Characterization of radiotherapy-induced Hsp72 reveals the presence of exosomes

The apoptogenic and pro-inflammatory cytokine TNF- α was measured to determine whether gamma irradiation upregulates pro-inflammatory cytokine release in a similar fashion as in prostate cancer patients (Fig. 1D, E). Exposure of mice to the PC-3 human xenograft induced the appearance of TNF- α (Fig. 3A; left panel). There appeared to be a delayed kinetics for serum TNF- α expression which peaked at 48 h post exposure to 0.5 and 5.0 Gy irradiation (Fig. 3A; left panel). Interestingly, in PC-3 human xenograft-bearing animals “mock” irradiated (0 Gy) there was an increase (albeit insignificant) in TNF- α (Fig. 3A; left panel). However, exposure of non-tumor bearing mice to gamma irradiation did not result in significant expression in serum TNF- α levels (Fig. 3B; right panel).

To determine the mechanism by which irradiation induces Hsp72 release, prostate cancer cell line PC-3 and DU-145 were exposed to 0.5 or 5.0 Gy irradiation *in vitro*. Exposure to both 0.5 and 5.0 Gy resulted in a significant increase in total Hsp72 expression in prostate cancer cells as judged by Western blot analysis (Fig. 3B). To determine if Hsp72 and Hsp27 release from tumors was solely due to necrotic cell death, prostate cancer cells were exposed to gamma irradiation and cell death measure using the classical lactate dehydrogenase (LDH) release assay. Recently, we and others have demonstrated that Hsp72 is released to the extracellular milieu within immunologically potent exosomes. To confirm that the released Hsp72 was indeed released within exosomes, exosomes from PC-3 and DU-145 cells were collected and probed. We demonstrate that tumor-derived exosomes express Hsp72, Hsc73, tubulin (a cytoplasmic protein), but not calnexin (an endoplasmic reticulum protein) or grp94 (Fig. 3C).

In a separate experiment, cells exposed to 5.0 Gy but not 0.5 Gy irradiation resulted in significant cell death ($p < 0.05$) starting at ~24 h post exposure in both PC-3 (Fig. 4A; left panel) and DU-145 (Fig. 4A; right panel) cells, as judged by an increase in LDH. These results correlate well with Hsp72 release data from PC-3 and DU145 cells; exposure of both prostate cancer cells to both 0.5 and 5.0 Gy irradiation resulted in a significant increase in Hsp72 release, as early as 24 h post exposure and remaining constant for up to 96 h post exposure (Fig. 4B; left and right panels).

Discussion

The current study provides the first insights into the impact of common clinical cancer therapies on circulating levels of Hsp72. While baseline serum levels of Hsp72 in cancer patients are of interest in both defining prognostic significance and identifying a potential target for new therapeutic strategies, understanding how common cancer treatments such as radiation impact on circulating levels provides potentially important information to clinicians.

The results of the present study build upon limited current knowledge of Hsp72 levels in prostate cancer patients prior to initiation of treatment. Cornford et al. assessed the expression of intracellular HSP in tissue obtained from patients with early prostate cancer either at the time of prostatectomy or as an incidental finding at the time of transurethral resection of the prostate (TURP) as well as from patients with advanced disease obtained at the time of TURP [18]. These samples were compared with those of control patients with tissue obtained at the time of cystectomy for bladder cancer not involving the prostate or TURP for benign prostatic hypertrophy. Immunohistochemical analysis of Hsp72 expression revealed similar expression of Hsp72 in early prostate cancers compared with non-neoplastic controls but diminished expression was noted in morphologically advanced cancers. No association was noted however with intensity of staining and Gleason score. Abe et al. examined the potential role of plasma Hsp72 as a biomarker for prostate cancer. Plasma Hsp72 levels were measured in 125 patients with localized/untreated or hormone refractory prostate cancer and compared with levels for 45 healthy male donor controls of similar age. Plasma Hsp72 levels in patients with localized untreated disease were significantly higher than those in the control group. While a primary cutoff point for plasma Hsp72 was defined that significantly distinguished the localized untreated patients from the control group, plasma Hsp72 was not more effective than PSA as a predictor for diagnosis or stratification of patients into established risk groups. The impact of treatment on plasma Hsp72 levels was not assessed [19].

The current study was therefore designed not only to define Hsp72 levels in prostate cancer patients at baseline but also to assess the impact of common treatments for prostate cancer on serum Hsp72 levels and to characterize its mechanism of release and biological significance using Mouse orthotopic xenograft of human prostate cancer tumors *in vivo* and *in vitro*. While no increase was noted with AST, a significant increase in circulating serum Hsp72 was noted in response to XRT (Fig. 1A). In addition, we demonstrated that following XRT there is a significant increase in cells phenotypically characterized at CD8⁺ cytotoxic T lymphocytes and CD56⁺ natural killer (NK) cells and a concomitant increase in the pro-inflammatory cytokines IL-6 and TNF- α (Fig. 1B–E). We hypothesized that XRT stimulates the passive and activate release of Hsp72 from tumors. Passive release is achieved by the direct XRT-induced necrosis of tumors. This liberates heat shock protein peptide complexes (HSP-PC) which bind to and stimulate antigen presenting cells (APC) to produce pro-inflammatory cytokines, chemokines and reactive oxygen species, increase the expression of costimulatory molecules and augments the maturation of dendritic cells, a process known as the chaperokine activity of Hsp72 found in the extracellular milieu [27–29]. Recently, it was demonstrated that Hsp72 can also be induced by an active process [21,30]. We propose that in addition to necrotic cell death, as a source for released Hsp72, XRT induces the active release of Hsp72.

To validate our human clinical data, experiments were performed using orthotopic xenograft of human prostate cancer tumors to examine how irradiation induces Hsp72 release from tumors. Consistent with our findings in prostate cancer patients, radiation exposure resulted in a significant increase in serum Hsp72 concentrations in both human xenografts and syngeneic tumor bearing mice (Fig. 2; top panels). The dose dependent nature of Hsp72 levels in response to radiation support our hypothesis that radiation directly results in Hsp72 release into the serum. Serum Hsp72 rose to a maximum level by 24 hours post exposure to radiation and

returned to base-line values by 96 hours in both human xenografts and syngeneic tumor bearing mice (Fig. 2; top panels). The finding of higher levels of Hsp72 release in human xenografts (Fig. 2; top left and middle panels) versus syngeneic tumor bearing mice (Fig. 2; top right panel) suggests that at least part of the increase in circulating Hsp72 is tumor specific, although further work is required to verify this important assertion.

The notion that this response is at least in part tumor specific was therefore further assessed by measuring serum Hsp27 levels (Fig. 2; bottom panels). Until recently, it was not possible to differentiate Hsp72 released from mouse or human tumors implanted into mice. To overcome this problem, we measured serum Hsp27 levels in the implanted tumors. Since Hsp27 is exclusively expressed in human and not mouse tissues, Hsp27 released into the blood can be correctly assumed to have originated only from the human xenograft. We demonstrated that Hsp27 was released into circulation in response to irradiation in a similar fashion as Hsp72, albeit to a lesser levels in DU-145 human xenograft (Fig. 2; bottom middle panel) as compared to PC-3 human xenograft (Fig. 2; bottom left panel). This point was further reinforced in the next experiment in which no significant serum Hsp27 was measured in 4T1 bearing mice exposed to radiation (Fig. 2; bottom right panel). This is expected since 4T1 cells are a breast adenocarcinoma cell line syngeneic in BALB/c mice and therefore it is expected that it will release Hsp25, not Hsp27. Interestingly, the kinetics of Hsp27 release induced by gamma irradiation peaked at 48 hours post exposure, whereas Hsp72 peaked at 24 hours in both PC-3 and DU-145 prostate cancer cells. The significance of this is currently unknown. However, we assume this is indicative of a difference in the mechanism by which Hsp27 is transported within the cell and subsequently released into the extracellular milieu, as compared with Hsp72. Experiments are currently underway in our laboratory to conclusively answer this question (Kaur et al, *in preparation*).

An interesting observation was the relatively small but significant increase in serum Hsp27 and Hsp72 levels in mice “exposed” to sham XRT, in which mice were placed in the irradiation chamber, but the irradiator not turned on (Fig. 2; left and middle panels). The reason for this is not known for certain, however, we speculate that the increase in serum Hsp72 is triggered by fear/psychological stress in a similar fashion as that observed during predatory stress [31], since there was no such increase in serum Hsp72 when mice were not placed in the irradiation chamber (data not shown).

Although the increase in Hsp72 concentrations in the blood following exposure to a variety of stressful stimuli has been clearly demonstrated, the physiological function of circulating serum Hsp72 remains a “black hole.” What is widely agreed upon is that Hsp72 can facilitate immunologic responses. The signals that mediate the *in vivo* elevation of circulating serum Hsp72 after stressor exposure are incompletely understood. Although our studies clearly demonstrate that released tumor-derived Hsp72 contributes to the stimulation of human DC (Table 1), the exact mechanism by which this occurs *in vivo* remains to be elucidated.

In the current study, we demonstrate that XRT induces the release of Hsp72 from tumors and that this increase is associated with increases in cells phenotypically characterized as CD8⁺ CTL and NK cells with a concomitant increase in pro-inflammatory cytokines including IL-6 and TNF- α . However, further investigation is required to determine if the excess serum Hsp72 levels seen in patients and tumor bearing animals as a result of radiation is associated with a tumor specific immune response. Our results demonstrating that gamma radiation induces the release of Hsp72 within highly immunologically potent exosomes are intriguing (Fig. 3C). Exosomes are nanometer sized membrane vesicles formed by invagination of multivesicular bodies and secreted from epithelial and hematopoietic cells. Zitvogel et al showed that exosomes produced by mouse DC pulsed with tumor peptides induce the rejection of established tumors in an antigen specific, T cell-dependent fashion in which the anti-tumor

effects were associated with long-term survival [32]. Indeed, this property of exosomes is currently being assessed for its potential as a cancer vaccine in phase I clinical trials [33].

There are admittedly several other limitations to the current studies including the relatively small sample size, single disease type assessed, and the question of whether there is a tumor specific component to the immune response observed remains to be directly answered. While the present series includes a relatively small number of patients, the pattern of increasing Hsp72 levels as well as increase in correlative markers of immune response with radiation therapy were remarkably consistent and the changes seen highly statistically significant. While the analysis was quantitative in nature, never the less the laboratory personnel responsible for processing and analysis of the samples were blinded to clinical sample information. The relatively small sample size does limit our ability to explore the predictive and prognostic significance of serum Hsp72 levels. In light of these considerations, a validation study is being performed to confirm and expand upon these findings. While it is unlikely that the patterns of change in Hsp72 and correlative immune markers are unique to prostate cancer, additional studies with patients undergoing radiation, hormonal therapy and/or chemotherapy for other malignancies is necessary to confirm the universal nature of this response across tumor types.

The finding that radiation therapy as routinely administered in the clinic leads to an increase in serum Hsp72 levels raises several intriguing questions. Provided this finding is validated, the question of immune specificity of this response will need to be answered. If an augmented tumor specific response is indeed associated with radiation therapy this would open up an important new area of investigation. Correlation of extracellular HSP levels with intracellular levels in tumors may also provide insights into mechanisms for radiation resistance and focus attention on strategies to overcome such hurdles to this commonly utilized cancer treatment.

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Abbreviations

Hsp72	stress-inducible seventy kilo-Dalton heat shock protein
APC	antigen presenting cells
AST	androgen suppressive therapy
CTL	cytotoxic T lymphocytes
DC	dendritic cells
<i>hsp72</i>	stress-inducible seventy kilo-Dalton heat shock gene
IL	interleukin
NK	natural killer
RT	radiation
XRT	radiation therapy

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Fig. 1.

Effect of gamma irradiation on circulating serum Hsp72, immune effector cells and cytokine expression. Patients were enrolled with clinically localized prostate cancer (clinical T1c–T3b, N0, M0) treated with 8 weeks of 3D conformal radiation therapy. Patients with low-risk disease defined as clinical T1–T2a, Gleason ≤ 6 and PSA < 10 ng/ml were treated with radiation alone while patients with adverse risk factors including bulky palpable disease, Gleason ≥ 7 , or PSA > 10 ng/ml typically received 8 weeks of neoadjuvant androgen suppressive therapy (AST) prior to initiation of radiation therapy (XRT) with AST continued through radiation therapy. A, Serum Hsp72 was measured at various time points pre-AST and post-XRT using the classical sandwich ELISA. Data represent the mean Hsp72 concentration pg/ml $\pm$ SD and is the sum of

three independent experiments performed in quadruplicates. *, $p < 0.001$ vs control, 0 Gy (Student's t -test). B, CD8⁺ (CTL) cells or, C, CD56⁺ (NK) cells were analyzed by flow cytometry as described in detail in the Methods section. Data are the mean percentage of cells expressing CD8 or CD56 $\pm$ SD and are the sum of two independently performed experiments. *, $p < 0.05$ vs respective control (Student's t -test). D, IL-6 and, E, TNF- α in serum was measured by the classical sandwich ELISA according to the manufactures instructions (BD BioScience). Data represent the mean cytokine concentration pg/ml $\pm$ SD and is the sum of two independent experiments performed in quadruplicates. *, $p < 0.001$ vs control (Student's t -test).

Fig. 2. Gamma irradiation stimulates the release of tumor-derived HSP into the systemic circulation. Homozygous athymic male BALB/c *nude* mice (8–10 week-old) were injected with PC-3 (10^6 cells), DU-145 (10^6 cells), or PBS into the right hind leg. When human xenografts reached approximately 100 mm^3 , mice were exposed to 0, Gy, 0.5 Gy or 5 Gy of γ -rays from a 60Co source at a rate of 2 Gy/minute. Blood was drawn at various times post irradiation exposure and serum was treated with 1% Lubrol WX for 10 min at 4°C with gentle rocking, and the concentration of serum Hsp72 (top panels) or Hsp27 (bottom panels) was measured by the classical sandwich ELISA according to the manufactures instructions (StressGen). Data represent the mean serum concentration ($\text{pg/ml} \pm \text{SD}$) and is the sum of three independent experiments performed in quadruplicates. *, $p < 0.001$ vs control (0 Gy) as determined by the Student's *t*-test.

Fig. 3.

Gamma irradiation induces the release of inflammatory mediators from tumor-bearing but not from non-tumor bearing mice. A, PC-3 (10^6) cells (left panel) or PBS (right panel) was injected into the right hind leg of 8–10 week-old homozygous athymic male BALB/c *nude* mice. When human xenografts reached approximately 100 mm^3 , mice were exposed to 0, Gy, 0.5 Gy or 5 Gy of γ -rays from a 60Cobolt source at a rate of 2 Gy/minute. Blood was drawn at various times post irradiation exposure and serum was treated with 1% Lubrol WX for 10 min at 4°C with gentle rocking, and the concentration of serum TNF- α was measured by the classical sandwich ELISA according to the manufactures instructions (Santa Cruz). Data represent the mean serum TNF- α concentration (pg/ml $\pm$ SD) and is the sum of two independent experiments

performed in quadruplicates. *, $p < 0.001$ vs control (0 Gy) as determined by the Student's *t*-test. B, PC-3 cells (10^6 ; left panel) or DU-145 cells (10^6 ; right panel) were exposed to 0 Gy, 0.5 Gy or 5 Gy and incubated for indicated times at 37°C with 5% CO₂ in air. Twenty-four hours later cells were lysed and total Hsp72 expression measured by Western blot analysis. The intensity of the bands were analyzed by densitometry with a video densitometer (ChemilmagerTM 5500; Alpha Innotech, San Leandro, CA) using the AAB software (American Applied Biology). Data are representative experiment from three independently performed experiments with similar results. C, Exosomes were isolated from PC-3 (10^7) cells (left panel) or DU-145 (10^7) cells (right panel) 96 h after exposure 0.5 Gy. Isolated exosomes were analyzed by Western blot using Hsp72, Hsc73, grp94 (StressGen), tubulin, calnexin (Santa Cruz Biotechnology) specific antibodies. The intensity of the bands were analyzed by densitometry with a video densitometer (Chemilmager5500) using the AAB software (American Applied Biology). Data are a representative experiment from two independently performed experiments with similar results.

Fig. 4.

Low dose gamma irradiation induces the release of Hsp72 by a mechanism independent of cell death. A, PC-3 (10^6) cells (left panel) or DU-145 (10^6) cells (right panel) were exposed to 0 Gy, 0.5 Gy or 5.0 Gy and at various times post irradiation exposure cells were assayed for viability using the CytoTox 96 Non-Radioactive Cytotoxicity Assay according to the manufactures instructions (Promega), and the percentage of LDH released versus total LDH was calculated. Bars are mean percentage cell death $\pm$ SD and represent three independently performed experiments. *, $p < 0.001$ vs control (Student's t -test). B, PC-3 (10^6) cells (left panel) or DU-145 (10^6) cells (right panel) were exposed to 0 Gy, 0.5 Gy or 5.0 Gy. Supernatant was extracted at various times post irradiation exposure and treated with 1% Lubrol WX for 10 min

at 4°C with gentle rocking, and the concentration of released Hsp72 was measured by the classical sandwich ELISA. Data represent the mean Hsp72 concentration pg/ml $\pm$ SD and is the sum of two independent experiments performed in quadruplicates. *, $p < 0.001$ vs. control, 0 Gy (Student's *t*-test).

Table 1

Tumor-derived Hsp72 contributes to the stimulation of human DC.

Tumor-derived supernatant ^a		CD83 surface expression (%) after following pre-treatment ^b		
Cells	Treatment	Control	Anti-IFN- γ	Anti-Hsp72
PC-3	Control (t=0h)	3.2 $\pm$ 2	4.8 $\pm$ 3	6.1 $\pm$ 2
	Control (t=96h)	17.3 $\pm$ 3	18.7 $\pm$ 3	5.8 $\pm$ 2*
	0.5Gy (t=96h)	45.2 $\pm$ 5	40.6 $\pm$ 5	9.2 $\pm$ 3*
	5.0Gy (t=96h)	46.8 $\pm$ 6	47.5 $\pm$ 7	8.8 $\pm$ 3*
DU-145	Control (t=0h)	4.2 $\pm$ 3	5.5 $\pm$ 3	5.9 $\pm$ 2
	Control (t=96h)	18.4 $\pm$ 2	15.1 $\pm$ 2	8.7 $\pm$ 3*
	0.5Gy (t=96h)	47.9 $\pm$ 6	40.2 $\pm$ 5	9.2 $\pm$ 3*
	5.0Gy (t=96h)	46.3 $\pm$ 7	38.7 $\pm$ 5	9.5 $\pm$ 3*

^a Supernatant was recovered from PC-3 cells (10^6) or DU-145 cells (10^6) after exposure to 0.5 Gy (sublethal) or 5 Gy (lethal) of γ -rays for 96 h or maintained at 37 C for 0 h (control t=0h) or 96 h (control t=96h). After recovery, the supernatant was centrifuged to clear floating cells and cellular debris and passed through a polymyxin B column five times to remove any residual endotoxin contamination, and only samples with less than <1.0 endotoxin units per 20 μ g of protein were used in subsequent experiments.

^b Recovered supernatant was either added directly to 10^5 immature DC (CD14⁺/CD83⁺) in 24-well plates for 3–5 days (control) or first depleted of IFN- γ using anti-human IFN- γ neutralizing antibody (1 μ g/ml; R&D Systems, Minneapolis, MN) or passed through a Hsp72 column containing anti-Hsp72 (StressGen Biotechnologies) five times to deplete Hsp72. After 3–5 days culture, the expression of CD83 was measured by flow cytometry using a FACScan with a Lysys II software program (Becton & Dickinson). Individual cells were gated based on forward (FSC) and orthogonal scatter (SSC). Cell debris was excluded by raising the FSC-height PMT threshold. Data are the mean percentage of cells expressing CD83 $\pm$ SD and is the sum of five independently performed experiments.

^{*} $p < 0.05$ vs control (Student's *t*-test).