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Hsp72 in prostate cancer

Radiation therapy induces circulating serum Hsp72 in patients with prostate cancer

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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 (DU-145 and PC-3) 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 PC-3 and DU-145 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 immune responses. These findings, if validated, may lead to new treatment paradigms for common human malignancies.

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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-alpha (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-inflamma-

Abbreviations: Hsp72, stress-inducible 70 kDa heat shock protein; APC, antigen presenting cells; AST, androgen suppressive therapy; CTL, cytotoxic T lymphocytes; DC, dendritic cells; *hsp72*, stress-inducible 70 kDa heat shock gene; IL, interleukin; NK, natural killer; RT, radiation; XRT, radiation therapy.

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tory cytokines TNF-α, interleukin-1 beta (IL-1β) 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 five 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 10^6 tumors into the right hind leg. When tumors became palpable, tumor volume was measured every day. When tumors reach 100 mm^3 mice were exposed to 0.5 Gy (sublethal) or 5 Gy (lethal) of γ-rays from a ^{60}Cobolt source at a rate of 2 Gy/min. 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% CO_2 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 ^{60}Cobolt source at a rate of 2 Gy/min. Cells were then incubated for an additional 96 h at 37 °C with 5% CO_2 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 μl 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 μg 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 h 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 chemilluminescence (Santa Cruz Biotechnology). The intensity of the bands were analyzed by densitometry with a video densitometer (Chemilmager™ 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.