Cross-Presentation of the Oncofetal Tumor Antigen 5T4 from Irradiated Prostate Cancer Cells—A Key Role for Heat-Shock Protein 70 and Receptor CD91

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Abstract

Immune responses contribute to the success of radiotherapy of solid tumors; however, the mechanism of triggering CD8⁺ T-cell responses is poorly understood. Antigen cross-presentation from tumor cells by dendritic cells (DC) is a likely dominant mechanism to achieve CD8⁺ T-cell stimulation. We established a cross-presentation model in which DCs presented a naturally expressed oncofetal tumor antigen (5T4) from irradiated DU145 prostate cancer cells to 5T4-specific T cells. The aim was to establish which immunogenic signals are important in radiation-induced cross-presentation. Radiation (12 Gy) caused G₂-M cell-cycle arrest and cell death, increased cellular 5T4 levels, high-mobility protein group-B1 (HMGB1) release, and surface calreticulin and heat-shock protein-70 (Hsp70) expression in DU145 cells. DCs phagocytosed irradiated tumor cells efficiently, followed by upregulation of CD86 on phagocytic DCs. CD8⁺ 5T4-specific T cells, stimulated with these DCs, proliferated and produced IFNγ. Inhibition of HMGB1 or the TRIF/MyD88 pathway only had a partial effect on T-cell stimulation. Unlike previous investigators, we found no evidence that DCs carrying Asp299Gly Toll-like receptor-4 (TLR4) single-nucleotide polymorphism had impaired ability to cross-present tumor antigen. However, pretreatment of tumor cells with Hsp70 inhibitors resulted in a highly statistically significant and robust prevention of antigen cross-presentation and CD86 upregulation on DCs cocultured with irradiated tumor cells. Blocking the Hsp70 receptor CD91 also abolished cross-presentation. Together, the results from our study demonstrate that irradiation induces immunologically relevant changes in tumor cells, which can trigger CD8⁺ T-cell responses via a predominantly Hsp70-dependent antigen cross-presentation process. Cancer Immunol Res; 3(6); 1–11. ©2015 AACR.

Introduction

Traditional treatments of cancer, such as surgery, chemotherapy, and radiotherapy, have been shown to trigger immune responses, which may contribute toward treatment outcome. Radiation is curative in up to 40% of patients with early-stage (localized) prostate cancer, but it is not yet clear what are the predictors of complete responses. Radiotherapy in prostate cancer has been shown to be associated with increased frequencies of tumor antigen-specific T cells (1). The abscopal effect of radiation (tumor regression at a distant site following localized radiation) has been shown to be immune mediated not only in mouse tumor models (2, 3) but also in patients with metastatic melanoma and lung adenocarcinoma (4, 5). Furthermore, CD8⁺ T-cell infiltration in the irradiated tumor tissue serves as a prognostic factor (4–7), indicating that radiation can switch the immunosuppressive tumor milieu to a proimmune environment.

For solid tumors, tumor antigen–specific CD8⁺ T-cell responses can be induced either by tumor cells entering lymph nodes (8) or dendritic cells (DC) cross-presenting tumor antigens either in lymph nodes or ectopic lymphoid tissues present in some tumors (9, 10). Efficient cross-presentation requires tumor cell damage or cell death and is associated with translocation or release of damage-associated molecular patterns (DAMP). The precise nature of immunogenic cell death (ICD) is not well defined but generally involves surface translocation of “eat me” signals, such as calreticulin (CRT), and stress-associated proteins, such as Hsp70. The release of chemoattractant molecules and HMGB1, representing DAMPs, also has been observed. However, there seems to be considerable plasticity in the combination and extent of these changes. The type of trigger causing cell damage and cell death may influence the relative proportions of key ICD events (11). Our study focuses on ionizing radiation, which is known to cause primarily DNA damage, cell-cycle arrest, and cellular-damage responses. These changes can trigger either DNA repair or cellular senescence and also apoptotic, necrotic.
or necroptotic cell death. The early release of IFNα/β by irradiated tumor cells can polarize antigen-presenting cells and aid their cross-presenting function (12). High-dose (10–100 Gy) in vitro irradiation of tumor cells enhances CRT translocation to the cell surface and dose-dependent release of HMGB1 and AIP by breast, colon, and prostate cancer cell lines (13). These typical ICD markers may facilitate phagocytosis of damaged/dead cells and provide maturation signals for DCs (14).

The aim of our study was to determine the relative importance of immunogenic signals in radiation-mediated tumor antigen cross-presentation. As antigen cross-presentation studies often use artificially overexpressed antigens, which may provide false-positive results, we established a model focusing on a naturally occurring oncofetal antigen, 5T4, which is expressed in most solid tumors (15). This cross-presentation model enabled us to study the effect of irradiated tumor cells on DC phenotypic and functional maturation, while the use of specific inhibitors revealed the main players of the cross-presentation process. We show here that in radiation-induced tumor antigen cross-presentation, the Toll-like receptor 4 (TLR4) pathway is not the major mechanism and the Asp299Gly TLR4 single-nucleotide polymorphism (SNP) is not associated with any impairment of the process. Instead, we found that Hsp70 is crucially important both in activating DCs and triggering CD8+ T-cell responses to DCs cocultured with irradiated tumor cells. Our results highlight the plasticity of tumor antigen cross-presentation and demonstrate the important immunologic role of Hsp70 following tumor irradiation.

Materials and Methods

Media and reagents

RPMI-1640 (Lonza) was supplemented with fetal bovine serum (FBS; PAA), AB-serum (Sigma) where indicated, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mmol/L L-glutamine ( Gibco), 25 mmol/L HEPES, and 1 mmol/L sodium pyruvate (Sigma). Lipopolysaccharide (LPS), oxaliplatin, and glycyrrhetinic acid were obtained from Sigma, VER155008 and 2-phenylethyl-5-nesofoxamidone (PES) from Tocris Bioscience (R&D Systems), inhibitory peptide (and control) of MyD88 from ProImmune (Oxford), and TRIF from Invivogen.

Tumor cells and treatment

DU145 prostate cancer cells were obtained from the European Collection of Animal Cell Cultures (ECACC) and maintained in culture with regular passaging for less than 6 months. Authentication was carried out by the supplier using cytogenetic, isoenzymatic, and DNA profile analysis. The HLA type of DU145 cells is HLA-A03/A33/B50/B57 (Welsh Blood Transfusion Service, Cardiff, United Kingdom). The cells were Mycoplasma free, as tested monthly using a MycoAlert Mycoplasma Detection Kit (Lonza). Irradiation was carried out using a 60Co-6 source (with dosimetry quality assurance) at a rate of 0.627 Gy/min. Oxaliplatin (Sigma) was used at a dosage of 20 µmol/L.

Donors and DC preparation

Ethical approval was granted and informed consent was obtained from healthy volunteers. HLA class-I typing was carried out as above. Peripheral blood mononuclear cells (PBMC) from venous blood, collected in EDTA vacutainers, were prepared by density gradient centrifugation. CD14+ monocytes were isolated by negative selection using the EasySep Human Monocyte Enrichment Kit without CD16 Depletion (STEMCELL Technologies). Average purity of CD14+ cells was 70% to 80%. Cells were incubated at 5 × 10^6 cells per well in 6-well trays in 5 mL/well of 10% FBS-RPMI plus 500 ng/mL human recombinant GM-CSF (ProSpec) and 500 U/mL IL4 (Gentaur) for 5 to 6 days.

T-cell and B-cell lines

A CD8+ T-cell line was developed from a HLA-A2+ healthy donor by repeated stimulation of nonadherent PBMCs with autologous DCs loaded with 2 µg/mL 5T417-25 peptide (RLAR-LALVI; ProImmune), as described previously (16). T cells (1–2×10^5) were expanded with a mixture of 5 × 10^4 peptide-pulsed autologous B lymphoblastoid cells (BLCL) irradiated with 40 Gy; 5 × 10^5 allogeneic PBMCs from 2 to 3 donors, irradiated with 30 Gy; 50 U/mL IL2 and 1 µL/mL OKT3 hybrid-oma supernatant in 50 mL RPMI, supplemented as above, and with 10% FBS and 1% AB-serum (16).

Immunocytochemistry

DU145 cells were seeded on coverslips and left untreated or were irradiated with 12 Gy. After 72 hours, cells were fixed with a 1:1 (v/v) mixture of ice-cold acetone:methanol for 5 minutes. After drying, cells were blocked in 1% BSA/PBS for 1 hour, then stained with an anti-Hsp70 antibody (Enzo Life Sciences), and a goat anti-mouse Alexa Fluor 488 secondary (Life Technologies). Nuclei were stained with DAPI. Images were gathered on a Zeiss Observer Z.1 microscope, fitted with an Apotome 2 module for structured illumination, using a 63×/1.4 numerical aperture oil immersion objective, and an Axiocam 506 monochrome camera system. Representative images from Z-axis sections were overlaid to generate maximum projection images.

Flow cytometry

Cells were labeled in flow cytometry buffer (PBS, 1 mmol/L EDTA and 2% FBS) with fluorochrome-conjugated 5T4, CRT, HMGB1 (R&D Systems), Hsp70 (Enzo Life Sciences), CD91 (BD Pharmingen), TLR4, HLA-DR, CD86, and CD83 (ebioscience) antibodies or unconjugated SREC-I (R&D Systems) followed by a goat anti-mouse Alexa Fluor 488 (Invitrogen) antibody and incubated on ice for 40 minutes. For intracellular labeling, the cells were fixed and permeabilized with eBioscience Fix/Perm reagents before antibodies were added for 40 minutes at room temperature. For cytokine flow cytometry, T cells were fixed and permeabilized as above and CD3, CD8, and IFNγ antibodies were added together for 40 minutes. Flow cytometry was carried out using a FACSCanto flow cytometer with FACSDiva software (BD Bioscience).

For cell-cycle analysis, Guava Cell Cycle Reagent (Millipore) was used; cell death was assessed using the Annexin-V-Propidium Iodide (PI) Apoptosis Detection Kit (BD Bioscience).

For phagocytosis assays, DU145 cells at 72 hours after irradiation were labeled with 5 µmol/L CFSE (eBioscience) and were cocultured with DCs (5 × 10^4 cells/well; 1:1 ratio) in a 96-well U-bottomed plate for 24 hours at 37°C. After incubation, the cells were surface labeled with HLA-DR or CD86 antibodies.

Western blotting

Cell pellets were resuspended in 1-mL cold RIPA Lysis Buffer (Santa Cruz Biotechnology) with freshly added protease and...
protein transduction sequence alone, which renders the peptides interactions (17, 18). The control peptides consist of the pro-
binding to the TIR domains and interfering with TLR adaptor
TRIF (FCEEFQVPGRGELH), respectively, serve as decoys by
to the sequence of the BB-loop of MyD88 (RDVLPGT) and
adaptor (Santa Cruz Biotechnology). Bands were developed by ECL on film (GE Healthcare). Relative density of the bands exposed was calculated using the ImageJ Software.

Inhibition of TRIF, MyD88, HMBG1, Hsp70, and its receptors

The MyD88 and TRIF inhibitory peptides that correspond to the sequence of the B-box domain of MyD88 (RDVLPGT) and TRIF (FCEEFQVPGRGELH), respectively, serve as decoys by binding to the TIR domains and interfering with TLR-adaptor interactions (17, 18). The control peptides consist of the protein transduction sequence alone, which renders the peptides cell-permeable. DCs were pretreated with 20 μmol/L of MyD88 or 25 μmol/L of TRIF inhibitory or control peptide, respectively, for 6 hours before LPS stimulation (100 ng/mL) or with 25 μmol/L of both when adding DCs to DU145 cells for the cross-presentation assay.

Glycyrrhizin, an HMBG1-inhibitor, was added at 50 μmol/L at the time of irradiation, while VER155008, an Hsp70-inhibitor, at 5 μmol/L to 0- and 12 Gy-irradiated DU145 cells at 0, 24, and 48 hours of the 72-hour incubation, respectively. PIS was added to DU145 cells at 20 μmol/L at the time of irradiation. Hsp70 receptor blocking was carried out by treating DCs with the SREC-I-specific purified goat IgG polyclonal antibody (R&D Systems) or CD91 mouse IgG1 monoclonal antibody (Thermo Scientific) or relevant isotypes (R&D Systems) at 1 μg/mL for 1 hour before adding DCs to DU145 cells.

Antigen cross-presentation

DU145 cells were plated in two 24-well plates at 5 × 10^5 cells per well. One plate was irradiated with 12 Gy before incubation for 72 hours. DCs were then added to the wells at a 1:1 DU145:DC ratio. After 48 hours, ST4-specific T cells were added at a 1:1:5 (DU145:DC:T cell) ratio. Golgi Plug (0.5 μL/500μL) and Golgi Stop (0.35 μL/500 μL; Sigma) were added to the wells 1 hour later and the cultures were incubated overnight. Cytokine flow cytometry was carried out to determine the percentage of IFNγ+CD8+ T cells.

T-cell proliferation

DU145 cells were plated in two 24-well plates at 1 × 10^5 cells per well. One plate was irradiated with 12 Gy and 10^5 DCs in 0.5 mL was added to the wells. After 4 hours, CSFE-labeled T cells (5 × 10^5 cells/well) were added and incubated for 5 days. Flow cytometry analysis of CSFE dilution in CD3+CD8+ cells was carried out.

SNP analysis

SNP analysis was performed by the Department of Medical Genetics (Cardiff and Vale NHS Trust, University Hospital of Wales, Cardiff, United Kingdom). DNA amplification was carried out from blood or established BLCLs by PCR followed by pyrosequencing optimized for the Asp299Gly sequence of the TLR4 polymorphism. Out of 67 samples tested, 4 donors were found to carry the Asp299Gly SNP.

Statistical analysis

Statistical analysis was carried out by applying the Student t test, paired t test, and ANOVA with the Tukey post hoc test (GraphPad InStat 3.06). Statistically significant differences are marked as *, P < 0.05; **, P < 0.01; ***, P < 0.001.

Results

Irradiation induces immunologically relevant changes in tumor cells

To establish the optimum minimal radiation dose causing significant changes in DU145 cells in vitro, dose-escalation and time-kinetics experiments were performed. A significant proportion of irradiated cells was arrested in the G2–M phase, detectable first at 24 hours after irradiation. There was a small but significant radiation dose-dependent increase at 48 hours in the proportion of cells in sub-G0 phase representing apoptotic cells with fragmented DNA (Fig. 1A). The type of cell death, detected with Annexin/PI-labeling, was mainly late apoptotic/necrotic and the proportion of cells with early apoptotic markers remained low throughout the 72-hour incubation (Fig. 1B). Other radiation-associated immunologically relevant changes were also observed. The total cellular HMBG1 content increased with early time kinetics (Fig. 1C, i), while significant amounts of HMGB1 were released from the cells, detectable from 48 hours after irradiation by ELISA (Fig. 1C, ii). Surface MHC class-I expression was not altered by irradiation (not shown), but the cellular content of the target antigen, ST4, was significantly elevated following 12-Gy radiation (Fig. 1D, i and ii) as detected by flow cytometry and confirmed by Western blotting (Fig. 1E).

Irradiation of tumor cells induces Hsp70 cytoplasmic translocation and surface expression

Radiation resulted in the significant upregulation of surface Hsp70 on DU145 cells (Fig. 2A). Multicolor flow cytometry analysis confirmed that upon irradiation in a large proportion of cells with cell surface Hsp70 expression CRT1 was also translocated to the cell surface; this double-positive subset was not observed without irradiation (Fig. 2B, right vs. middle plot). Immunocytochemistry confirmed that while Hsp70 expression was predominantly nuclear in untreated DU145 cells, 72 hours after irradiation, cytosolic and cell surface expression became dominant (Fig. 2C and Supplementary Fig. S1). Although a slight increase in Hsp70 content was observed 3 hours after irradiation.
in DU145 cells by Western blotting (Fig. 2D), it was not as significant as that observed 2 hours after 42°C heat treatment; by 72 hours after irradiation, no increase was detectable. This suggests that Hsp70’s cellular localization may be important in antigen cross-presentation.

DC activation and tumor antigen cross-presentation following uptake of DU145 cells

Next, we studied whether irradiated tumor cells are taken up by DCs and if they activate DCs and trigger their antigen-presenting function. Tumor cells were labeled with CFSE 72 hours after irradiation and added to DCs at a 1:1 ratio. Phagocytosis was measured 24 hours later by determining the proportion of HLA-DR^+CFSE^+ cells. Although some uptake of nonirradiated tumor cells was observed (Fig. 3A, i), the proportion of phagocytic DCs increased significantly upon encountering irradiated DU145 tumor cells (Fig. 3A, ii and iii). DC phenotype following phagocytosis of irradiated or nonirradiated tumor cells was also studied 24 hours after DC:tumor cell coculture. CD86 expression was significantly elevated on DC cultured with irradiated, but not with nonirradiated, tumor cells (Fig. 3B). Furthermore, CD86 expression...
was significantly higher on those DCs that phagocytosed tumor cells, that is, those positive for CFSE (Fig. 3C, cells in Q2).

Figure 3.

TLR4 polymorphism does not affect antigen cross-presentation from irradiated tumor cells

To elucidate whether TLR4 SNP results in impaired antigen cross-presentation, as it has been shown to do with oxaliplatin-treated tumor cells (19), we conducted a series of experiments with DCs generated from monocytes of donors carrying the normal (Asp299; n = 5) or the polymorphic Gly299 (n = 4) TLR4 allele. All donors were HLA-A2+. The general characterization of monocytes and DCs revealed that TLR4 expression levels (Fig. 5A, i and ii) and LPS-induced TNFα production were comparable between the two groups (Fig. 5A, iii). Phenotypic maturation of DCs (CD86, HLA-DR, and CD83) carrying the SNP allele following coculture with irradiated tumor cells was also unimpaired (Fig. 5B). Most importantly, cross-presentation of 5T4 from irradiated tumor cells was similarly efficient by DCs carrying the Asp299 or the Gly299 TLR4 allele, respectively (Fig. 5C). To see whether TLR4 SNP was affecting chemotherapy-induced but not radiation-induced tumor antigen cross-presentation, T-cell responses were also studied with oxaliplatin-treated tumor cells. T-cell responses were generally weaker than those induced by DCs cocultured with irradiated tumor cells, but significantly elevated responses were observed in one out of three normal subjects and 2 out of 3 TLR4 SNP donors (Fig. 5C). Despite the low number of donors, the experiments clearly show that donors with TLR4 SNP are able to cross-present tumor antigen from either irradiated or chemotherapy-treated tumor cells.
Hsp70 inhibition blocks antigen cross-presentation

The contribution of heat-shock proteins to antigen cross-presentation has been demonstrated in several models; we performed experiments to establish whether Hsp70 plays a role.

Figure 3.
DC maturation and antigen cross-presentation by irradiated tumor cells. A, representative dot plot showing uptake of CFSE-prelabeled DU145 cells (x-axis) after 0-Gy (i) or 12-Gy (ii) irradiation by DCs (HLA-DR⁺ cells). Phagocytic DCs are in the top right quadrant (Q2). iii, summary of results from 5 donors; each symbol represents the mean percentage of DCs in Q2 from triplicate samples per donor. B, flow cytometry analysis of CD86 expression after 24-hour coculture of DCs without (Nil) or with 0-Gy or 12-Gy irradiated DU145 cells. Mean ± SEM of CD86 mean fluorescence intensity (mfi) from triplicate cultures is shown. C, flow cytometry of CD86 expression, analyzed on DCs gated as Q1 or Q2 DCs, respectively, after DCs coculture with DU145 cells. D, i, proliferation of CFSE-labeled 5T4-specific T cells 5 days after stimulation by autologous DCs cocultured with DU145 cells, as indicated. Mean ± SEM of percentage of IFN⁺ T cells from triplicates from an individual donor. F, i, 5T4 antigen cross-presentation is inhibited by HLA-class I blocking antibody; ii, 5T4⁺ (DU145, PC3, and M38) but not 5T4⁻/C0 (LNCaP) tumor cells stimulate T cells in the cross-presentation assay. Mean ± SEM of percentage of IFN⁺ T cells from triplicates are shown. NS, not statistically significant.
Antigen Cross-Presentation from Irradiated Tumor Cells

**Figure 5.**

TLR4 polymorphism does not affect tumor antigen cross-presentation. A, i, TLR4 expression on monocytes from 5 donors with the Asp299 (299A) and 4 donors with the polymorphic Gly299 (299G) allele; ii, TLR4 expression on day 5 DCs. iii, day 5 DCs were stimulated with LPS and TNFα production was measured by flow cytometry. Symbols represent percentage of positive cells from individual donors. The boxes show the 25% and 75% percentiles of the combined data, and the lines represent the medians. B, DC phenotyping from donors as in A. DCs were cocultured in cross-presentation of irradiated tumor cells. To test this, first we applied to tumor cells before irradiation the small-molecule inhibitor VER155008, which inhibits the activity of both the induced and constitutive forms of Hsp70 (21, 22). Tumor cell numbers after 72 hours were only slightly lower when VER155008 was applied to irradiated cells, presumably because cell proliferation was already slowed down by the irradiation. However, as expected, the treatment inhibited the growth of nonirradiated tumor cells by approximately 70% (Fig. 6A). Similarly, VER155008 significantly increased cell death of nonirradiated but not irradiated DU145 cells (Fig. 6B). The inhibitor did not impair Hsp70 cell-surface expression on irradiated tumor cells (Fig. 6C). After establishing that VER155008 delivers the expected effects to untreated tumor cells, we studied its effect on the ability of irradiated tumor cells to upregulate CD86 on DCs. CD86 upregulation was partially inhibited (Fig. 6D) when DCs encountered irradiated tumor cells pretreated with VER155008. Finally, we applied VER155008 in the cross-presentation model to test its effect on T-cell activation. The inhibitor significantly decreased both the background cross-presentation of tumor antigen from nonirradiated tumor cells and the enhanced level of cross-presentation observed from irradiated tumor cells (Fig. 6E), as detected by decreased T-cell IFNγ production. The results were confirmed with DCs derived from 2 donors. To test that the inhibition of cross-presentation with VER155008 was not due to an off-target effect, we applied PES, another Hsp70-inhibitor. PES binds only to stress-induced but not constitutive Hsp70 (23). Interestingly, PES only inhibited T-cell stimulation induced by irradiated tumor cell-loaded DCs but not by the addition of nonirradiated tumor cells (Fig. 6E). These experiments indicate a crucial role for radiation-induced Hsp70 in tumor antigen cross-presentation.

Irradiated tumor cell-derived Hsp70 signals mainly via CD91 on DCs

To further elucidate the importance of Hsp70 in the cross-presentation model, we tested the expression of potential receptors CD91 and SREC-I on day 5 DCs. Significant surface expression of both receptors was observed (Fig. 7A). When neutralizing antibodies against these receptors were applied in the cross-presentation experiments, T-cell activation was completely inhibited in the presence of the anti-CD91- but not the SREC-I-specific neutralizing antibody (Fig. 7B). These results demonstrate that CD91-mediated effects in DCs, such as tumor cell–derived Hsp70 binding, are necessary for efficient antigen cross-presentation from irradiated tumor cells.

**Discussion**

Antigen cross-presentation has been indicated as an important mechanism for generating CD8+ T-cell responses against solid tumors that do not migrate into lymph nodes or viruses that do not infect professional antigen-presenting cells. Although chemotherapy-induced antigen cross-presentation has been studied extensively, there is a paucity of information about ionizing radiation–mediated antigen cross-presentation. The abscopal effect, observed in patients undergoing radiotherapy, has been demonstrated to be immune-mediated and is likely to involve antigen cross-presentation from irradiated tumors (4, 5). Further studies in this field would aid better understanding of how radiotherapy could be made more successful.

We studied the relative importance of immunogenic signals in antigen cross-presentation from irradiated human tumor cells. We established a model using a tumor-specific T-cell line as a detector of cross-presentation of a naturally expressed tumor antigen from irradiated, HLA-mismatched prostate cancer tumor cells by DCs. The radiation dose (12 Gy) applied to tumor cells in these experiments is in line with the latest technical developments of radiotherapy in prostate cancer and other malignancies. High-dose
Brachytherapy and intensity-modulated radiotherapy offer treatments with fewer fractions but higher doses, delivered more precisely to the cancer. The cellular effect of radiation is complex, resulting in growth arrest, senescence, and different types of cell death. We observed cell-cycle arrest in the G2–M phase, as reported by others (24), and a gradual increase of cell death with time following irradiation. Cell death was predominantly of the late apoptotic/necrotic type. The p53 gene is mutated in DU145 cells, which may affect radiation-mediated repair response and apoptosis (25). As p53 mutations are frequent in prostate cancer, our observations are likely to be representative of the physiologic behavior of the majority of prostate cancer cells.

Figure 6.
Hsp70 inhibition abolishes antigen cross-presentation. A, the effect of VER155008 on DU145 cell numbers after 72-hour culture. B, different types of cell death as detected by Annexin/PI staining in the absence or presence of VER155008. C, surface expression of Hsp70 (gray) versus isotype (black) in the absence or presence of VER155008: (i) summary from triplicates; (ii) representative histograms. D, effect of VER155008-treated or untreated DU145 cells on CD86 expression of DCs following a 24-hour coculture: (i) representative histograms; (ii) summary from triplicates. E, stimulation of 5T4-specific T cells in a cross-presentation experiment with DCs loaded with VER155008 or PES-treated or untreated DU145 cells. This experiment was carried out with DCs derived from 2 donors. A–E, mean ± SEM of results from triplicate samples are shown.
Radiation-induced upregulation of CRT from the endoplasmic reticulum to the cell surface is one of the typical stress responses with an important immunologic impact, such as the facilitation of phagocytosis (26). The results show a partial contribution by the TLR signaling pathway and HMGB1 to antigen cross-presentation. HMGB1 is both a nuclear factor and a secreted protein. In the nucleus, it acts as an architectural chromatin-binding factor that influences DNA tertiary structure. When released from dying cells, it functions as a proinflammatory cytokine (27). However, its effects are pleiotropic and they depend not only on its redox status but also on the particular receptor it binds, such as RAGE, TLR2, or TLR4. Glycyrrhizin, which binds directly to HMGB1 and inhibits its chemokine function and autophagy induction (20), among other potential effects, was proved inhibitory in the cross-presentation model. HMGB1 has been shown to associate with TLR4 (28). The Asp299Gly SNP of TLR4 causes structural changes of the TLR4 extracellular domain, with a potential impact on LPS binding (29). Cross-presentation has been implied to be affected by this SNP, similar to the effect observed in TLR4 (29). Cross-presentation has been implied to be affected by this SNP, similar to the effect observed in TLR4 (29). Cross-presentation has been implied to be affected by this SNP, similar to the effect observed in TLR4 (29). Cross-presentation has been implied to be affected by this SNP, similar to the effect observed in TLR4 (29).

Hsp70 has well-described immunologic roles as well, as tumor tissue–derived Hsp70 has been shown to be protective against tumor challenge in mice (33). We observed a predominantly nuclear expression of Hsp70 in untreated DU145 cells, while in irradiated cells nuclear expression seemed lower and cytosolic and cell-surface expression increased significantly. This translocation observed at 72 hours after irradiation may have been associated with enhanced repair activity in the irradiated cells. Although Hsp70 inhibition with VER155008, a specific Hsp70-family inhibitor (21), did not influence tumor cell growth and cell death following irradiation, it significantly inhibited the growth of nonirradiated tumor cells. VER155008 also did not influence Hsp70 cell-surface expression on irradiated DU145 cells; however, it partially inhibited the ability of irradiated DU145 cells to activate CD86 upregulation on DCs. The reason behind this observation is not clear. The inhibition of antigen cross-presentation was complete when VER155008 was added either to irradiated or control DU145 cells. Another Hsp70 family inhibitor, PES (23), only affected the enhanced T-cell response observed with irradiated but not that with untreated tumor cells.

Hsp70 can bind to TLR2 or TLR4, CD91, CD40, or to scavenger receptors such as SREC-I and LOX-1. Hsp70 binding to TLR4 can upregulate HMGB1 in DCs (34), providing cross-talk between the heat shock and the HMGB1–TLR4 pathways. On the other hand, SREC-I and LOX-1 were shown to be both responsible for antigen cross-presentation in a murine system (35). To determine which receptor is important in this model, DC phenotyping and antibody-blocking experiments were carried out. These results are consistent with those from previous studies (36, 37), showing that CD91 is the dominant receptor for Hsp70 during the cross-presentation process.

We have not addressed directly whether Hsp70’s role in this model lies mainly in antigen-chaperoning (33, 38, 39) or enhancing autophagy (40) or triggering DC maturation (41, 42) with subsequent increase in T-cell stimulation. All of these known functions can be important and they likely play a synergistic role. Our model provides an opportunity to study the fine details of...
heat-shock protein–mediated chaperoning of a naturally expressed antigen in a human tumor model. Such an analysis would represent an interesting follow-up to the work presented here. Despite the long list of players necessary for “optimal” antigen cross-presentation, the plasticity of the process has also been demonstrated, as for example, highly polarized (type I) DCs can efficiently prime T cells even when cocultured with apoptotic cells (43). Furthermore, DCs can acquire antigen from live cells for antigen cross-presentation in both tumor and viral settings (44–46). In the latter, while apoptosis is inhibited by the virus, Hsp70 expression is significantly upregulated (47). These examples illustrate that if any key player of the antigen cross-presentation process is overexpressed or hyperactivated, it can generate a shortcut leading to antigen cross-presentation even if not all the elements, as discussed earlier, are present. Taken together, results from our study provide strong evidence that preexisting tumor antigen-specific T cells can be reactivated as a consequence of irradiation of tumor cells. We also demonstrate that Hsp70 plays a crucial role in antigen cross-presentation from irradiated tumor cells. These observations have practical implications for the design of future immuno-radiotherapy combinations.

Disclosure of Potential Conflicts of Interest

J. Staffurth has received speakers bureau honoraria from Janssen and Astellas, and is a consultant/advisory board member for Janssen. No potential conflicts of interest were disclosed by the other authors.

References


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Conception and design: J. Salimu, M.D. Mason, Z. Tabi
Development of methodology: J. Salimu, Z. Tabi
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): J. Salimu
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): J. Salimu, I.K. Spary
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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): J. Salimu

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