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 present a naturally expressed oncofetal tumor antigen (ST4) from irradiated DU145 prostate cancer cells to ST4-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 ST4 levels, high-mobility protein-group B1 (HMG-B1) 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+ ST4-specific T cells, stimulated with these DCs, proliferated and produced IFNγ. Inhibition of HMG-B1 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): 678–88. ©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 HMG-B1, 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 necrotic 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 ATP by blood, 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 oncotical antigen, ST4, 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 glycyr rhizin were obtained from Sigma, VER155008 and 2-phenylethynesulfonamide (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 137Cs-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/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-LAILVI; ProImmune), as described previously (16). T cells (1–2 × 10^6) were expanded with a mixture of 5 × 10^6 peptide-pulsed autologous B lymphoblastoid cells (BLCL) irradiated with 40 Gy; 5 × 10^6 allogeneic PBMCs from 2 to 3 donors, irradiated with 30 Gy; 50 U/mL IL2 and 1 μL/mL OKT3 hybridoma 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 (Lifetech). Nuclei were stained with DAPI. Images were gathered on a Zeiss Observer.2 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 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).

Western blotting
Cell pellets were resuspended in 1-mL cold RIPA Lysis Buffer (Santa Cruz Biotechnology) with freshly added protease and

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protein transduction sequence alone, which renders the peptides cell-permeable. DCs were pretreated with 20 μmol/L of TRIF inhibitory or control peptide, respectively, for 6 hours before LPS stimulation (100 ng/mL). Hsp70 on DU145 cells (Fig. 2A). Multicolor 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 HMGB1 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, 5T4, 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 CRT 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...
HLA-A2^+ cells, that is, that were positive for CFSE (Fig. 3C, cells in Q2 was significantly higher on those DCs that phagocyted tumor cells, that is, that were positive for CFSE (Fig. 3C, cells in Q2 of Fig 3A, ii) compared with those in Q1. ST4 antigen cross-presentation from DU145 tumor cells by HLA-A2^+ DCs was measured by assessing proliferation and intracellular cytokine production of ST4-peptide-specific T cells (16). Although some T-cell activation was triggered by DCs cultured with nonirradiated DU145 cells, significantly more T-cell proliferation and IFNγ production was observed when DCs were cocultured with irradiated DU145 cells (Fig. 3D and E). T-cell stimulation in the cross-presentation model was HLA class-I restricted, as a class-I blocking antibody completely inhibited T-cell stimulation (Fig. 3F, i). DU145 cells were not unique in their ability to serve as antigen donors for ST4-specific T-cell activation, as ST4-positive PC3 (prostate) and M38 (mesothelioma) cells behaved the same way (Fig. 3F, ii). LNCaP cells, expressing no or little ST4, triggered no significant T-cell responses. The experiments demonstrate that DCs are able to present a naturally expressed tumor antigen to specific T cells and that this process is significantly enhanced by tumor cell irradiation.

**TLR4-MyD88 signaling is not the critical pathway in antigen cross-presentation from irradiated tumor cells**

Tumor antigen cross-presentation from tumor cells treated with chemotherapy, especially with anthracyclines, has been studied more extensively than that from irradiated tumor cells. However, it has been suggested that in both cases, antigen cross-presentation crucially depends on the TLR4–HMGB1 interaction and consequently patients with TLR4 polymorphism are unable to mount immune responses to tumor antigens (19). To study TLR4–HMGB1 signaling in antigen cross-presentation from irradiated tumor cells, we applied Glycyrrhizin (GA), an HMGB1-inhibitor (20), to tumor cells before irradiation. HMGB1 inhibition resulted in a small decrease in CD86 expression on DCs after their coculture with tumor cells (Fig. 4A, i), and also a small but significant decrease in T-cell IFNγ production (Fig. 4A, ii). HMGB1 can bind to multiple receptors, such as TLR2 and TLR4, so next we targeted the MyD88/TRIF signaling pathway with inhibitory peptides. We established that 20 to 25 μmol/L of the inhibitory peptides significantly reduced LPS-induced TNFα production by DCs compared with that of control peptides (Fig. 4B). Although neither TRIF nor the MyD88 inhibitory peptide alone (25 μmol/L each) had any effect on T-cell stimulation (not shown), when applied together, they resulted in a small inhibitory effect (Fig. 4C), indicating the importance of pathways other than TLRs in antigen cross-presentation.

**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 and consequently patients with TLR4 polymorphism are unimpaired (Fig. 5B). Most importantly, cross-presentation of ST4 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 chemotherapytreated 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 4.
Partial effect of HMGB1–TLR4 pathway inhibitors on DC maturation and antigen cross-presentation. A, DCs were cocultured with 0-Gy (i) or 12-Gy (ii) irradiated DU145 cells in the presence or absence of 50 μmol/L glycyrrhizin (GA) for 72 hours. CD86 upregulation on DCs (i) and 5T4-specific T-cell stimulation (ii) were analyzed by flow cytometry. The columns show mean ± SEM of results from triplicate cultures. B, DCs were treated with LPS in the presence of inhibitory peptides targeting MyD88 (20 μmol/L) or TRIF (25 μmol/L). Control peptides (cell-permeable domain of the inhibitory peptide) were used at the same concentrations. Mean ± SEM of the percentage of TNFα-producing DCs are shown, as detected by cytokine flow cytometry. C, DCs were cultured in a cross-presentation assay in the presence of MyD88 and TRIF inhibitory peptides together or with control peptides (25 μmol/L each). Mean ± SEM of percentage of IFNγ T cells from triplicate cultures are shown. The experiments were repeated two to three times.

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...
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) summary from triplicates; (ii) representative histograms. E, stimulation of ST4-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 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−/− knockout mice (19). However, LPS-induced cytokine production is not affected by the TLR4 SNP even when present in a homozygous form (30). Our results are in agreement with this, as we observed no influence of Asp299Gly TLR4 SNP on LPS-induced TNFα production in DCs. However, we also found no effect by the TLR4 SNP on radiation-induced antigen cross-presentation. Furthermore, contrary to the observation by others (19), we also did not find any evidence of impaired antigen cross-presentation from oxaliplatin-treated tumor cells by DCs carrying the TLR4 SNP. The possible explanation behind this discrepancy may be related to donor variation as the previously published observation (19) was based on results from a single donor with TLR4 SNP.

Heat-shock proteins represent another group of damage-associated molecules, upregulated by irradiation and released into the extracellular space from dying cells or secreted from live cells (31). In prostate cancer, Hsp70 has been shown to be protective, 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.

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