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Mini-review

The role of heat shock protein 70 (Hsp70) in radiation-induced immunomodulation

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ABSTRACT

Despite enormous progress in radiation technologies (high precision image-guided irradiation, proton irradiation, heavy ion irradiation) and radiotherapeutic concepts (hypofractionated irradiation schemes), the clinical outcome of radiotherapy in locally advanced and metastasized tumors and in hypoxic tumors which are radiation-resistant remains unsatisfactory. Given their key influence on a number of biological and immunological parameters, this article considers the influence of irradiation-induced stress proteins on radiation-induced immunomodulation. Depending on its location, the major stress-inducible Heat shock protein 70 (Hsp70) has been found to fulfill multiple roles. On the one hand, increased intracellular Hsp70 levels have been found to play a key role in the recovery from stress such as radio(chemo)therapy, and on the other hand extracellular Hsp70 proteins are potent stimulators of the innate immune system and mediators of anti-tumor immunity. Furthermore, if loaded with tumor-derived peptides, members of the Heat Shock Protein 70 (HSP70) and 90 (HSP90) families can stimulate the adaptive immune system via antigen cross-presentation. An irradiation-induced enhancement of the selective expression of a membrane form of Hsp70 on the surface of tumor cells which can act as a recognition structure for activated NK cells might have significant clinical relevance, in that the outcome of irradiation therapy for advanced tumors could be improved by combining it with cell-based and other immunotherapies that target this membrane form of Hsp70.

Major stress-inducible Heat Shock Protein 70 (Hsp70): function and subcellular localization

Heat Shock Proteins (HSPs) were firstly described by Ferruccio Ritossa in 1962 [1] as a set of evolutionary highly conserved genes in *Drosophila melanogaster* that are activated upon heat stress. Six of the eight members of the HSP70 family predominantly reside in the cytosol, where they maintain protein homeostasis by supporting the folding, refolding, and assembly of nascent polypeptides, preventing protein aggregation, and assisting the transport of other proteins across membranes [2]. Apart from heat, the synthesis of different HSP family members is increased by a large variety of different stressors including chemo- and radiation therapy which cause the production of reactive oxygen species (ROS) and also during cell proliferation and differentiation. A comparison of two highly homologous members of the HSP70 family, the constitutive Hsc70 (Hsp73, HSPA8, Hsp70-8) and the major stress-inducible Hsp70 (Hsp72, HSPA1A, Hsp70-1), has revealed that, under physiological conditions, the constitutive Hsc70 is expressed at higher levels than Hsp70, whereas the synthesis of Hsp70 is more rapid and accumulates in different subcellular compartments after stress [3,4]. Furthermore, in contrast to normal cells, tumors frequently overexpress Hsp70 in the cytosol, present Hsp70 on their plasma membrane [5,6], and actively release Hsp70 [7–9]. Elevated cytosolic levels of Hsp70 and Hsp27 have been found to mediate protection against apoptosis, promote malignant transformation, senescence and metastatic spread [10–15], whereas extracellular, tumor-derived HSPs are considered to act as danger signals [16] that can elicit anti-tumor immune responses [13,17–19]. A summary of the key actions of Hsp70 depending on their localization is shown in Table 1.

Membrane localization of Hsp70 on tumor cells is enabled by tumor-specific lipid components. Under physiological conditions Hsp70 co-localizes with the lipid raft glycolipid

Abbreviations: APC(s), antigen-presenting cell(s); ATP, adenosine triphosphate; Ca, calcium; Gb3, globoyltriaosylceramide; Grp, glucose-regulated protein; Gy, gray; HSP, Heat Shock Protein (refers to the family of Heat Shock Proteins); Hsp, Heat shock protein (refers to a specific member of the family); IFNγ, interferon gamma; mAb, monoclonal antibody; MHC, major histocompatibility molecules; NK cell, natural killer cell; NSCLC, non-small lung cell cancer; PS, phosphatidylserine; Treg cell, regulatory T cell.

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Table 1
Key actions of Heat shock protein 70 (Hsp70).

Localization	Function	References
Cytosolic	Binds to polypeptides in an ATP dependent manner	[20] [21]
	Prevents aggregation of unfolded peptides and transports proteins	[18]
	Regulates intercellular signaling	
	Mediates antigen cross-presentation	
Membrane-bound	Acts as a tumor-specific recognition structure for activated NK cells on plasma membranes	[22] [10]
	Mediates anti-apoptotic functions on lysosomal membranes	
Extracellular free and lipid-bound	Induces inflammatory and anti-inflammatory responses	[23] [24] [25] [26]

globoyltriaosylceramide (Gb3) [27], whereas Hsp70 is predominantly associated with phosphatidylserine (PS) outside of lipid rafts after stress. In non-stressed cells, the ATP-dependent aminophospholipid translocase [28] enables the exclusive localization of PS to the inner side of the plasma membrane. However, PS translocates from the inner to the outer plasma membrane leaflet via an activation of the ATP and Ca^{2+} dependent phospholipid scramblase after stress and thus provides an early marker of apoptosis [29]. Although the role of PS in the outer membrane leaflet is still not completely clear, it is assumed that oxidatively modified PS provides a phagocytic "eat-me" signal [30]. However, PS positivity does not necessarily mean that cells are no longer viable, as Hsp70/PS membrane-positive tumor cells are viable and can be grown in cell culture [31,32]. Furthermore, viable T cells also have been found to present PS on the outer leaflet following activation [33].

HSPs as targets for adaptive and innate immune responses

The potential of using members of families (particularly the 70 kDa and 90 kDa families) as potential chaperones for immunogenic peptides in the context of cancer immunotherapeutics has been considered by the group of Srivastava for many years [18]. Although the clinical success of this approach has been variable, the use of tumor-derived gp96 has been introduced into the clinical setting (Prophage Series of vaccines from Agenus: www.agenus.com/science/prophage.php). These studies were based in the concept that isolated from tumor tissue chaperoned tumor-specific peptides and, on administration, could induce the generation of peptide-specific effector cells. The specificity of the immunity that was generated was reported as being toward the antigenic peptides that were carried by the HSP, rather than the HSP itself. It was therefore proposed that this avoided potential cross reactivity with other tissues expressing the relevant. Some subgroups responded very well, whereas others have not profited from the therapy. Potential problems with this approach involved the limited amount of tissue from which the HSP (and therefore the vaccine) could be generated. Notwithstanding this, a number of clinical trials evaluating the clinical potential approach have been performed, and some success has been obtained [34]. As with all such studies, the translation of such therapies has been slow due to the fact that initial clinical trials are usually performed in patients with advanced disease.

Furthermore, the search for tumor-specific targets which are located intracellularly in normal cells, but are expressed on the cell surface of tumor cells and can be recognized by immune competent effector cells, has resulted in the identification of such as Hsp70, Hsp90, or Grp78 [35]. Herein, we concentrate on the major stress-inducible Hsp70 which is exclusively expressed on the cell surface of tumor cells, but not normal cells [5]. Ionizing irradiation, even at sublethal doses (below 5 Gy), induces the synthesis of Hsp70 in the cytosol of normal and tumor cells [36]. However, due to differences in the lipid composition of the plasma membrane only tumor cells have the capacity to present Hsp70 on their cell surface [27]. Irradiation as well as other stress factors such as heat, chemotherapeutic agents, Hsp90 inhibitors, amino acid analogues, glucose deprivation, hypoxia and reoxygenation, and drugs [37] further increases the cell surface density of Hsp70 on tumor cells. The cell surface bound form of Hsp70 on viable tumor cells is detectable using a mouse monoclonal antibody (cmHsp70.1, patent multimmune GmbH) [38], but not by other commercially available Hsp70-specific antibodies. The cmHsp70.1 monoclonal antibody (mAb) antibody detects the conformation of Hsp70 in the plasma membrane of tumor cells and does not cross-react with the highly homologous Hsc70.

Low dose irradiation up-regulates a number of immunologically-relevant antigens, such as major histocompatibility molecules (MHC), tumor-associated antigens (Carcinoembryonic Antigen (CEA)), mucin 1 [39], as well as the expression of the Intercellular Adhesion Molecule-1 (ICAM-1) on endothelial cells [40] and the apoptosis inducer Fas (CD95), all of which have immunomodulatory properties [41,42]. In addition to the direct killing of cancer cells by ionizing irradiation which is mediated by DNA damage can also induce non-targeted abscopal or bystander effects that can elicit the stimulation of T and NK cell mediated immune responses [42]. Most of these antigens are also relevant for the induction and regulation of T cell mediated immune responses and the generation of protective anti-tumor immunity [43]. T cells can be roughly grouped into $CD4^+$ T helper cells that recognize antigenic determinants presented by MHC class II molecules on antigen presenting cells (APCs), and $CD8^+$ cytotoxic T cells which recognize their target antigenic peptides that are presented in the context of MHC class I molecules [44]. $CD4^+$ T helper cells exist in different epigenetic states such as Th1, Th2, Th17, fork-head box 3 ($Foxp3^+$) T regulatory cells, T follicular helper, Th9, and Th22 cells [45] that are determining their different functions. Th17 cells show a high plasticity and thus can acquire pro-inflammatory characteristics of Th1 cells. T cells recognize MHC–peptide complexes via clonal T cell receptors which exhibit an enormous heterogeneity which is generated by variable diversity joining gene recombination and crossover events. T cell mediated immune responses, and the memory which they display, play a crucial role in immunosurveillance of cancer. However, effective anti-cancer immune responses can be blocked or down-regulated by regulatory T (Treg) cell populations which can be characterized, in part at least, on the basis of their expression of FoxP3 transcription factor, a high constitutive cell surface expression of the IL-2 receptor alpha chain (CD25), expression of the glucocorticoid induced TNF-receptor related protein GITR and the cytotoxic T lymphocyte associated antigen 4 (CTLA-4), as well as low levels of the IL-7 receptor (CD127) in humans [46]. Another level of control of anti-cancer immunity – so called "checkpoint inhibitors" – is also currently attracting a lot of attention. Under normal circumstances, immune checkpoints are important for maintaining self-tolerance by preventing autoimmunity and protecting the tissue from damage when the immune system is activated. However, it is now apparent that the expression of immune checkpoint proteins can be used to establish resistance mechanisms by tumor cells, thus allowing progressive tumor growth [47]. Attention has therefore turned to the potential therapeutic value of antibodies that target CTLA-4 and the programmed cell death protein 1/programmed cell death protein 1 ligand (PD1/PD-L1) to block such interactions and inhibit this protective response [48–50]. Immune checkpoint blockade is believed to have enormous therapeutic potential and integrative immunotherapies that incorporate immune checkpoint blockade should result in durable clinical responses and increased cure rates [51].

Two major antibody-based therapies are directed against the PD-1/PD-L1 (Pembrolizumab, Nivolumab) and the CTLA-4 (Ipilimumab) pathway. Early results are showing impressive response rates in advanced melanoma patients with manageable toxicity profiles.

Myeloid-derived suppressor cells (MDSCs) are key elements of the cancer-related inflammation with potential to support tumor growth, invasion and metastatic spread [52]. Apart from MDSCs, also T cell derived subpopulations such as $CD3^+CD56^+$ double-positive natural killer T (NKT cells) and $CD11b^+Gr1^+$ NK cells that are known to support tumor control can also exert a negative impact on the generation of protective anti-cancer immunity [53].

NK cells are a part of the innate immune system which is responsible for the first line of defense against viral and bacterial infections, as well as malignantly transformed cells and tumors. In a similar way to T cells, NK cell subpopulations have a number of immunoregulatory functions and can kill their targets via the secretion of interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα) and T cell recruiting chemokines (RANTES, MIP1α, MIP1β), and the induction of apoptosis via Fas–Fas ligand and TRAIL–receptor interactions, and the release of granzyme B and perforin [54–61]. The recognition of the Fc component of antibodies which have bound to tumor-specific or tumor-associated antigens by NK cells triggers antibody-dependent cellular cytotoxicity (ADCC), and thereby establishes a link between B cell and NK cell-mediated immune responses. In contrast to T cells, NK cells do not express a single clonal receptor type rather they express a large variety of different receptors on one cell. The balance between the expression density of activating receptors with a short immunoreceptor tyrosine-based activation motif and inhibiting receptors with a long intracellular tyrosine-based inhibition motif determines whether an NK cell exerts a cytolytic or inhibitory function. NK cell receptors can be grouped into different families – the immunoglobulin like, C-type lectin (NKG2D, CD94, NKG2A, NKG2C) and natural cytotoxicity (NKp30, NKp44, NKp46) receptors [62]. Membrane Hsp70 serves as a target for activatory C-type lectin NK cell receptors [63].

As indicated above, and in contrast to T cells, pre-activated NK cells have the capacity to recognize membrane-bound Hsp70 on tumor cells even in the absence of immunogenic peptides [64]. We have been able to demonstrate that an incubation of NK cells with Hsp70 protein or an Hsp70-derived peptide (TKD) plus low dose interleukin-2 (IL-2) for 3–5 days [65] can stimulate the proliferative and migratory capacity of NK cells toward highly aggressive Hsp70 membrane-positive tumor cells [22,66]. We have also shown that it can initiate apoptotic cell death in these tumor cells via an increased secretion of granzyme B. A mammalian glycosylation pattern has been found to be key for the internalization of granzyme B into membrane Hsp70 positive tumor cells [40,67–69]. A schematic representation of the stimulation of NK cells with Hsp70 peptide TKD plus low dose IL-2, and also the recognition of Hsp70 membrane-positive tumor cells by pre-activated NK cells, is shown in Fig. 1. This scheme indicates that Hsp70 can be released either actively from viable tumor cells in liposomes or from dying cells as free Hsp70, which might exert immunostimulatory functions. The stimulation of NK cells with TKD/IL-2 is associated with an up-regulation in the expression density of activatory C-type lectin NK receptors such as CD94, NKG2C, and NKG2D [63,66,70,71]. Since the increased expression density of these receptors on NK cells is associated with increased cytolytic activity, the mean fluorescence intensity of these markers serves as a surrogate for NK cell cytotoxicity. Furthermore, tumor cells that have been irradiated or treated with drugs [37] and thus exhibit an increased cell surface expression of Hsp70 [22,72] are lysed significantly better by NK cells that had been stimulated with the pro-inflammatory cytokine IL-2 and the Hsp70 peptide TKD than tumor cells with a lower Hsp70 expression *in vitro* [65] and *in vivo* [73]. In this context, it should be noted that resting NK cells from patients with cancer showed no cytolytic activity against irradiated tumor cells (unpublished observation). Therefore, we speculate that anti-tumor immune responses against membrane Hsp70 positive tumor cells is selectively mediated by activated effector cells of the innate immune system.

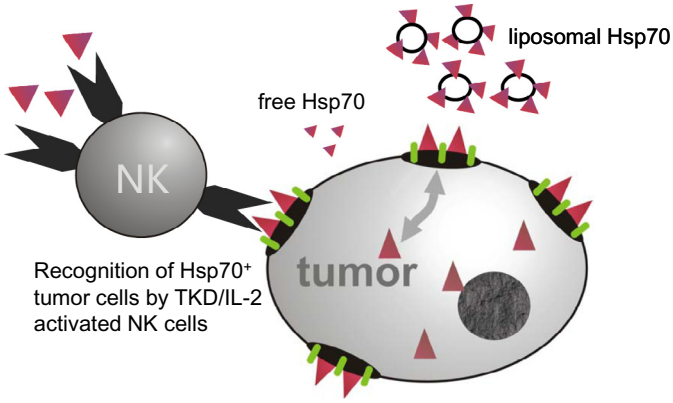

Fig. 1. NK cell stimulation with TKD/IL-2. Schematic representation of the *ex vivo* stimulation of patient-derived NK cells with Hsp70-peptide TKD plus low dose IL-2 (large red triangles) and recognition of membrane Hsp70 positive tumor cells. Upon stimulation, the density of activatory C-type lectin receptors (black symbols on NK cells) such as CD94, NKG2C, NKG2D is upregulated on NK cells. These receptors mediate recognition of membrane Hsp70 on tumor cells, which resides in lipid rafts. Hsp70 is transported from the cytosol to the plasma membrane (double-headed arrow) in tumor cells via a non-ER/golgi transport pathway. Hsp70 can be actively released by viable tumor cells that express Hsp70 on their plasma membrane in lipid vesicles (small circles with small red triangles). Free Hsp70 (small red triangles) is released by dying tumor cells. In general the amount of liposomal Hsp70 from viable tumor cells in the extracellular milieu is much higher than that of free Hsp70 which is released by dying cells. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Promising results demonstrating the capacity of pre-activated human NK cells to induce the regression of Hsp70 membrane positive human tumors in immunodeficient mice [73] prompted the initiation of a phase I clinical trial. In this trial, patients with colorectal and non-small lung cell cancer (NSCLC) who were refractory to therapy and exhibiting widespread metastatic disease were treated with an escalating dose of *ex vivo* TKD/IL-2 activated, autologous NK cells [71]. The study showed an excellent tolerability and safety profile for *ex vivo* stimulated, autologous NK cells in patients even after 6 repeated re-infusions of complete leukapheresis products. Two out of 5 patients showed clinical responses (1 stable disease and 1 mixed response) after only 4 infusion cycles. Despite the low patient numbers, this result was not expected since all of the treated patients had not previously shown any response to standard radio(chemo)therapies. Based on these findings, a clinical phase II proof-of-concept trial involving the administration of *ex vivo* stimulated, autologous NK cells to patients with NSCLC after radio(chemo)therapy is initiated in late 2014 (Ms submitted). For this study, radio(chemo)therapy is administered prior to starting the immune therapy in order to reduce the tumor mass and increase the density of Hsp70 expression on the surface of surviving residual tumor cells.

As indicated earlier, membrane Hsp70 positive tumor cells can actively release Hsp70 in lipid vesicles termed exosomes [66]. As the amount of liposomal Hsp70 is much higher than that of free Hsp70 which is derived from dying cells (Fig. 1), it was proposed that serum levels of liposomal Hsp70 might predict the presence of Hsp70 membrane-positive tumors, *in vitro*. Mouse studies have indicated that serum Hsp70 levels could correlate with the tumor volume and that an irradiation-induced tumor regression was associated with a reduction in Hsp70 serum levels [74]. These findings have now been confirmed in patients with hepatocellular carcinomas (HCC) [9].

Importantly, these studies have shown that Hsp70 levels are significantly higher in the serum of tumor patients compared to healthy individuals and patients with inflammatory diseases such as chronic hepatitis. We therefore propose that serum Hsp70 levels will provide a useful biomarker and a minimally invasive approach for predicting the presence of tumors and for monitoring the outcome of a therapy in the near future.

In addition to acting as a target recognition structure for activated NK cells, Hsp70 can act as a vehicle for the delivery of antigenic peptides to T cells via indirect antigen presentation pathways. It is generally accepted that the primary sequences of HSP70 and HSP90 proteins are unable to activate a T cell-mediated immune response. However, it has been shown that members of the HSP70 and HSP90 family have the capacity to bind immunogenic tumor peptides that can be cross-presented on MHC molecules of APCs [75]. Tumor mouse models have revealed that HSP–peptide complexes isolated from tumor cells, but not those isolated from normal cells, can elicit protective T cell mediated immunity against the same type of tumor from which the HSP–peptide complexes were derived [76]. This concept has also been tested in phase I–III clinical trials in patients with cancer and infectious diseases [77], advanced melanomas [78,79], metastatic colorectal cancer [80], and non-Hodgkin lymphomas [81,82]. The results of the HSP–peptide vaccination trials revealed biological responses such as immune stimulation of T cells and some clinical responses in certain tumor subgroup analyses.

Inhibition of HSP with molecular weights of 90 kDa can induce immunomodulatory activities. Hsp90 inhibition leads to a compensatory increase in cytosolic Hsp70 levels which reduces the antitumor capacity of the Hsp90 inhibitor and induces a release of Hsp70 from tumor cells (unpublished observation). This concept has recently been reviewed by the Garrido group [83]. It is therefore important to develop inhibitors of Hsp70 which can be used in combination with the Hsp90 inhibition. At present a number of new Hsp70 inhibitors that can increase the sensitivity of tumors to therapeutic agents and radiation therapy, and also enhance the efficacy of established Hsp90 inhibitor strategies, have been tested in preclinical models. Aptamers which bind to the peptide-binding and ATP-binding domains of Hsp70, and thereby act as Hsp70 inhibitors, have been shown to trigger anti-tumor immune responses and reduce the growth of subcutaneous B16F10 melanoma tumors *in vivo* via a mechanism which is associated with the recruitment of macrophages and T cells into the tumor bed [84]. Furthermore, a small molecule inhibitor of Hsp70 (Pifithrin-μ) can effectively inhibit the growth of human prostate cancer cells *in vitro*, and increase the anti-tumor effects of hyperthermia in a murine xenograft model [85]. A study by Schlecht and colleagues [86] suggests that the simultaneous inhibition of Hsp70 and the constitutive member of the 70 kDa heat shock family, Hsc70, using siRNA approaches is required in order to reduce the viability of human MD-MB-468 breast carcinoma cells. Despite the plethora of roles that Hsp70 has in the maintenance of cellular homeostasis, Hsp70 inhibitors do not appear to exhibit toxicity in animals [83–85].

Further to the above, Hsp70 can be directly applied as an antitumor therapeutic. Work of the Margulis group has shown that rat glioblastomas can be successfully treated with an intratumoral delivery of exogenous Hsp70 [87]. The reduction in tumor size, as confirmed by magnetic resonance imaging, was associated with a significant infiltration of tumors by NK and T cells, as well as an increased production of granzyme B and interferon-γ. With regard to these promising novel data from pre-clinical studies, a pilot study in children with brain tumors has been initiated. Intratumoral injections of recombinant Hsp70 (up to five times) were well tolerated and one patient out of 12 exhibited a complete clinical response, as documented using radiological imaging [88]. Immunologically, a shift toward an inflammatory Th1 type immune response was observed in the peripheral blood, as was a reduction in the prevalence of Treg cells.

Concluding remarks

Despite enormous progress in radiation technologies (high precision image-guided irradiation, proton irradiation, heavy ion irradiation), and radiotherapeutic concepts (hypofractionated irradiation schemes), the clinical outcome of radiotherapy in locally advanced and metastasized tumors and in hypoxic tumors which are radiation-resistant remains unsatisfactory [89–92]. Given their key influence on a number of biological and immunological parameters, this article considers the influence of irradiation-induced stress proteins on radiation-induced immunomodulation. It is well established that, among other stress factors, irradiation initiates the synthesis of Hsp70 via the production of ROS in eukaryotic cells and causes the translocation of PS from the inner to the outer plasma membrane leaflet [93,94]. Depending on its location, Hsp70 has been found to fulfill multiple roles. On the one hand, increased intracellular Hsp70 levels have been found to play a key role in the recovery from stress [2,95] including radio(chemo)therapy. Therefore, in bladder cancer an inhibition of cytosolic Hsp27 and Hsp70 has been found to exert beneficial effects for radio(chemo)therapy [96].

On the other hand, membrane-bound and extracellular Hsp70 proteins are potent stimulators of the innate immune system [97]. Fractionated radiation (5×2 Gy) enhances the release of Hsp70 from dying tumor cells and thus stimulates immune responses that are mediated by NK cells and dendritic cells [78,98]. When loaded with tumor-derived peptides, members of the HSP70 and HSP90 families also have been shown to stimulate the adaptive immune system via antigen cross-presentation [75–77]. Furthermore, intratumorally applied Hsp70 initiates immune stimulation in patients with brain tumors [87], and the efficacy of radiotherapy and intratumoral dendritic cell therapy in CT26 tumor mouse models has been shown to be boosted by a co-injection with recombinant Hsp70 [99].

Although the mechanism by which Hsp70 is exported is not completely understood, several laboratories have reported on an active release of Hsp70 from cultured viable cells into the supernatant, and this is predominantly in the context of lipid vesicles [97,100–102]. An alternative vesicular pathway for the export of Hsp70 that does not involve the classical ER Golgi compartment has been proposed for the export of liposomal Hsp70 [103]. These findings are in line with other groups who demonstrated that inhibitors which perturb ER Golgi transport, including brefeldin A or monensin do not affect extracellular Hsp70 release [104]. It has been shown that environmental stress, including fractionated irradiation, causes a translocation of Hsp70 from the cytosol into the extracellular milieu, where it can stimulate the innate immune system in the presence of pro-inflammatory cytokines [6]. Furthermore, Hsp70 has been found to co-localize with PS in the outer membrane leaflet of stressed tumor cells [32], whereas PS is restricted to the inner plasma membrane leaflet in non-stressed cells [105]. Given that Hsp70 and PS are co-localized in the membrane of stressed tumor cells, we speculate that Hsp70 is translocated to the outer membrane leaflet by flipping together with PS during the process of early apoptosis or by a fusion of Hsp70/PS containing lipid vesicles with the plasma membrane.

Immunologically, an irradiation-induced enhanced Hsp70 membrane expression on highly aggressive tumor cells could serve as a recognition structure for activated, but not for resting, NK effector cells. Taken together, these findings might have significant future clinical relevance, in that the clinical outcome of irradiation therapy for advanced tumor diseases could be improved by combining it with cell-based and other immunotherapies that target membrane Hsp70.

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Conflict of interest

None.

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