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Invited Review

Relevance of the chaperone-like protein calreticulin for the biological behavior and clinical outcome of cancer

Jitka Fucikova[a,b], Lenka Kasikova[a,b], Iva Truxova[a,b], Jan Laco[c], Petr Skapa[a], Ales Ryska[c], Radek Spisek[a,b,*]

[a] Department of Immunology, Charles University, 2nd Faculty of Medicine and University Hospital Motol, Prague, Czech Republic
[b] Sotio, Prague, Czech Republic
[c] Fingerland Department of Pathology, Charles University Medical Faculty and University Hospital, Hradec Kralove, Czech Republic

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ABSTRACT

The death of cancer cells can be categorized as either immunogenic (ICD) or nonimmunogenic, depending on the initiating stimulus. The immunogenic processes of immunogenic cell death are mainly mediated by damage-associated molecular patterns (DAMPs), which include surface exposure of calreticulin (CRT), secretion of adenosine triphosphate (ATP), release of non-histone chromatin protein high-mobility group box 1 (HMGB1) and the production of type I interferons (IFNs). DAMPs are recognized by various receptors that are expressed by antigen-presenting cells (APCs) and potentiate the presentation of tumor antigens to T lymphocytes. Accumulating evidence indicates that CRT exposure constitutes one of the major checkpoints, that determines the immunogenicity of cell death both in vitro and in vivo in mouse models. Moreover, recent studies have identified CRT expression on tumor cells not only as a marker of ICD and active anti-tumor immune reactions but also as a major predictor of a better prognosis in various cancers. Here, we discuss the recent information on the CRT capacity to activate anticancer immune response as well as its prognostic and predictive role for the clinical outcome in cancer patients.

1. Introduction

The immunogenicity of tumor cells depends on their antigenicity (i.e., the expression of specific tumor antigens) and their adjuvanticity provided by the expression or release of danger-associated molecular patterns or DAMPs [1]. Immunogenic cell death (ICD) involves changes in the cell surface expression profile as well as the release of soluble mediators from the tumor cells and therefore represents a particular way to deliver DAMPs into the tumor microenvironment [1–3]. Moreover, ICD constitutes a prominent pathway for the activation of immune system against cancer, which in turn determines the long-term success of anticancer therapies as documented by numerous reports in last ten years [1,4–9]. To date, four major types of ICD have been described: (1) **ICD driven by immunogenic chemotherapy agents routinely used in the clinic** (doxorubicin, mitoxantrone, oxaliplatin and bortezomib) [4,7,8,10,11]; (2) **ICD activated by physical modalities** (radiotherapy, hypericin induced photodynamic therapy and high hydrostatic pressure) [9,12–17]; (3) **ICD driven by pathogens** [2]; and (4) **necroptotic ICD** [18,19] as described recently in detail by Galluzzi and colleagues [2]. All of these types of ICD rely on the pre-apoptotic activation of several stress response pathways that are associated with the expression and release of DAMPs [5,9,14]. So far, four DAMPs have been described to have essential functions in ICD: (1) pre-apoptotic exposure of CRT and various heat-shock proteins (HSPs) on the outer leaflet of the plasma membrane; (2) the production of type I interferons (IFNs); (3) the secretion of ATP, which relies on the activation of autophagy and (4) the release of the non-histone chromatin-binding protein high mobility group box 1 (HMGB1) into the extracellular space. A detailed overview of these markers is beyond the scope of this review and has recently been extensively described elsewhere [1,20–24]. Among the well described DAMPs, CRT exposure represents one of the major checkpoints [1]. When localized to the ER, CRT is critical for ensuring the proper conformation of proteins and glycoproteins, as well as for the homeostatic control of cytosolic and ER calcium levels [25]. Once translocated to the cell surface, CRT represents a crucial “eat-me” signal and facilitates the engulfment of tumor cells by dendritic cells (DCs), which leads to tumor antigen presentation and tumor-specific cytotoxic T lymphocyte responses [12]. In addition to studies that have documented this role of CRT in experimental mouse models and in vitro human studies, analysis of the

* Corresponding author at: Sotio, Jankovcova 1518/2, 170 00 Prague 7, Czech Republic.
E-mail address: spisek@sotio.com (R. Spisek).

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clinical samples reveals an intriguing prognostic and predictive role of CRT exposure for the clinical outcome of various cancers. Here, we therefore discuss the role of CRT in the ER-mediated stress response, summarize the recent findings on its role in cancer immunosurveillance and review its role as a predictor of the outcome of cancer treatment.

2. Biological functions of CRT

CRT represents an endoplasmic reticulum (ER) Ca^{2+}-binding chaperone that has multiple functions inside and outside the ER [26,27]. Since its discovery in 1974, it has been demonstrated to be present in many other cellular structures including the cytoplasm, the cell membrane and the extracellular matrix [28]. This highly conserved, 46 kDa Ca^{2+}-binding protein consists of three distinct structural and functional domains: [1] a lectin-like globular N-terminal domain that interacts with α-integrins [29] and contains a steroid receptor-like a DNA-binding site [30,31] that plays a role in its chaperone activity; [2] the proline-rich P domain that also takes part in the chaperone activity [32] and is also a high-affinity and low-capacity Ca^{2+}-binding region [33] and [3] the C domain, a highly acidic region that is important for its Ca^{2+}-buffering functions [28,34,35]. These domains are followed by a four-amino acid ER retention sequence (KDEL) at the carboxyl terminus [28]. Within the ER, CRT is involved in the quality control of newly synthesized proteins and glycoproteins by interacting with various other ER chaperones, mainly calnexin and the 57-kDa ER protein (ERp57) in the calreticulin/calnexin cycle [26]. In addition the critical functions of CRT within ER, CRT regulates a variety of diverse and important biological processes when localized intracellularly, on the cell surface and in extracellular compartments [25,28,31,36,37]. To date, CRT has been shown to play important roles in: (1) antigen processing and presentation for adaptive immune response [3,12], (2) phagocytosis of CRT-expressing tumor cells by DCs [38], (3) cell adhesion and migration [39–41], cellular proliferation [39] and thrombospondin 1 (TSP1) mediated local adhesion disassembly (for cell migration) [25,40,42]. Therefore, CRT represents a critical mediator of physiological and pathological processes, such as wound healing, fibrosis, activation of the immune response and cancer [25,28,31,36,37].

3. ER stress and CRT exposure on dying tumor cells

In addition to its functions within the ER, CRT plays a crucial role in the process of immunogenic apoptosis or ICD, which has important implications for the immune systems anti-tumorigenic role [4,12,43,44]. Tumor cells that are undergoing stress, damage or injury, expose CRT on their surfaces or release it into the extracellular space, where it acts as an important immunostimulatory danger signal [1,10]. The immunogenic properties of CRT were first described in 2007 by Obeid and colleagues [12] (Fig. 1). They reported that ecto-CRT, exposed on the surface of cancer cell lines that are undergoing ICD in response to anthracyclines, oxaliplatin, UVC and γ-radiation, facilitates the engulfment of these cells by DCs, which leads to tumor antigen presentation and a tumor-specific cytotoxic T lymphocyte response [12]. Ecto-CRT expressing apoptotic tumor cells, that were implanted into immunocompetent mice are able to elicit an immune response that protects the mice against a subsequent challenge with live tumor cells of the same type in the absence of any adjuvant [12]. However, for the induction of immunogenic apoptosis, the ecto-CRT exposure must be accompanied by additional apoptotic signals, because the presence of ecto-CRT alone is not sufficient to activate the immune response [3,45]. The complete danger-signaling pathway that lead to translocation of CRT to the cell surface in cancer cells that are undergoing immunogenic cell death has been characterized by Panaretakis and colleagues [5] (Fig. 1). When elicited by immunogenic chemotherapy, the exposure of CRT depends on the formation of reactive oxygen species (ROS) and nitric oxide and the activation of ER stress response. Early activation of the ER-sessile kinase PERK leads to phosphorylation of the eukaryotic translation initiation factor EIF2α, which is followed by partial activation of caspase-8. Caspase-8 mediates the cleavage of the ER protein BAP31 and the activation of the pro-apoptotic proteins BAX and BAK. These processes are followed by subsequent anterograde transport of CRT from the ER to the Golgi apparatus and vSNARE-dependent exocytosis to the plasma membrane surface [5]. In contrast, the hypericin-PDT-mediated ER-stress apoptotic pathway that leads to the subsequent translocation of CRT to the cell surface, which was described by Garg and colleagues in 2012, requires only PERK, BAX, BAK and the secretory pathway [9]. Moreover, ERp57, an ER luminal thiol-disulfide oxidoreductase, was not found to be associated with ecto-CRT translocation in hypericin-PDT therapy, as has been shown to be critical for the chemotherapy-mediated translocation pathway [12,46]. Most recently, we dissected the molecular mechanism of ecto-CRT exposure induced by another immunogenic physical modality, high hydrostatic pressure (HHP), we showed that the PERK-dependent eIF2α phosphorylation and subsequent activation of caspase-2 play an important role in the HHP-mediated CRT exposure in both mice and human tumor cells [14]. Moreover, we reported for the first time the critical role of caspase-2 in the CRT trafficking during ICD as indicated by the decreased phagocytosis of HHP-treated sh-caspase-2 tumor cells by DCs. In conclusion, we characterized the molecular mechanism of the ecto-CRT exposure pathway that is induced by HHP, which is more analogous to that induced by anthracycline treatment than to the Hyp-PDT-mediated apoptotic pathway [14]. Importantly, cancer cells with genetic or experimentally enforced defects in the ER stress-associated pathways fail to die in an immunogenic manner in response to the described ICD inducers [1,5]. Moreover, multiple strategies and approaches were identified to convert non-immunogenic instances into ICD, which has possible clinical implementations with respect to the use of combinatorial immuno(chemo)regimens that efficiently promote ICD and therefore mediate complete tumor regression in patients [47]. Consistent with this statement, the knockdown of CRT completely abolishes the immunogenicity of cell death induced by multiple ICD inducers and conversely, the absorption of recombinant CRT to the surface of cells that succumb to non-ICD inducers can restore the immunogenicity of cell death [5,12,48].

4. Perception of CRT exposure by immune effectors

Surface-exposed CRT interacts with various receptors on the surface of innate immune cells, including pattern recognition receptors (PRRs) especially toll-like receptors (TRLs); phagocytosis or scavenger receptors (CD91); purinergic receptors such as P2 × 7R or P2Y2R and the nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) [25]. However, CRT is best characterized for its prominent function as an **"eat-me" signal,** that promotes the uptake of cell corpses and debris by APCs [10,38,49]. The surface translocation of CRT occurs before the cells expose phosphatidylserine (PS) on the outer leaflet of the plasma membrane [10]. However, although both ecto-CRT and PS serve as "eat-me" signals, only CRT is intimately linked to ICD [50,51]. Furthermore to provide phagocytic functions, ecto-CRT has been found to interact with various proteins such as (1) thrombospondin [52]; (2) complement component 1, q subcomponent (C1q); (3) mannose-binding lectin (MBL) [53] and especially (4) CD91 (LDL-receptor-related protein or LRP) on the professional phagocytes, which stimulates Rac-1 and drives the engulfment of apoptotic cells [9]. The crucial role of CD91 in the function of phagocytes has been demonstrated by evidence that shRNA-dependent downregulation of LRP1 reduces the immunogenicity of cancer cells during hypericin-based PDT [9]. LPR1 also represents the main ER chaperone receptor and is expressed not only by human but also by mouse myeloid cells [38,54], and macrophages that lack LRP1 exhibit limited phagocytic potential [55]. Moreover, in human cells, it has been shown that LRP1 silencing through RNA interference or an LRP-1 targeted monoclonal antibody can inhibit the activation of DCs by immunogenic tumor cells [56,57].