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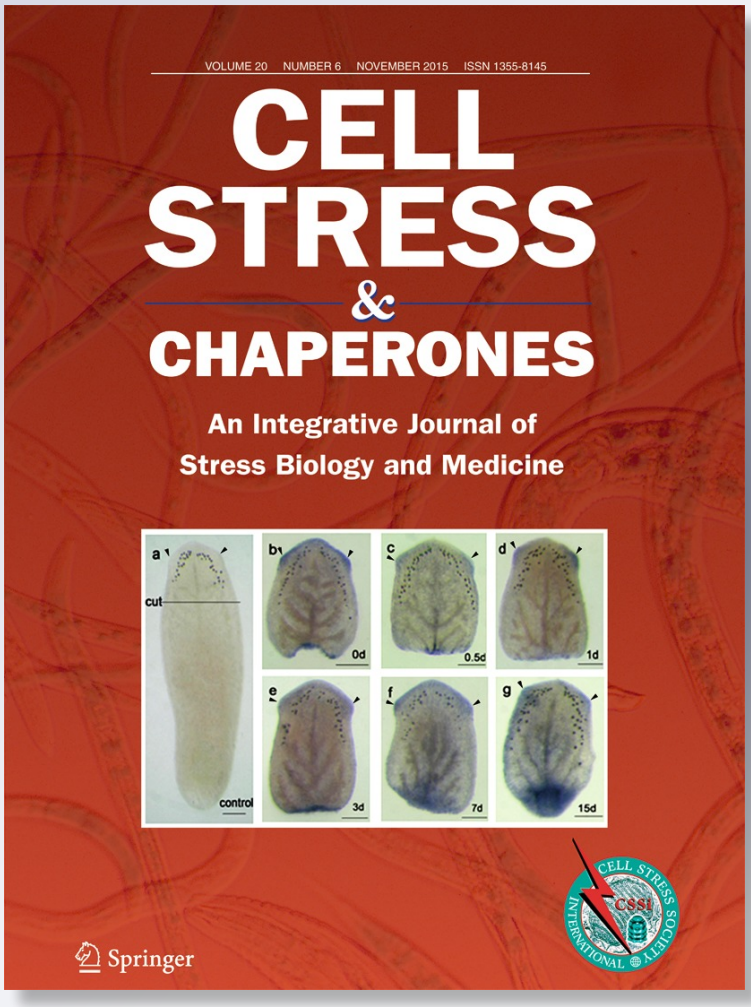

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MEETING REVIEW

Unfolding the complexities of ER chaperones in health and disease: report on the 11th international calreticulin workshop

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Abstract The 11th International Calreticulin workshop was held May 15–18, 2015 at New York University School of Medicine-Langone Medical Center, New York. The meeting highlighted many of the new discoveries in the past 2 years involving the important role of molecular chaperones in physiological and pathological processes. Crucial to the understanding of these disease processes was the role of chaperones in maintaining quality control of protein processing in the endoplasmic reticulum, the importance of Ca^2 regulation acting through its action in stress-related diseases, and the trafficking of glycoproteins to the cell surface. Central to maintaining healthy cell physiology is the correct ER-associated protein degradation of specific misfolded proteins. Information on different mechanisms involved in the degradation of misfolded proteins was revealed. This was a landmark meeting for the chaperone field in terms of new insights into their roles in physiology. These insights included the unfolded protein response, innate/adaptive immunity, tissue repair, the functions of calreticulin/chaperones from the cell surface, and extracellular environment. Diseases included neurodegenerative disorders, prion disease, autoimmunity, fibrosis-related disease, the host immune response to cancer, and hematologic diseases associated with calreticulin mutations. The 12th calreticulin workshop is planned for the spring of 2017 in Delphi, Greece.

Keywords Calreticulin · Cancer modulation · ER associated protein degradation · ER stress · Glycoprotein folding · Unfolded protein response · Immune regulation · Wound healing

Introduction

The International Calreticulin Workshop derives its name from a chaperone–calreticulin (CRT) initially described in 1974 by D.H. MacLennan (Toronto) as a high affinity Ca^{2+} binding acidic protein (HACBP) in the skeletal muscle sarcoplasmic reticulum. That was an end to it or so many thought, the description of the protein located in one organelle with an important role in Ca^{2+} homeostasis. Driven by a passion for this protein, Marek Michalak took on the mantle of "Übermensch" and organized the first international CRT workshop held in Banff, Canada in 1994, 20 years after the initial discovery of CRT. By the mid-1990s, the protein's intracellular location and important biological functions had expanded exponentially (see Fig. 1). CRT has matured into a full-fledged chaperone located in the endoplasmic reticulum (ER) where it oversees glycoprotein folding for protein relocation to the cell surface or for export. Currently, CRT has evolved into a multifunctional protein with evidence of its presence in other organelles. The former discovery of the involvement of CRT in MHC class I protein assembly highlighted its crucial role in antigen presentation and hinted at the protein's additional roles in immunopathology. CRT remains a sophisticated regulator of Ca^2 homeostasis and storage both in and outside of the ER and is

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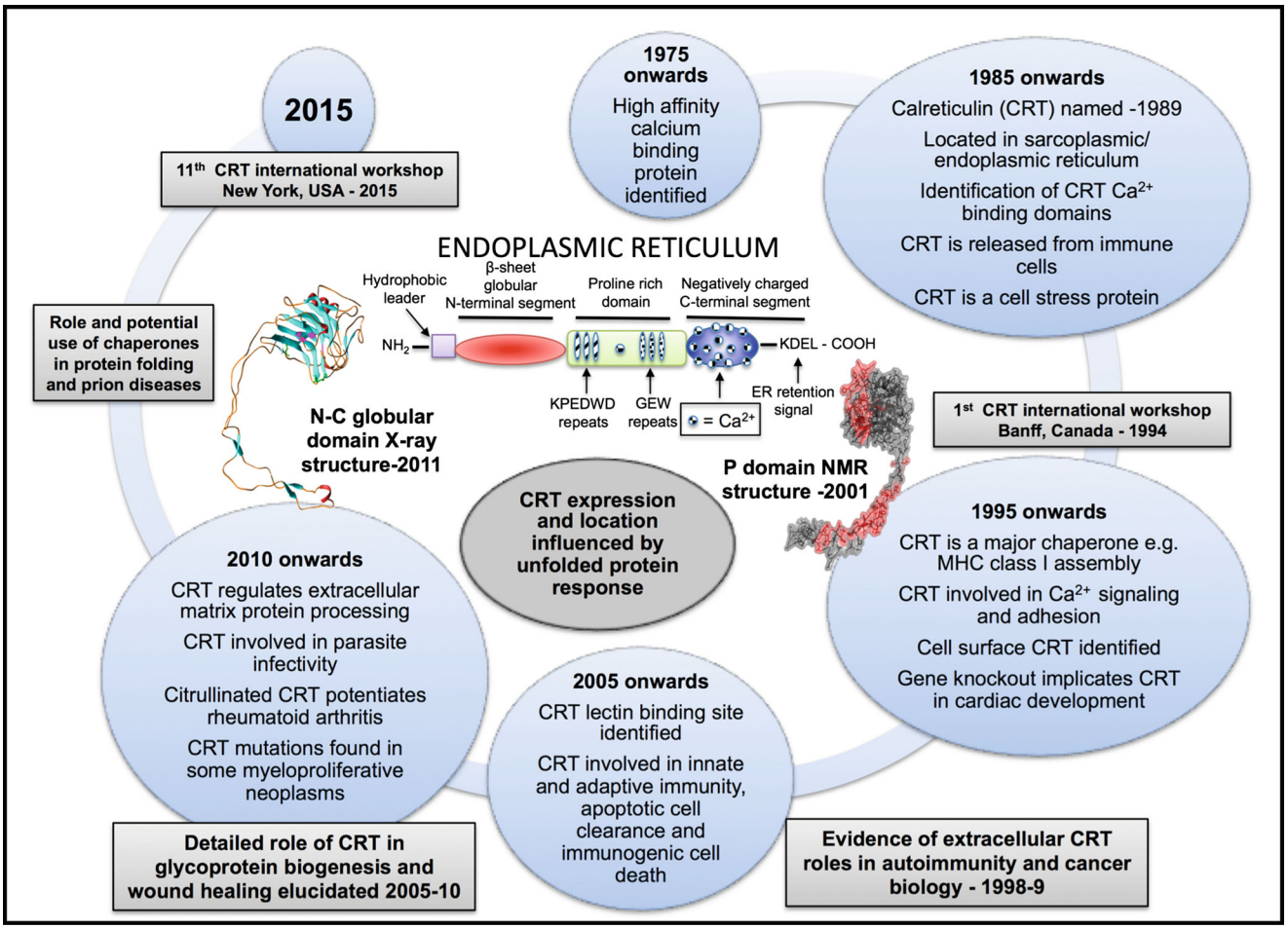

The evolution of calreticulin: a chaperone and cell stress protein

Fig. 1 A chronology of the "evolution" of calreticulin from a Ca^{2+} binding protein to a complex chaperone involved in multiple aspects of cell development, physiology, and pathology. Many of the novel functions of CRT and other chaperones have been revealed at the International CRT workshops over the past 21 years

overexpressed in the presence of stress such as heat (i.e., heat shock response) and heavy metals.

The underlying theme of the 11th CRT workshop was the role of CRT and other chaperones in immunity, tissue regeneration, neurodegenerative diseases and cancer, and its related therapeutic potential. Many of the functions and pathways now described involve the interaction of extracellular chaperones with cells, a theme that was highly contentious in previous workshops. In this meeting, there was an expanding interest in the evolving extracellular roles of CRT and its associate molecules in the amiable environment hosted by the local organizer Leslie Gold (New York, USA) and her colleagues. However, Marek Michalak (U of Alberta, Edmonton, Canada) set the tone in his keynote address, reminding us how the ER oxidoreductase ERp57/PDI3 exquisitely responds to cell stress to affect ER Ca^{2+} homeostasis and how PDIA6 impacts on ER stress coping responses together with microRNA-322 (MiR-322). He described how disruption of ER Ca^{2+} homeostasis can reduce MiR-322 levels, which in turn can regulate the unfolded protein response (UPR), an ER stress coping response. Normally, when the UPR is activated, the cell makes one of two choices; prepare for death or alleviate the damage and restore homeostasis and normalization of cell function. A third way was proposed in which the cell averts cell death but is retained in a constant state of flux, with ER stress being consistently adjusted leading to metabolic dysfunction and risk of organ damage. A potential role of cyclosporine and other ER associated oxidoreductases in the regulation of UPR and ER homeostasis was also discussed. This set the scene for the meeting that focused on the role of chaperones in diverse health and disease processes. Thus, outside the ER as presented herein, in its newer exciting role in functioning from the cell surface inward, an explosion of crucial functions has emerged for CRT and other chaperones. As research in this field unfolds, a plethora of new questions remains, many of which have not been answered. These queries were discussed in a final session of this workshop.

Session I: Calreticulin biochemistry, ER quality control and calcium homeostasis

The first session revealed new insights into some of the most established functions of chaperones, namely protein folding, ER quality control, and proteostasis in disease states. The first speaker, Maurizio Molinari (Institute for Research in Biomedicine, Switzerland), described a new quality control checkpoint for proteins with a defective charged transmembrane segment but an otherwise correctly folded ER luminal domain. Using a chimera of α_1-antitrypsin fused to the negatively charged transmembrane segment of CD3δ, it was shown that this protein did not succumb to canonical ER-associated protein degradation (ERAD) mechanisms but was retained in the ER in a BiP—and calnexin (CNX)—independent manner. Retention in a post-ER compartment was mediated by interactions with the cytosolic AAA-ATPase p97 and luminal quality control factor, UDP-glucose:glycoprotein glucosyltransferase (UGGT1). Depletion or inhibition of these components permitted export of the chimera along the secretory pathway. These studies suggest a potential means, whereby proteins with minor structural defects that retain function may be rescued from quality control rejection in certain disease situations. Daniel Hebert (University of Massachusetts, USA) shifted the focus to chaperone-assisted folding pathways. Using influenza hemagglutinin and neuraminidase as examples, he explained how the location of Asn-linked glycans and their interactions with the lectin-chaperones CNX and CRT delay the folding of N-terminally located polypeptide segments, thereby allowing time for critical distal segments to be synthesized. Then, it was shown that successful folding of the nonviral serpin protein, antithrombin III was involved in the recruitment of lectin chaperones to N-terminal N-glycans to delay the formation of two disulfides located therein. This provides time for a C-terminal disulfide to form, thereby lessening the probability of formation of incorrect disulfide intermediates with cysteines synthesized earlier during translation. These studies explain both how protein folding defects can arise and how they can be ameliorated.

A critical property of neurodegenerative disease is the dysfunctional biogenesis, folding, trafficking and degradation of proteins within brain cells. Using a mutant superoxide dismutase 1 (SOD1) animal model of amyotrophic lateral sclerosis (ALS), Claudio Hetz (U of Chile) showed that knocking down two arms of the unfolded protein response (UPR), Xbp1 and ATF4, prolonged survival of the animals. With the Xbp1 knockout, by including small molecule enhancers of autophagy, an increase in autophagy was shown to be protective. Surprisingly, transgenic expression of spliced Xbp1 was also protective, presumably due to enhanced production of chaperones and ERAD components. Four ALS-linked mutations were identified in two protein disulfide isomerase (PDI) genes, PDAI1 and PDIA3/ERp57, which were subsequently phenotypically characterized and shown to be associated with altered motoneuron branching. In addition, specific deletion of ERp57 in the murine nervous system led to severe motor dysfunction. Notably, PDI and ERp57 thiol oxidoreductases are upregulated in the SOD1 mouse and in ALS patients. Collectively, the data identifies ER proteostasis imbalance as a risk factor in ALS. Jody Groenendyk (U of Alberta, Canada) described that increased expression of CRT in adult mouse heart is associated with severe cardiac fibrosis. Gene expression profiling revealed increased expression of collagens, pro-inflammatory cytokines, TGF-β1, and the downstream Smad2/3 transcription factors, known to transactivate collagen, fibronectin, integrins, and other adhesive/migration-related genes. Remarkably, tauroursodeoxycholic acid (TUDCA; inhibitor of ER stress) treatment reduced TGF-β1 mRNA and profoundly reduced cardiac fibrosis in mice. These studies link impaired ER proteostasis to activation of the TGF-β1/Smad2/3 signaling pathway, which may lead to abnormal thickening of the heart valves and cardiac fibrosis.

Session II: unfolded protein response (UPR), neurodegenerative diseases, and cell death

Neuronal cells have a nonessential cellular protein PrP-C on their surface, while scrapie prions possess a pathogenic isoform of PrP-C (PrP-Sc) associated with neurodegenerative disease. David Williams (U of Toronto, Canada) discussed a new therapeutic concept to prevent the aggregation of scrapie prion protein. Interestingly, these aggregates require the interaction of PrP-Sc with PrP-C, and elimination of PrP-C prevents pathological aggregate formation. Thus, he proposed targeting the biogenesis of PrP-C to block its cell surface expression by preventing a necessary biosynthetic step, namely proline isomerization by peptidyl prolyl isomerase (PPI). This was achieved using immunosuppressive drugs such as the PPI inhibitor, FK506, or by silencing a particular PPI, the ER resident immunophilin, FKBP10. The involvement of FKBP proteins in PrP-C translocation was shown, and FKBP10 was designated as a potential therapeutic target for prion disease. The UPR from ER stress adaptively responds to resolve protein misfolding in the ER via three transmembrane sensors, IRE1α, ATF6α, and PERK, each with independent signaling pathways. Randal Kaufman (Sanford-Burnham Medical Research Institute, USA) explained that certain types of liver disease involve UPR activation. Hepatic ER stress involves a sequential pathology beginning with nonalcoholic fatty liver disease (NAFLD) that progresses to inflammatory liver disease (NASH) and, finally, hepatocellular carcinoma (HCC). Disrupting lipid homeostasis in the liver of mice by knocking down the IRE1α pathway caused a defect in very low-density lipoprotein (VLDL) secretion, leading to lipid overload in hepatocytes. This defect is associated with a decrease in PDI-1

concomitant with loss of lipid microsomal transfer protein (MTP) complex, disrupted triglyceride assembly into nascent VLDL, and secretion of ApoB that is insufficiently saturated with lipids. Prevention of lipid accumulation in hepatocytes and thus ER stress is a distinct function of pathways involved in the UPR. Jeffrey Kelly (Scripps Research Institute, USA) discussed the chaperone-mediated folding and assembly of transthyretin (TTR) tetramers. TTR is predominantly found in the serum and cerebrospinal fluid and acts as a carrier for hormones and vitamins. These tetramers exist in two states with differing stability based on side chain packing/mobility. The more stable state can be promoted by the Hsp70-Hsp40 chaperone pathway. The link between chaperone-mediated protein folding and chaperone-mediated kinetic stability of the folded tetramer elucidated a new concept that has implications for protein folding diseases, including the amyloidosis that can occur with aggregated TTR. Interestingly, differences in a protein whether folded in the cold or at room temperature, and spontaneously versus biologically, were found to affect the time to protein inherent crystallization. This subtlety provides information about the physics of protein aggregation/polymerization in the pathogenesis of "protein folding" disease. Jeffrey Brodsky (U of Pittsburgh, PA, USA) described the individual steps in ERAD, namely recognition, ubiquitination, retrotranslocation, and degradation. Yeast provides a powerful system to study each of these steps and determine which factors ("chaperones") are necessary for individual steps and the particular stress responses involved. Examples of proteins with these unique requirements include the epithelial sodium channel, CFTR, and the potassium channel, Kir2.1. The importance of the uniqueness of each of these proteins and the diseases that they can cause when mutated is well known. The perplexing question is how to identify luminal and cytosolic chaperones that contribute to ERAD selection for these biochemically different disease-associated mutated proteins including the transcriptional responses associated with each chaperone system.

Session III: ER stress and cancer, CRT, and the immune response to cancer

Cell stress and especially ER stress have long been considered a means by which cells tolerate and adapt to environmental insults to prevent cell death. CRT in its Ca^{2+} storage capacity and regulation of Ca^{2+} levels and signaling is believed to play a role in maintaining cell survival. Lorrie Kirshenbaum (University of Manitoba, Canada) presented data explaining the regulation of cardiac cell death and survival. Often due to loss of cardiac cell function, cardiovascular disease remains a major cause of morbidity. Notably, a side effect of Doxorubicin (DOX) treatment of cancer patients is a higher rate of heart failure. The observation that cardiac myocytes in mice treated with DOX display disrupted mitochondria led to the discovery that DOX disrupts protein complexes in the mitochondrial respiratory chain. Using a yeast 2—hybrid screen with Bcl-2—Bnip3 was identified. Bnip3 (not present in the ER) is increased by hypoxia and induces cardiac cell death by provoking mitochondrial cell death with features of apoptosis and necrosis. Interestingly, NIPLET, a splice variant of Bnip-3 containing the Delta exon 3 (transmembrane domain), is cytoprotective. The studies showed that DOX initiates the loss of mitochondrial uncoupling protein 3 and cytochrome c oxidase via a mechanism dependent on Bnip3, ultimately leading to necrosis. In contrast, NIPLET contains the C-terminal ER retention signal that suppresses mitochondrial permeability transition pore opening and cell death. These data implicate a novel intrinsic survival mechanism for cardiomyocytes involving BNIP3 splice variants and mitofusin 2 (bridges mitochondria to ER). Interestingly, NIPLET is highly expressed in certain cancers, which when knocked down results in cell death. Immunotherapy is currently a popular topic as a new understanding of immune-regulated tumoricidal activity is rapidly emerging. The ability of macrophages to target tumor cells for cell death and removal is referred to as programmed cell removal (PrCR), as described by Mingye Feng (Irving Weissman's Lab, Stanford University, CA, USA). Critical to this process is the presence of CRT on the surface of macrophages and the downregulation of the "don't eat me" signal, CD47 from the cell surface of the cancer cell. Activation of macrophage Toll-like receptor (TLR3, 4, 7) signaling via Bruton's tyrosine kinase (Btk) increases trafficking of CRT to the cell surface of macrophages and together with the decrease in CD47 from the cancer cell surface is involved in live tumor cell killing by PrCR. The immune potency of CRT for anticancer therapy was further revealed by Edwin Bremer (University Medical Center Groningen, the Netherlands) who presented data indicating that exogenous CRT binds directly to granulocytes and monocytes (mainly PMNs), inducing cell survival and triggering the innate immune response characterized by the release of numerous proinflammatory cytokines that in turn promoted adaptive immunity implemented by clonal expansion of antigen-specific T cells and tumor cell killing by dendritic cells (DCs). CRT-mediated innate and adaptive immunity can potentially be exploited for anticancer therapy. One of the complex issues concerning how chaperones can modulate immunity in tumor pathological conditions in vivo was presented by Nasrin Mesaeli (Weill Cornell Medical College, Doha, Qatar), who is employing Tie2-CRT transgenic mice to focus on the analysis of endothelial cell-specific gene targeting for lung cancer therapy.

Session IV: chaperone functions at the cell surface

Several chaperones including CRT are often referred to as "alarmins," comprised of endogenous molecules that are released from cells and elicit various immune responses.

Chaperones can be released from cells via environmental and drug-induced signals by nonclassical secretory pathways and following necrosis. Patrizia Agostinis (KU Leuven, Belgium) discussed her finding on the role of CRT in immunogenic cell death (ICD) in the context of cellular responses to chemotherapy and anticancer vaccines. Anticancer immunotherapies, based on the concept of ICD, rely on induction of ER stress with accompanying increased exposure of cell surface CRT to trigger "eat me" signals. ICD was presented as being organized into Type I and Type II, which were characterized in the context of three modules (ER stress, apoptosis, and translocation). Certain chemotherapies mobilize CRT to the surface as a part of their effectiveness in tumor cell killing and accordingly, failure to induce cell surface CRT was associated with reduced tumor cell susceptibility to ICD and failure of anticancer cell vaccination approaches. In fact, a retrospective meta-analysis showed that high tumoral CRT expression level is predictive of positive responses to ICD-inducing therapy, such as radiotherapy and paclitaxel, in lung and ovarian cancer patients. Importantly, a threshold level of CRT in the ER is required for its translocation to the cell surface, and ER stress is an apparent driver, which is supported by partial prevention of tumor cell death by TUDCA. Incubation of cancer cells with recombinant CRT greatly increases phagocytosis and responses to anticancer vaccines and thereby defines another level for the therapeutic potential of CRT. The perpetuation of ICD is dependent on the presentation of tumor cell antigens by DCs to the appropriate T cell. Karl-Gösta Sundqvist (Karolinska Institute, Stockholm, Sweden) addressed the role of cell surface CRT in opposing functions of T cell adhesion and motility versus T cell activation. Endogenous thrombospondin-1 (TSP-1) together with the CRT-LDL receptor-related protein 1 (LRP1) co-complex and CD47 provides a mechanism for integrated regulation of motility, adhesive contacts, and T cell activation by different plasma membrane cell surface receptors and downstream signaling pathways. Interestingly, antigen stimulation of T cells induces upregulation of TSP-1 via T cell receptors and CD28, concomitantly downregulating LRP1 expression, which converts the TSP1 signal to a pro-adhesive signal, which is immunosuppressive. A peptide from CRT, which binds TSP-1, increases T cell-antigen presenting cell binding to decrease T cell motility. It was suggested that the TSP-1 axis could be exploited as a target for therapy of inflammatory diseases.

Session V: ER chaperones and disease pathogenesis

Chaperones with intact protein structure are essential "housekeeping" proteins that maintain the normal physiological working of the cells. Discoveries underlying mutations in Janus Kinase 2 (JAK2) that are associated with myeloproliferative neoplasms are now complemented with mutations in the CRT gene (all in exon 9, consisting of deletions or insertions, but not point mutations) recently discovered by Tony Green (University of Cambridge, UK). These disorders, arising in the hematopoietic stem cell compartment, are experimentally tractable, permit clonal analysis, and enable the study of early stages of tumorigenesis. Among the best characterized hematological malignancies, JAK2 mutations occur in 90 % of myeloproliferative neoplasms. Mutant CRT activates the MAPK pathway in murine 32D cells and also undergoes proteasomal degradation. Although gain-of-function mutations in JAK2 provide clues for the pathogenetic mechanism, mutations in the CRT gene are just beginning to be understood in terms of the mechanism(s) of action (see below). Nevertheless, the newly discovered CRT mutations in patients with nonmutated JAK2 implicate a new cancer gene linking ER and cancer biology. From a therapeutic perspective, the discovery of alterations in JAK2 has rapidly led to the successful clinical use of JAK inhibitors in myeloproliferative neoplasms. Similarly, mutant CRT has the potential to be a tumor-specific therapeutic target because the mutations are in the C-terminus, converting this region from acidic to basic, as well as modifying its localization in the ER, as the KDEL motif is affected. Insight into mechanisms of action of CRT mutants in myeloproliferative neoplasms was described by Stefan Constantinescu (Ludwig Institute for Cancer Research and University of Louvain, Belgium). Using mouse embryonic fibroblasts, his group showed different subcellular localization of transfected mutant CRT with del52 or ins5 compared to transfected wild-type CRT. In addition, using protein fragment complementation, it was shown that CALR (gene encoding CRT) mutants induce dimerization of the C-terminal ends of JAK2 kinase causing its activation. This requires recruitment and activation by CALR mutants of a cytokine receptor in the secretory pathway and on the cell surface. The latter are dependent on the presence of N-glycosylation sites on the receptor and the novel CALR sequence derived from the frame-shift. Thus, the novel CALR mutants become oncogenic proteins by their ability to induce persistent activation of the JAK-STAT pathway in myeloid progenitors.

The intestine is a harsh environment requiring stringent protection from stress and cell death. Luis Agellon (McGill University, Canada) presented an overview of ER coping responses that are very active in intestinal tissues. For example, the intestine is regularly exposed to large amounts of diet-derived lipids; our Western diets contain high amounts of fat composed largely of palmitic acid, a known and potent inducer of ER stress. The small intestine contains three distinct fatty acid binding proteins (Fabps), which exhibit asymmetric distribution along the length of the intestine. Accumulating evidence indicates that Fabps cooperate in order to protect enterocytes of the small intestine from ER stress, caused by fatty acid overload, so that they can function properly. Increased hepatic glucose production and defective

hepatocyte insulin signaling contribute to the two key features of type 2 diabetes (T2D), hyperglycemia, and insulin resistance. Ira Tabas (Columbia University, NY, USA) presented an overview of his recent work on Ca^{2+} signaling and ER stress in T2D with a link to obesity (associated with T2D). One mechanism of increased hepatic glucose production is the increase in the ratio of glucagon to insulin signaling in hepatocytes. Glucagon-activated protein kinase A in hepatocytes activates inositol trisphosphate receptor (IP_3R) and phospholipase C (PLC) thereby leading to an increase in Ca^{2+}. Elevated cytosolic Ca^{2+} activates the phosphatase, calcineurin and the kinase, and Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII). By different mechanisms, calcineurin and CAMKII lead to nuclear translocation of the transcription factors CREB-regulated transcription coactivator 2 (CRTC2) and FoxO1 respectively, involved in gluconeogenesis and glycogenolysis. This Ca^{2+} activated pathway is associated with high hepatic glucose in obesity. CaMKII also triggers a separate signaling pathway that activates the PERK branch of the UPR. The downstream effector ATF4 induces the gene Trb3, which causes a defect in hepatocyte insulin signaling thereby contributing to insulin resistance in obesity. Furthermore, ATF4, via inducing another factor, exacerbates adipose tissue inflammation, an important metabolic defect associated with obesity. Activation of PERK by CAMKII involves a histone de-acetylase and a co-repressor, which were confirmed in liver biopsies from obese individuals. The common upstream CaMKII node that regulates both insulin resistance and high hepatic glucose pathways may offer novel therapeutic targets for T2D.

Session VI: chaperones as therapeutics

The translocation of CRT and other chaperones to the cell surface has been shown to enhance both the innate and adaptive immune response to cancer cells including increasing the sensitivity of cancer cells to chemotherapies. In the session, Encouse B. Golden (Silvia Formenti's Lab, New York University School of Medicine, USA) discussed the effect of radiotherapy on the immune response to cancer aside from direct killing of cancer cells by radiation. The discovery that radiotherapy enhanced the immune response to cancer stemmed from the observation of "the abscopal" effect meaning the (immune-mediated) shrinking of tumors distant to the radiation site. Subsequently, this effect appears to be mediated by radiation-induced exposure of CRT on apoptotic cancer cells, which enhances the cross-priming phase of the immune response by DCs to T cells. Employing clinically relevant doses of radiation used for breast cancer combined with carboplatin or paclitaxel, it was shown in vitro using a mouse cell line that radiotherapy produced both a dose-dependent induction and chemotherapeutic enhancement of ICD, as measured by the three components of cytotoxic agent-induced stress namely, cell surface exposure of CRT, and release of HMGB1 and ATP. These results suggest that ICD stimulated by either high dose radiotherapy alone or concurrent chemotherapy may establish a pro-immunogenic tumor microenvironment. The radiotherapy effect on immune response was continued by Sofia Gameiro (Laboratory of Tumor Immunology and Biology, NCI, MD, USA) who stated that a common problem of sublethal doses of radiotherapy, devised to minimize toxicity to normal cells, results in the survival of tumor cells that foster disease progression. This NIH group examined the molecular and immunogenic consequences of radiation and docetaxel exposure in multiple human carcinomas and investigated the possible mechanisms that augment antigen-specific cytotoxic T lymphocyte (CTL)-mediated lysis of surviving cancer cells. It was shown that whereas only radiation induced ICD, both treatment modalities induced immunogenic modulation in various tumor cell types, rendering the surviving cells more susceptible to CTL killing. Augmented CTL lysis specific for several cancer cell antigens was largely dependent on cell surface expression of CRT, ER stress, and the UPR (PERK). In addition, exogenous CRT also increased CTL killing and did so in the absence of irradiation. The direct interaction of exposed CRT with CTLs has important implications for immunotherapy and can be a marker indicating response to immunotherapy.

Gabriela Chiosis (Memorial Sloan Kettering Cancer Center, NY, USA) focused on the ER glucose-regulated protein 94 (GRP94), a molecular chaperone often overexpressed in tumors and associated with poor survival. She stated that under stress, chaperones become biochemically "rewired" from their housekeeping role to a stress role with its own respective proteome. Exploiting this difference as a unique therapeutic opportunity, a chemical genetics in silico approach was taken using the inherent molecular flexibility paralogs of Hsp70 inhibitors (e.g., geldanamycin) to identify small molecule hits that inhibit Grp94 activity by blocking its ATP-binding site. Pharmacologic inhibition of HER-2+, EGFR+, and triple negative breast cancers (TNBC) was obtained with these inhibitors. The main advantage of these compounds is that they directly bind to tumor tissue and not normal cells as only cancer cells have cell surface GRP94. As GRP94 is vital for functioning of receptor tyrosine kinase receptors (RTKs), inhibitors of this chaperone are also proposed as a novel target therapy for the treatment of cancers, particularly those that function via RTK signaling.

Session VII: ER chaperones in immunity and host defense

After several iterations of chaperone-assisted folding attempts, misfolded proteins in the ER enter the ER-associated

degradation (ERAD) pathway. In antigen presenting cells (APC)s, some components of the ERAD machinery are localized to phagosomes where they are thought to translocate antigen to the cytosol, facilitating MHC class I-restricted cross-presentation of antigens by DCs to CD8-positive T cells. This is crucial for the induction of adaptive immunity. To investigate the translocation mechanism, Peter Cresswell (Yale University, USA) described the development of a fluorescence complementation assay that utilizes a split version of the Venus protein that becomes fluorescent only after deglycosylation in the cytosol. Although it was yet not possible to use the system for the analysis of cross-presentation, the probe worked successfully to investigate the ERAD pathway. Using an siRNA genome wide screen, three novel proteins potentially involved in ERAD (TMUB2, TMTC4, TFP12) were identified as well as known numerous ERAD components. To confirm the ERAD requirement for the known and unknown candidates, the CRISPR/CAS9A technique was used to knock out the genes encoding these proteins in HEK 293 T cells. This was still in progress, but interestingly, ablation of the known ERAD genes Hrd1, HERP, or AUP1 did not affect cell viability or the rate of cell division. For the adaptive immune response, CRT is an integral component of the major histocompatibility complex (MHC) class I peptide loading complex (PLC) essential for antigen processing and cross presentation. Using computational and experimental approaches including amino acid substitution mutants of the CRT molecule, Malini Raghavan (University of Michigan, MI USA) showed that the heretofore uncharacterized ATP-binding site on CRT is located within its globular domain in proximity to the high affinity Ca^{2+}-binding site. Furthermore, these studies identified an important regulatory role for the ATP-binding site in calcium binding, in CRT-substrate interactions, and in the stability of the PLC. ATP binding destabilizes CRT interaction with monoglucosylated substrates. Conversely, mutants that affect ATP binding reduce MHC class I cell surface expression, prolong CRT binding to cellular MHC Class I, and stabilize the interaction of CRT with monoglucosylated forms of MHC-I within the PLC. Robert Binder (University of Pittsburgh, PA, USA) focused on the heat shock protein (HSP) family of chaperones, particularly on HSP70, gp96 and 90, with specific attention to their role in tumor immunosurveillance. Studies from the Binder lab demonstrate that HSPs can provide a crucial amplification pathway for antigen presentation since they target the efficient endocytic and signaling receptor CD91/LRP1. The tumor antigen peptides are approximately eight amino acids and bind directly to MHC class I molecules. HSPs are on the surface of cancer cells and also in the tumor microenvironment following cancer cell death. Tumor cell-derived peptides chaperoned by HSPs ligate CD91/LRP1 on DCs (APCs), which increases p38 and NFκB intracellular signaling thereby raising CD86 and CD40 cell surface antigens along with HSP/peptides for recognition by NK cells and T cells located in lymph nodes. Using knock-out models and a T cell read-out of cytokine release, it was demonstrated that the LRP1 on DCs is essential for adaptive immune responses involving T cells to enable tumor cell killing. It was shown that the presence of chaperone increases the efficiency of T cell cross-priming by five orders of magnitude. In contrast to smaller tumors with the presence of low antigen plus HSPs to transfer to DCs, larger tumors, apparently releasing an overwhelming amount of HSPs target DCs generating regulatory T cells through expression of neuropillin thereby dampening the immune response.

Session VIII: ER chaperones phagocytosis and autoimmunity

Antibodies to CRT and other ER chaperones are produced in autoimmune-related diseases implicating a role for chaperones in autoimmunity. In previous meetings, Joseph Holoshitz (University of Michigan, USA) reported a role for cell-surface CRT in rheumatoid arthritis (RA) pathogenesis. The RA "shared epitope" (SE) sequence is a five amino acid sequence present on disease associated human leukocyte antigen (HLA) class II DR molecules. Interactions of this epitope with cell surface CRT triggers innate immune signaling. More recent studies using a collagen-induced arthritis model showed that a cell-free synthetic SE ligand mimetic increased osteoclast abundance in joints, enhanced bone damage, and caused severe arthritis in mice. QKRAA was agonistic, while DKCLA was antagonistic. Two experimental approaches were used to find mimetics with antagonistic effects to block SE-CRT interaction for inhibition of SE signaling in vitro as a potential therapeutic modality for arthritis: (1) a rationally designed SE antagonistic peptidomimetic library (SEAL) and (2) small molecule virtual library screening. SAR studies showed that Glu257 in CRT P domain interacts with SE for antagonistic effect, whereas Asp209, Glu223, and Glu223 interact for an agonistic effect. Two lead compounds have shown potent SEAL activity in vitro and in vivo (reduced the number of osteoclasts in the joint in a dose/response manner and delayed onset of arthritis in mice). The possibility that citrullinated modified isoforms of CRT that bind to the SE of HLA DR $B1^{+ve}$ subjects was highlighted by Paul Eggleton (University of Exeter Medical School, UK). He proposed that patients with lung bronchiectasis (BR) that have from 2 to 5 chronic bacterial infections per year cause ER stress. He suggested that these clinical conditions can manifest in citrullinated CRT being released from cells into the circulation. Citrullinated CRT detected in the serum of some patients with bronchiectasis progressed to developing RA. High CRT autoantibody and extracellular CRT titers were present in the plasma of patients with BR with and without RA. Interestingly, these patients also have a greater abundance of the shared epitope (SE), and it has been shown by Holoshitz

and colleagues that citrullinated CRT binds to SE. These studies suggest that BR is a chronic lung disease that may be the initial trigger for developing RA, via a CRT-dependent pathology. Exogenous and cell surface CRT activate macrophages and is considered the universal mediator of phagocytosis of apoptotic cells. Similarly, CRT is important in the phagocytosis of both dead and live cancer cells by DCs, as described in this report. However, CRT effects on phagocytosis of bacterial and macrophage bactericidal activities are not known. Rekha G Panchal (USAMRIID, MD, USA) showed that recombinant CRT induces the expression of pro-inflammatory and immunomodulatory cytokines by mouse and human bone-marrow derived macrophages and induces NF-κB nuclear translocation. Genes induced by CRT in macrophages are associated with the innate immune response and specifically related to the host defense against bacteria, such as antimicrobial peptide (AMP). Preliminary studies suggest that exogenous CRT induces macrophage reactive oxygen species (ROS) and killing of *Klebsiella pneumonia* and methicillin-resistant *Staphylococcus aureus* (MRSA). Earlier studies by Arturo Ferreira (Faculty of Medicine, University of Chile) showed that *Trypanosoma cruzi* (*T. cruzi*) CRT (TcCRT), the causative agent of Chagas Disease, is located on the parasite surface at the site of flagellum emergence, where it recruits host complement component C1q to enhance parasite infectivity. Interestingly, patients infected with *T. Cruzi* demonstrate tumor regression, and it was shown that TcCRT is antiangiogenic, inhibits proliferation, migration, and capillary morphogenesis, in vitro and in vivo, and inhibits mammary adenocarcinoma growth. TcCRT has higher antiangiogenic activity than human CRT as it interacts indirectly with C1qR on endotheliocytes and directly with scavenger receptors. Anti-TcCRT antibodies block the antitumor effects of *T cruzi* infections, providing a molecular link between *T. cruzi* infections and tumor protection.

Session IX: chaperones in adhesion, fibrosis, and wound healing

CRT, other chaperones, and HSPs have been shown to be involved in wound healing and fibrosis of different organs. However, little is known about the molecular level functions and signaling pathways involved in these processes. This session presented new data in this important area of translational medicine. Joanne Murphy-Ullrich (University of Alabama at Burlignton, USA) showed that TGF-β-induced collagen I expression was attenuated in vascular smooth muscle cells (VSMCs) isolated from mice with floxed CRT alleles and transfected with cre-recombinanse to knock-down CRT. Furthermore, TGF-β stimulation of type I collagen production was shown to be mediated by calcineurin-dependent NFAT dephosphorylation. In vivo, using a mouse carotid artery ligation injury model, high expression of CRT was observed in the neointima. Both neointima formation and ECM collagen deposition were reduced in arteries where CRT levels were knocked-down by a unique locally targeted plasmid delivery method. These data provide a novel role for CRT in mediating neointimal formation through regulating collagen I production following arterial injury. A consequence of diabetes is poor wound healing manifested as diabetic foot ulcers, a global serious unmet medical need for patients without access to hyperbaric oxygen therapy and one with no effective topical treatments. Wei Li (University of Southern California, USA) discovered that a secreted form (via exosomal release) of Hsp-90α enhances wound healing in vivo in mice essentially by mediating re-epithelialization through the induction of keratinocyte migration, shown in vitro. It was further shown that this activity resides in a 115 amino acid fragment of Hsp90α, fragment 5 (F5), an outside loop shown by the crystal structure. F5 binds to LRP1 and signals via the PI3K/AKT pathway. F-5 is being developed for clinical application of various types of wounds. Chronic kidney disease often involves renal fibrosis concomitant with upregulation of CRT. Using a mouse model of unilateral ureteric obstruction to induce renal fibrosis, with subsequent 2D-gel proteome analysis of fibrotic tissue, Aristidis Charonis (Academy of Athens, Greece) showed that CRT was upregulated primarily in tubular epithelial cells during the early stages of disease development. Overexpression of CRT in cultured tubular epithelial cells increased the expression of several protein families: type IV collagen, fibronectin, vinculin, SNAIL, and the 14-3-3 proteins. By ChIP analysis, the NR5A2 transcription factor was implicated in transcriptional regulation of CRT, and NR5A2 was required to be sumoylated for promoter activity. Importantly, elevated CRT levels have been found in tubular epithelial cells in IgA nephropathy kidney biopsies. This observation suggests that CRT and the molecules involved in its upregulation may constitute potential targets for antifibrotic intervention.

Michal Opas (University of Toronto, Toronto, Canada) uses embryonic stem (ES) cells as models to study the signaling pathways of cell differentiation. He showed that the absence of CRT in 3T3L1 cells influenced ES differentiation toward adipogenic lineages. This is achieved through a negative feedback loop, by which the initial stimulation of CRT by peroxisome proliferator-activated receptor γ2 (PPARγ) negatively regulates the expression of PPARγ, lipoprotein lipase, CCAAT enhancer-binding protein α, and aP2α. In this way, CRT functions as a Ca^{2+}-dependent molecular switch that regulates commitment to adipocyte differentiation. Inversely, overexpression of CRT suppresses adipogenesis and switches the cell toward an osteogenic lineage by a mechanism involving the transcription factor Runx2. CRT regulates the threonine kinase, GSK3β and tyrosine phosphatase SHP-2, which have negative or positive effects, respectively, via their effect on STAT1, which normally retains Runx2 in the cytosol but translocates to the nucleus to induce osteogenesis. The

presence or absence of CRT in lineage determination in terms of its ER chaperone activity is interesting to pursue.

Posters

Over 20 poster presenters showcased their data in brief oral presentations covering a wide range of topics, from embryonic development, protein purification, stem cell development, and various chaperone mouse models. Several presentations focused on *Trypanosoma cruzi*, ER stress, and quality control and were recurring topics in the poster session. CNX-deficient mice conferred significant resistance to developing multiple sclerosis in an experimental autoimmune encephalomyelitis model. Other posters described point or frame shift mutations in CRT that were recently identified to be involved in myeloproliferative neoplasms and MHC I antigen presentation. Numerous posters focused on the role of CRT during stem cell differentiation and epithelial to mesenchymal transition, key events during cardiogenesis. In vitro, exogenous CRT induced the expression of collagen, fibronectin, and other matrix proteins in fibroblasts, thereby explaining in part how topical application of CRT healed wounds in a diabetic mouse model. Finally, a poster showed the importance of the Ca^{2+} capacity of the ER and ER-resident proteins in the regulation of STIM1, a transmembrane protein that senses ER Ca^{2+} concentration and transmits this information to the plasma membrane to trigger store-operated Ca^{2+} influx.

Conclusion

The first CRT international workshop in 1994 focused on the structure and function of the CRT protein. The recent 11th workshop highlighted additional complexities of CRT, other chaperones, and HSPs within and outside the cell. Particularly notable and revealed at the workshop was the exquisite complexity of the ERAD system. Not only are misfolded proteins responsible for many diseases, but how chaperones deal with unfolded proteins appears to be a more refined and complex response than originally thought. This meeting brought together a variety of expertise within the basic science and clinical community to further our knowledge of CRT and other chaperones in the fields of cell differentiation, hematological disorders, extracellular matrix-related fibrotic disorders, wound healing, heart disease, diabetes, the host immune response to cancer, and immunity and autoimmunity. We greatly look forward to the continued research in these areas but will have to wait until the 12th International Calreticulin Workshop to be held in Delphi, Greece in 2017.

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