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Imiquimod-induced autophagy is regulated by ER stress-mediated PKR activation in cancer cells

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ABSTRACT

Background: Autophagy is a highly conserved cellular catabolic pathway for degradation and recycling of intracellular components in response to nutrient starvation or environmental stress. Endoplasmic reticulum (ER) homeostasis can be disturbed by physiological and pathological influences, resulting in accumulation of misfolded and unfolded proteins in the ER lumen, a condition referred to as ER stress. Imiquimod (IMQ), a Toll-like receptor (TLR) 7 ligand, possesses anti-tumor and anti-viral activities in vitro and in vivo.

Objective: IMQ has been reported to promote the apoptosis of THP-1-derived macrophages through an ER stress-dependent pathway. However, the role of ER stress in IMQ-induced autophagy is unknown. In this study, we investigated the relationship between ER stress and IMQ-induced autophagy.

Methods: The expression of LC3, P62, p-PERK, Grp78, p-elF2α and IRE1α proteins were determined by immunoblotting. The relationship between ER stress and IMQ-induced autophagy were analyzed by ER stress inhibitors, a PERK inhibitor and the genetic silencing of PERK. The role of double-strand RNA-dependent protein kinase (PKR) activation in IMQ-induced autophagy was assessed by inhibiting PKR and genetically silencing PKR. The IMQ-induced autophagy was evaluated by immunoblotting and EGFP-LC3 puncta formation.

Results: IMQ induced reactive oxygen species (ROS) production in cancer cells. Additionally, IMQ markedly induced ER stress via ROS production and increased autophagosome formation in a dose- and time-dependent manner in both TLR7/8-expressing and TLR7/8-deficient cancer cells. Pharmacological or genetic inhibition of ER stress dramatically reduced LC3-II expression and EGFP-LC3 puncta formation in IMQ-treated cancer cells. IMQ-induced autophagy was markedly reduced by depletion and/or inhibition of PKR, a downstream effector of ER stress.

Conclusion: IMQ-induced autophagy is dependent on PKR activation, which is mediated by ROS-triggered ER stress. These findings might provide useful information for basic research and for the clinical application of IMQ.

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1. Introduction

Endoplasmic reticulum (ER) homeostasis can be disrupted by physiological and pathological conditions, resulting in accumulation of misfolded and unfolded proteins, referred to as ER stress [1]. Under ER stress conditions, activation of the unfolded protein response (UPR) alleviates the unfolded protein load through several pro-survival mechanisms, including expansion of the ER membrane and selective synthesis of key components of the protein-folding and quality control machineries [2]. In response to ER stress, three critical transmembrane ER signaling proteins are activated to initiate adaptive responses for restoring ER homeostasis. These signal transducers are the protein kinases inositol-requiring kinase 1 (IRE1) [3,4] and double-stranded RNA-activated protein kinase-like ER kinase (PERK) [5] as well as the transcription factor activating transcription factor 6 (ATF6) [4,6]. IRE1 is released from Kar2p/BiP and undergoes homodimerization and trans-autophosphorylation to activate its RNase activity for selective splicing of XBP1 mRNA, which targets UPR-related gene expression. PERK attenuates mRNA translation by phosphorylating eukaryotic translation initiation factor 2 (eIF2) at Ser51, and eIF2 subsequently activates ATF4. After ATF6 is sequentially cleaved by site-1 and site-2 proteases, the processed forms of ATF6 translocate to the nucleus and bind to ER stress-response element-1 (ERSE-1) to promote the expression of target genes under stress conditions. When cells experience irreversible ER stress, the chronic accumulation of unfolded proteins triggers an ER stress-related apoptotic (programmed cell death) response [1]. Many diseases are caused by the mutations of chaperones or protein foldases that disrupt protein-folding pathways [7].

Autophagy is an evolutionarily conserved process in which damaged organelles or macromolecules are packaged into acidic isolation membrane (autophagosome/lysosome) for bulk degradation under stressful conditions. Autophagy can promote cell survival or death, and its mechanisms have been fully elucidated. Several studies have demonstrated correlations between ER stress and autophagy: ER stress triggered autophagy initiation via IRE1-mediated kinase activity. Through this activity, autophagy serves as a prosurvival mechanism that removes damaged organelles under conditions of nutrient starvation [8]. Excessive polyglutamine 72 repeat (polyQ72) aggregates stimulate ER stress (PERK/eIF2a phosphorylation)-mediated cell death, and LC3 conversion, an essential step in autophagosome formation, has a protective effect against polyQ72-induced cell death [9]. However, stimulation of ER stress can induce autophagic cell death upon glucosamine treatment in human glioma cancer cells [10]. PERK stimulates the expression of the transcription factors ATF4 and CHOP to activate LC3 and Atg5 protein expression in response to hypoxia [11]. These data suggest that autophagy plays important roles in cell survival and cell death after ER stress activation.

Imiquimod (IMQ), a TLR7 agonist, is nucleotide-like imidazoquinoline family and possesses both anti-tumor and anti-viral activity in vitro and in vivo[12–14]. IMQ is presently used as a noninvasive topical therapeutic agent for the treatment of superficial basal cell carcinoma, and IMQ also serves as an effective clinical antagonist for the treatment of viral warts and other skin lesions [12–14]. IMQ promotes innate immune response by directly invoking CCL2-dependent recruitment of plasmacytoid dendritic cells (pDCs) and transforming DCs into a set of "killer DCs" to eliminate tumor cells. IMQ also triggers anti-tumor immunity by activating tumor-specific cytotoxic T cells to induce killing of tumor cells in TLR7-dependent pathways [15,16]. IMQ induced autophagic cell death in a basal cell carcinoma cell line (BCC/KMC1) and in colon cancer-derived Caco-2 cells [17,18]. IMQ also promotes the Bcl-2-mediated translocation of cytochrome c to the cytosol and caspase-dependent apoptosis through the intrinsic apoptotic pathway in vitro and in vivo [19,20]. Indeed, IMQ exerts its anti-tumor activity not only by inducing apoptosis but also by activating autophagy to eliminate tumor cells.

In our previous study, we demonstrated that IMQ simultaneously induced autophagy and apoptosis in human basal cell carcinoma cells [18]. IMQ has been reported to promote the apoptosis of THP-1-derived macrophages through an ER stress-dependent pathway [21]. Another recent study has indicated that IMQ-induced apoptosis of melanoma cells is mediated by ER stress-dependent induction of Noxa and is enhanced by NF-κB inhibition [22]. However, the role of ER stress in IMQ-induced autophagy is unknown, and its mechanism of action is not well understood. In this study, we investigated the relationship between ER stress and IMQ-induced autophagy. We found that IMQ markedly induced ER stress through reactive oxygen species (ROS) production and increased autophagosome formation in both TLR7/8-expressing and TLR7/8-deficient cancer cells. IMQ also activated double-stranded RNA-dependent protein kinase (PKR), a downstream effector of ER stress, to promote autophagy progression. These findings support a mechanism of IMQ-induced autophagy and provide novel evidence demonstrating that IMQ can induce TLR7-independent autophagy progression.

2. Materials and methods

2.1. Reagents and antibodies

Imiquimod (IMQ, R837) was obtained from InvivoGen (San Diego, CA, USA). N-acetyl-L-cysteine (NAC), GSK2606414, C16 and 4-phenylbutyrate were obtained from Sigma (St. Louis, MO, USA). Antibodies specific to PERK, phospho (p)-PERK Thr980, PKR and SQSTM1/p62 were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibodies specific to eIF2α, p-eIF2α Ser52, GADD153/CHOP, Grp78, IRE1α and p-PKR Thr451 were purchased from Santa Cruz (Santa Cruz, CA, USA). The antibody specific to LC3 was purchased from Novus Biologicals (Littleton, CO, USA).

2.2. Cells and culture conditions

Our studies included the human basal cell carcinoma cell line BCC/KMC-1 which was established as previously described [23]. Human gastric adenocarcinoma cell line AGS were cultured in RPMI medium. Human melanoma cell lines A375 was maintained in MEM medium. All mediums were supplemented with 10% FBS and all cells were incubated at 37 °C, 5% CO_2.

2.3. Immunoblotting

Cells were harvested, washed twice with PBS, and then collected by centrifugation. Cells were lysed by PRO-PREP protein extraction solution (iNtRON, Kyungki-Do, Korea). Cell lysate extracts were vigorously shaken at 4 °C for 15 min, followed by centrifugation. The supernatants were collected, and the protein concentrations were determined using Bio-Rad assay reagent. A 30-μg sample of each lysate was subjected to electrophoresis on a SDS-polyacrylamide gel. Then, the samples were transferred to PVDF membranes. After blocking, the membranes were incubated with primary antibodies in TBST at 4 °C overnight. Then PVDF membranes were washed four times and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-mouse or anti-rabbit IgG (Upstate, Lake Placid, NY, USA) in TBST at 4 °C for 6 h. After washing four times, the membranes were incubated for 5 min with ECL Western blotting reagent (Pierce Biotechnology, Rockford, IL, USA), and chemiluminescence was detected by exposing the membranes to Kodak X-OMAT film for 30 s to 30 min.

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