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FADD regulates NF-κB activation and promotes ubiquitination of $cFLIP_L$ to induce apoptosis

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Tumor Necrosis Factor-α canonically induces the activation of NF-κB and associated gene product cellular FLICE-like inhibitory protein ($cFLIP_L$) to promote cell survival. Previously, we demonstrated that ectopic expression of the Fas associated death domain (FADD) diminishes the expression of $cFLIP_L$ and transduces caspases-8 mediated apoptosis, independent of FasL stimulation in HEK 293T cells. However, the underlying molecular mechanism of FADD mediated ablation of cFLIP and NF-κB signaling to determining the fate of cell death or survival remains elusive. Here, we explored a novel molecular mechanism of FADD mediated apoptotic cell death that was directed by ubiquitination of $cFLIP_L$ and inhibition of NF-κB activation, independent of TNF-α stimulation. We found that induced expression of FADD firmly interacts with procaspase-8 and precludes $cFLIP_L$ to from the death inducing signaling complex (DISC). In addition, FADD negatively regulates cellular inhibitor of apoptosis protein 2 (cIAP2) and Bcl-2. Furthermore, FADD restrains cIAP2 expression and interacts with RIP1 and procaspase-8 to accomplish apoptotic cell death signaling. Interestingly, FADD was also found to promote JNK1 mediated activation of E3 ubiquitin ligase ITCH to degrade $cFLIP_L$ that may lead to commencement of apoptosis. Thus, FADD is an important regulator for determining the fate of cell death or survival.

Fas associated death domain (FADD) is a pivotal signaling component of death receptor (DR) mediated apoptosis. DRs such as Fas (CD95/Apo) and tumor necrosis factor receptor 1 (TNFR1) (p55/CD120a), belongs to the TNF receptor super family that contain cytoplasmic death domain (DD) to execute downstream signal transduction[1]. Upon binding of ligand to the cell surface receptors, the DD of cell surface receptor homophilically interacts with the DD of FADD and induces oligomerization of DED (death effector domain) of FADD with apical caspases such as, procaspase 8/10 to form a death-inducing signaling complex (DISC)[2]. In the downstream, DISC facilitates processing and catalytic activation of caspases-8/10 to transduces downstream signaling of apoptosis[3]. However, the catalytic activation of caspase-8/10 has been negatively regulated by the anti-apoptotic protein Cellular Flice like inhibitory protein (cFLIP) to abrogate apoptotic instigation[4]. Although FADD is a multifunctional protein and its Fas ligand mediated proapoptotic function has been well studied[5,6]. However, the cellular dynamics of FADD and cFLIP in the regulation of cell death and survival by TNFR signaling remains elusive. TNF receptor (TNFR) signaling elicits both non-apoptotic and apoptotic response by the formation of two sequential complexes depending upon the stimulation of the TNF-α. The components of complex I constituted with TRADD, TRAF2, cIAPs and RIP1 activates NF-κB signaling for promoting cell survival. However, the subsequent dissociation of RIP1 from complex I and association with FADD and procaspase-8 initiates formation of pro-apoptotic complex II that substantiates apoptotic cell death[7]. Although, TNF-α augments the activation of transcription factor NF-κB in tumor cells and promotes cell proliferation by impeding apoptosis[8]. The TNF-α-induced NF-κB activation confers upregulation of several anti-apoptotic genes such as *Bcl-xL*, *A1/Bfl-1*, (*c-IAP)1/2*, X-chromosome-linked IAP (*XIAP*; also known as *hILP*) and *cFLIP*[9–11]. Surprisingly, upregulation of $cFLIP_L$ also provides strength to TNFR1 signaling for stability of complex I formation and constitutive NF-κB activation along with cell proliferation and survival[12,13]. Indeed, the constitutive activation of NF-κB suppresses immune surveillance in both adaptive and innate response and supports tumorigenesis, cancer chemoresistance and chronic inflammation[14–16]. Moreover, the growth and cell survival connecting signaling pathways such as

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Expression of cFLIP$_L$ Determines the Basal Interaction of Bcl-2 With Beclin-1 and Regulates p53 Dependent Ubiquitination of Beclin-1 During Autophagic Stress

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ABSTRACT

Autophagy and apoptosis are two different physiological processes, which is required for the maintenance of cellular homeostasis. The apoptosis associated proteins such as Bcl-2 and p53 have a close association with autophagic proteins HMGB1 and Beclin-1 to modulate autophagic signaling. We demonstrate here the involvement of anti-apoptotic protein cFLIP$_L$ in the regulation of autophagy during cellular stress. We found that ectopic expression of cFLIP$_L$ decreases the sensitivity of HEK 293T cells against rapamycin and H_2O_2 induced autophagic stress. Notably, the selective knockdown of cFLIP$_L$ augments autophagic stress in the cells accompanied with JNK1 activation and p53 dependent ubiquitination of Beclin-1. However, re-expression of cFLIP$_L$ in cFLIP knockdown cells restores autophagic equilibrium collectively with reversible effects on JNK1 and Beclin-1 integrity. The co-immunoprecipitation analysis suggests that cFLIP$_L$ is essential to maintain the canonical interaction of Bcl-2 with Beclin-1 to regulate autophagic stress and cell death. Altogether, our findings suggest that expression of cFLIP$_L$ regulates the basal interaction of Bcl-2 with Beclin-1 and substantiates p53 dependent ubiquitination of Beclin-1 during autophagic stress to determine the fate of cell death or survival. J. Cell. Biochem. 9999: 1–12, 2016. © 2015 Wiley Periodicals, Inc.

KEY WORDS: cFLIP; AUTOPHAGY; APOPTOSIS; OXIDATIVE STRESS; UBIQUITINATION

Autophagy is an evolutionarily conserved process in multicellular organism to maintain cellular homeostasis. Autophagy generally referred to as "self-eating" process that tends to remove the unwanted cytosolic constituents and transporting them to the lysosome. Basal level of autophagy helps to maintain homeostasis in normal condition, while during stress autophagy also supports cell survival [Bergamini et al., 2004; Levine and Klionsky, 2004]. Consequently, a defects in autophagy has been associated with various diseases, including neurodegenerative disorder, diabetes, aging, infection, autoimmunity, and cancer [Levine and Kroemer, 2008; Livesey et al., 2009; Choi et al., 2013]. In the setting of cancer, the cytoprotective process of autophagy mitigates the induction of apoptosis (type I cell death) to enhance tumor cell growth [Ouyang et al., 2012]. Indeed, the process of autophagy initiates with the formation of a double–membrane structure known as the autophagosome that engulfs the toxic cellular components and subsequently degrade them by using lysosomal machinery [Mizushima et al., 2010]. However, the formation of excessive autophagosomes may induce cell death (type II cell death) [Kuma et al., 2004; Yousefi et al., 2006]. The cellular process of autophagy and apoptosis are critically regulated by various sets of gene responsible for cell death and survival [Levine and Yuan, 2005]. For instance, the interaction between anti-apoptotic protein Bcl-2 and the autophagic protein Beclin-1 regulates the formation of autophagosome during autophagic stress [Pattingre et al., 2005; Maiuri et al., 2007; Oberstein et al., 2007]. The interaction of Bcl-2 with Beclin-1 get dissociated during stress induced cellular response [Pattingre et al., 2005; Marquez and Xu, 2012]. Although, some earlier reports advocate that during stress condition the nuclear binding protein HMGB1 translocates to the cytosol and dissociates Bcl-2 from Beclin-1 to sustain autophagy [Kang et al., 2010; Tang et al., 2010a]. However, the involvement of anti-apoptotic regulators

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RESEARCH ARTICLE

Apoptotic potential of Fas-associated death domain on regulation of cell death regulatory protein cFLIP and death receptor mediated apoptosis in HEK 293T cells

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Abstract Fas-associated death domain (FADD) is a common adaptor molecule which plays an important role in transduction of death receptor mediated apoptosis. The FADD provides DED motif for binding to both procaspase-8 and cFLIP molecules which executes death receptor mediated apoptosis. Dysregulated expression of FADD and cFLIP may contribute to inhibition of apoptosis and promote cell survival in cancer. Moreover elevated intracellular level of cFLIP competitively excludes the binding of procaspase-8 to the death effector domain (DED) of FADD at the DISC to block the activation of death receptor signaling required for apoptosis. Increasing evidence shows that defects in FADD protein expression are associated with progression of malignancies and resistance to apoptosis. Therefore, improved expression and function of FADD may provide new paradigms for regulation of cell proliferation and survival in cancer. In the present study, we have examined the potential of FADD in induction of apoptosis by overexpression of FADD in HEK 293T cells and validated further its consequences on the expression of pro and anti-apoptotic proteins besides initiation of death receptor mediated signaling. We have found deficient expression of FADD and elevated expression of $cFLIP_L$ in HEK 293T cells. Our results demonstrate that over expression of FADD attenuates the expression of anti-apoptotic protein cFLIP and activates the cascade of extrinsic caspases to execution of apoptosis in HEK 293T cells.

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Keywords Apoptosis · Fas associated death domain (FADD) · cFLIP · Death receptor mediated apoptosis

Abbreviations

DED	Death Effector Domain
DISC	Death-Inducing Signaling Complex
cFLIP	Cellular fas-associated death domain-like interleukin-1-β- converting enzyme-inhibitory protein
CHX	Cycloheximide
CD 95L	CD 95 ligand

Introduction

Apoptosis is a programmed mechanism of cell death that is essential for proper embryonic development and tissue homeostasis. Apoptosis is mainly triggered by activation of caspases through complex signaling, which include death receptor (extrinsic) and mitochondrial dependent (intrinsic) (Taylor et al. 2008; Vaux et al. 1994). The death receptor mediated apoptosis is initiated by binding of death inducing signals to their cognate receptors at the cell surface which trigger the signals for activation of initiator and effector caspases for cell death (Holler et al. 2003). The death receptor mediated apoptosis is mainly contributed by group of receptors like CD95, TRAIL-R1 and TRAIL-R2 which belongs to the tumor necrosis factor-1 (TNF-1) receptor superfamily-1. These receptors have a characteristic death

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Research Communication

Regulation of HA14-1 Mediated Oxidative Stress, Toxic Response, and Autophagy by Curcumin to Enhance Apoptotic Activity in Human Embryonic Kidney Cells

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Abstract

An alteration in susceptibility to apoptosis not only contributes to promotion of malignancy but can also enhance drug resistance in response to anticancer therapies. HA14-1 is a small molecule which has the potential of inducing apoptosis in cancerous cells. HA14-1 manifests an antagonistic effect on antiapoptotic protein Bcl-2 and consequently induces cell death in various cancerous cell lines. However, it is also known to generate ROS and toxic response in the cells upon decomposition. Elevated level of ROS is responsible for oxidative stress and other pathological consequences, if not metabolized properly. The aim of the present study was to examine the synergistic effect of curcumin in promoting apoptosis by regulating the HA14-1 mediated ROS generation, toxicity, oxidative stress, and autophagy in human embryonic kidney cells. Our study demonstrates that curcumin efficiently scavenges HA14-1 mediated generation of ROS and toxic response resulting in augmentation of apoptosis in HEK 293T cells by promoting inhibition of antiapoptotic proteins and process of autophagy. Thus curcumin along with HA14-1 regulates cell proliferation by disruption of the antiapoptotic signaling mechanism. This approach could serve as a promising strategy for therapeutic potential to overcome their adverse effects. © 2013 BioFactors, 00(00):000–000, 2013

***Keywords:** curcumin; HA14-1; ROS; cytotoxicity; oxidative stress; autophagy; apoptosis*

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***Abbreviations:** ROS, reactive oxygen species; MMP, mitochondrial membrane potential; LC3, microtubule-associated protein light chain 3; LDH, lactate dehydrogenase; SOD, superoxide dismutase.*

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1. Introduction

Apoptosis is one of the most important mechanism of cell death. Alteration in apoptosis not only contributes to the promotion of malignancy but can also enhance drug resistance in response to anticancer therapies [1]. Over the last decade, major attention has been focused on the regulation of apoptosis during various pathological conditions. Ethyl-2-amino-6-bromo-4-(1-cyano-2-ethoxy-2-oxoethyl)-4H-chromene-3-carboxylate (HA14-1), is a small cell permeable organic molecule that exhibits antiproliferative potential [2–4]. HA14-1, alone or in combination with other anticancer agents have been shown to induce apoptosis effectively in different types of cancer cells [5].

HA14-1 induces apoptosis by inhibiting Bcl-2 function [6]. It acts by inhibiting the Bcl-2/Bax interaction, resulting in the loss of mitochondrial membrane potential and leading to an