c-Jun NH₂-terminal kinase (JNK)/stress-activated protein kinase-associated protein 1 (JSAP1) attenuates curcumin-induced cell death differently from its family member, JNK-associated leucine zipper protein (JLP)

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SUMMARY Curcumin, a major component of turmeric, is known to exhibit multiple biological functions including antitumor activity. We previously reported that the mitogen-activated protein kinase (MAPK) scaffold protein c-Jun NH₂-terminal kinase (JNK)-associated leucine zipper protein (JLP) reduces curcumin-induced cell death by modulating p38 MAPK and autophagy through the regulation of lysosome positioning. In this study, we investigated the role of JNK/stress-activated protein kinase-associated protein 1 (JSAP1), a JLP family member, in curcumin-induced stress, and found that JSAP1 also attenuates curcumin-induced cell death. However, JSAP1 knockout showed no or little effect on the activation of JNK and p38 MAPKs in response to curcumin. In addition, small molecule inhibitors of JNK and p38 MAPKs did not increase curcumin-induced cell death. Furthermore, JSAP1 depletion did not impair lysosome positioning and autophagosome-lysosome fusion. Instead, we noticed substantial autolysosome accumulation accompanied by an inefficient autophagic flux in JSAP1 knockout cells. Taken together, these results indicate that JSAP1 is involved in curcumin-induced cell death differently from JLP, and may suggest that JSAP1 plays a role in autophagosome degradation and its dysfunction results in enhanced cell death. The findings of this study may contribute to the development of novel therapeutic approaches using curcumin for cancer.

Keywords autophagy, lysosome, MAP kinase

1. Introduction

Curcumin, a polyphenolic compound extracted from turmeric (Curcuma longa), has been reported to have antitumor activity in various types of cancer (1). Curcumin is known to have a hormetic character, in which at lower concentration it possesses antioxidant activity but induces reactive oxygen species (ROS) at higher concentration (2). The antitumor activity of curcumin seems to be attributed to its ability to induce ROS, mitogen-activated protein kinase (MAPK) activity, and/or apoptosis (3-5). Autophagy, a lysosome-dependent intracellular degradation system, is regarded as one protective mechanism against a curcumin-induced stress (3,6). Cargo transport in autophagy is often mediated by c-Jun NH₂-terminal kinase (JNK)-interacting proteins (JIPs), a family of MAPK scaffold proteins, which can also function as motor adaptors. These include JNK/stress-activated protein kinase-associated protein 1 (JSAP1, also known as JIP3) (7,8) and the structurally related JNK-associated leucine zipper protein (JLP, also known as JIP4 or SPAG9) (9,10).

JSAP1 has been identified as a scaffold protein for the JNK MAPK signaling pathway (7,8), as well as an adaptor for kinesin-1 and dynein motor proteins (11-13). Hill et al. (14) reported that Unc-16 (an ortholog of JSAP1 and JLP in Caenorhabditis elegans) is involved in the transport and clearance of autophagosomes. In mammalian neurons, Gowrishankar et al. (15) demonstrated that JSAP1 plays a role in axonal transport of lysosomes and that its dysfunction causes axonal accumulation of organelles, including...
lysosomes. In addition, recent human genetic studies strongly suggested that JSAP1 mutation is associated with several neurological disorders, such as spastic diplegia and intellectual disability (16,17). Despite mainly expressed in neurons, several studies have reported that JSAP1 plays an important role in cancer cells (18,19).

We recently showed that JLP modulates p38 MAPK and autophagy in response to curcumin, leading to an overall protective effect from curcumin-induced cell death (4). To date, however, whether JSAP1 is involved in curcumin-induced stress remains unknown. In this study, we explored the role of JSAP1 in curcumin-induced cell death, focusing on MAPK signaling, lysosome transport, and autophagy.

2. Materials and Methods

2.1. Cell culture and reagents

Human colon carcinoma HCT116 and DLD-1 cells were cultured in Dulbecco’s Modified Eagle Medium (Wako, Tokyo, Japan) and Roswell Park Memorial Institute 1640 (Wako), respectively, as described previously (20). Curcumin, puromycin, 4',6-diamidino-2-phenylindole (DAPI), and chloroquine were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plasmids and viral vector preparation

The following annealed oligonucleotides were inserted into pLVTH lentivirus plasmid vector (4) to express short hairpin RNAs (shRNAs) against human JSAP1: shJSAP1#1, forward, 5′- GATCCCCGTTTGAAGAT GCTCTGGAATTCAGAGATCCAGACATCTTC AAACCTTTTGAA-3′; reverse, 5′- AGCTTTTCC AAAAAGTTTGAAGATGCTTGAAATCTTTGA ATTCAGAGATCTCCAAACGGG-3′; shJSAP1#2, forward, 5′-GATCCCCGAACAAAGCTTTGCAGCAT TTCAAGAGATGCGAAAGCTTTTG AAAGGAAAGGAAAGCTTTTGAA-3′; reverse, 5′-AGCTTTTCCAAAAAAGAA ACAAAAGCTTTCCGATCTCTCTTTGAAGATGCG GAAAGCCTTTGGTACCGG-3′. The pLVTH expression vectors for scramble shRNA (pLVTH-shScr) and JLP shRNA (pLVTH-shJLP), and the pCL20c expression vectors for tandem monomeric red fluorescent protein (mRFP) and green fluorescent protein (GFP)-tagged microtubule-associated protein 1 light chain 3 (LC3) (pCL20c-CMV-mRFP-GFP-LC3) and hemagglutinin-tagged wild-type human JLP (pCL20c-CMVΔ4-HA-JLP) were described previously (4). A human JSAP1 cDNA (RefSeq accession number NM_001040439) encoding the full-length JSAP1 protein was prepared as for the human JLP cDNA (20), and inserted into pCL20c-HA (21) to generate pCL20c-CMV-HA-JSAP1. To express the HA-JSAP1 at suboptimal levels in cells, the CMV promoter/enhancer region was deleted and the resulting plasmid, named pCL20c-ΔCMV-HA-JSAP1, was used for the analysis in Figure 2C. The CRISPR/Cas9-based vector for targeting autophagy-related protein 5 (ATG5), PX459-sgATG5, was previously described (4). The following annealed oligonucleotides were inserted into BbsI-digested PX459 V2.0 (Addgene; Plasmid #62988, Cambridge, MA, USA), and the resulting plasmid PX459-sgJSAP1 was used to knockout JSAP1: forward, 5′-CACC GCGGCGCGTGTTGTTACGACCAAGCAGGC-3′; reverse, 5′- AAACACGTACCACCCGACGCGCCGGC-3′. Lentiviral vectors were produced as previously described (21).

2.3. Lactate dehydrogenase (LDH) assay and immunoblotting

LDH assay using the LDH-Cytotoxic Test (Wako) was performed as described previously (4). Total cell lysates were prepared and analyzed by immunoblotting as previously described (22), using anti-ATG5 (12994), anti-Phospho-JNK (9251), anti-Phospho-p38 (4631) (each diluted 1:1,000, from Cell Signaling, Boston, MA, USA), anti-HA (1:1,000; 11867423001, Sigma-Aldrich), and anti-α-tubulin (1:3,000; T5168) (two from Sigma-Aldrich) antibodies. Anti-JLP and anti-JSAP1 antibodies were affinity purified as described previously (20), and used for immunoblotting at 0.25 μg/mL and 0.33 μg/mL, respectively.

2.4. Immunocytochemistry, fluorescence, and quantification

Immunocytochemistry was carried out following standard protocols as described previously (22), using anti-HA (1:100; 11867423001, Sigma-Aldrich) and anti-LAMP-1 (1:300; H4A3, Developmental Studies Hybridoma Bank, Iowa City, IA, USA) antibodies. The secondary antibodies were goat Alexa Fluor 568- and 647-conjugated anti-mouse IgG antibody (both 1:500; Thermo Fisher Scientific, Waltham, MA, USA). For the mRFP-LC3 analysis in Figure 5E, the cells were fixed with 4% paraformaldehyde (Wako) and washed with 1X phosphate-buffered saline without detergent. Fluorescent images were captured using a confocal laser scanning microscope (TCS SP8; Leica). Quantitative analyses of lysosome distribution (Figure 2B), and colocalization of mRFP and GFP signals with LAMP-1 (Figures 5C and 5D) were performed as described previously (22,23). For quantification of mRFP-LC3 puncta, the area of mRFP-LC3 in each cell was measured using ImageJ (NIH).

2.5. Statistical analysis

Significance was determined using a two-tailed unpaired Student’s t-test. Values of p < 0.05 were considered statistically significant.
3. Results

3.1. JSAP1 knockdown enhances curcumin-induced cell death

We first examined whether JSAP1 is involved in curcumin-induced cancer cell death. To this end, we infected cells with lentiviral vectors encoding control scramble shRNA (shScr) or two distinct JSAP1 shRNAs, shJSAP1#1 and shJSAP1#2. As shown in Figure 1A, the protein levels of JSAP1 were substantially reduced in HCT116 and DLD-1 cells expressing shJSAP1s, as compared to their respective parent cells and control cells expressing shScr. We then evaluated the effect of JSAP1 depletion on curcumin-induced cell death. Significantly increased cell death was observed in JSAP1 knockdown cells (Figure 1B), indicating that JSAP1 plays a protective role in curcumin-induced cell death.

3.2. JSAP1 is involved in curcumin-induced cell death differently from JLP

We previously showed that JLP, a family member of JSAP1, suppresses curcumin-induced cell death (4). We therefore investigated whether JSAP1 acts in a similar manner to JLP or via a different mechanism. To this end, we first generated JSAP1 knockout HCT116 cells using CRISPR/Cas9 system (Figure 2A and Figure S1, http://www.ddtjournal.com/action/getSupplementalData.php?ID=70), and confirmed that JSAP1 knockout enhanced curcumin-induced cell death (Figure 2B), as observed in JSAP1 knockout cells (Figure 1B). Next, we performed rescue experiments by lentivirally expressing hemagglutinin-tagged JSAP1 (HA-JSAP1) or JLP (HA-JLP), and found that HA-JSAP1, but not HA-JLP, reversed the increased cell death (Figures 2C and 2D). The expression levels of HA-JSAP1 and HA-JLP were comparable in the HCT116 cells (Figure 2C), in which the percentage of hemagglutinin-positive cells were almost 100% (Figure S2, http://www.ddtjournal.com/action/getSupplementalData.php?ID=70). In addition, a further increase of cell death was observed by JLP knockdown in JSAP1 knockout cells (Figure S3, http://www.ddtjournal.com/action/getSupplementalData.php?ID=70). Taken together, these results indicate that JSAP1 inhibits curcumin-induced cell death in a different manner to JLP.

3.3. JSAP1 protects cell death induced by curcumin independently from JNK and p38 MAPK signaling pathways

As curcumin is known to activate JNK and p38 MAPK.
pathways (24), we asked whether JSAP1-mediated activation of JNK and/or p38 contributes to curcumin-induced cell death. We first performed immunoblotting with anti-phospho-JNK and -p38 antibodies. The activation of both JNK and p38 was observed in control HCT116 cells after curcumin challenge as expected, however, which was not attenuated by JSAP1 knockout (Figure 3A). Furthermore, we examined the effects of JNK and p38 inhibitors, SP600125 and SB203580, on cell death induced by curcumin. The JNK inhibitor SP600125 reduced cell death in control and JSAP1 knockout cells (Figure 3B), suggesting a pro-cell death role of JNK pathway. The p38 inhibitor SB203580 showed no significant difference of cell death in control and JSAP1 knockout cells (Figure 3C). Together, these results strongly suggest that JSAP1 protects curcumin-induced cell death independently from JNK and p38 MAPK signaling pathways.

3.4. JSAP1 may play a role in autophagosome degradation

It is known that curcumin alters lysosomal positioning (4,24) and activates autophagy to counteract curcumin-induced cell death (4). Indeed, the gene inactivation of ATG5, an essential gene for autophagy, enhanced curcumin-induced death of HCT116 cells (Figure S4, http://www.ddtjournal.com/action/getSupplementalData.php?ID=70).

We first assessed the distribution of lysosomes in curcumin-treated control and JSAP1 knockout cells by immunostaining with an antibody against LAMP-1, a lysosomal marker. As shown in Figure 4, there was no significant difference in the distribution between control and JSAP1 knockout cells, suggesting that JSAP1 is not involved in the regulation of lysosomal positioning. Next, we asked whether autophagosome-lysosome fusion is impaired in JSAP1 knockout cells. To examine this possibility, we lentivirally expressed mRFP-GFP-LC3 (25), a tandem fluorescent-tagged LC3 protein useful for dissecting the autophagosome maturation process, in control and JSAP1 knockout cells, treated cells with curcumin, and analyzed by immunostaining with an anti-LAMP-1 antibody. As shown in Figures 5A-5D, the percentage of colocalization of mRFP with LAMP-1 was much higher, compared to that of GFP with LAMP-1 in both control and JSAP1 knockout cells. Besides, most of GFP-positive puncta were mRFP-positive in control cells, which was consistent in JSAP1 knockout cells (Figures 5A-5D). These results suggest that JSAP1 is dispensable for the autophagosome-lysosome fusion.

During analysis with mRFP-GFP-LC3, we noticed that the number of mRFP puncta in curcumin-treated JSAP1 knockout cells was higher than that in curcumin-treated control cells. Indeed, quantitative analysis showed a significant increase of the number of mRFP puncta in JSAP1 knockout cells (Figures 5E and 5F). However, when we blocked the autophagic flux by impairing autophagosome-lysosome fusion with chloroquine, no significant difference in number of mRFP puncta was observed between control and JSAP1 knockout cells (Figures 5E and 5F). Taken together, these results may indicate that JSAP1 plays a role in autophagosome degradation and its dysfunction results in enhanced cell death.

Figure 3. JSAP1 protects cell death induced by curcumin independently from JNK and p38 MAPK signaling pathways. (A) JSAP1 WT#1 (control) and KO#1 (JSAP1 KO) HCT116 cells were treated with or without 40 μM curcumin for the indicated time points and subjected to immunoblotting using anti-Phospho-JNK (P-JNK) and -Phospho-p38 (P-p38) antibodies. (B, C) Control and JSAP1 KO cells in (A) were treated with 40 μM curcumin in the presence or absence of 40 μM JNK inhibitor SP600125 (B) or SB203580 (C), as indicated, and subjected to a cell death assay, as in Figure 1B. Quantitative data are expressed as means ± S.E.M of three independent experiments. *p < 0.01; n.s., not significant.

Figure 4. JSAP1 knockout does not impair lysosome positioning in response to curcumin. (A) JSAP1 WT#1 (control) and KO#1 (JSAP1 KO) HCT116 cells were treated with 40 μM curcumin for 12 hours, fixed and immunostained using an anti-LAMP-1 antibody. Nuclei were stained with DAPI. Z-stack images were obtained by confocal microscopy. Scale bar, 10 μm. (B) Quantification of the lysosome distribution results shown in (A). Quantitative data are expressed as means ± S.E.M of three independent experiments. The perinuclear region was defined as 0-5 μm from nuclear rim. At least 20 cells per experiment were analyzed. n.s., not significant.
4. Discussion

In the present study, we explored the role of JSAP1 in curcumin-treated cells and found that JSAP1 depletion enhances curcumin-induced cell death, indicating a protective role of JSAP1 in response to cell death stimulation. We recently reported that JLP, a JSAP1 family member, also possesses a similar function for curcumin (4). In addition, several studies have shown functional redundancy between JSAP1 and JLP in some cellular processes (26-28). However, due to the following observations, it is unlikely that JSAP1 attenuates curcumin-induced cell death in a similar way with JLP: (1) JLP knockdown in JSAP1 knockout cells further increased curcumin-induced cell death (Figure S3), and (2) exogenously expressed JLP, but not JSAP1, was unable to cancel the increase of cell death seen in curcumin-treated JSAP1 knockout cells, although the
expression levels of both the exogenous JSAP1 and JLP proteins were comparable (Figures 2C and 2D).

It is known that JIP family proteins, including JSAP1, can function as scaffolding proteins for JNK signaling cascades (29,30). However, upon curcumin challenge, no or very little effect of downregulation of JNK activation was observed in JSAP1 knockout cells (Figure 3A). Furthermore, the results of experiments using SP600125, a JNK inhibitor, did not support the involvement of JSAP1-JNK pathways in curcumin-induced cell death, but rather suggest a pro-cell death role of JNK pathways (Figure 3B). Other JIP family proteins, such as JIP1 and JIP2, might mediate JNK signaling pathways. To date, it is an open question how appropriate MAPK scaffold proteins would be selected for proper MAPK activation in response to extracellular and intracellular stimuli.

Autophagy consists of several sequential steps, including autolysosome formation. We previously demonstrated that JLP plays a role in lysosome positioning through retrograde transport along microtubules and its dysfunction leads to the inhibition of autophagosome-lysosome fusion (4). However, our results in this study showed that JSAP1 depletion did not impair lysosome positioning and autophagosome-lysosome fusion in curcumin-treated cells (Figures 4 and 5A-5D). Instead, we noticed substantial autolysosome accumulation accompanied by an inefficient autophagic flux in JSAP1 knockout cells (Figures 5E and 5F). During the preparation of this manuscript, another study by Cason et al. (31) reported the accumulation of stationary autolysosomes in the proximal axon of hippocampal neurons after JIP3/JSAP1 depletion. Together, it is likely that JSAP1 is involved in the later step of autophagy, i.e., the steps after autolysosome formation. Further study is needed to clarify the molecular mechanism of how JSAP1 depletion leads to the accumulation of autolysosomes.

The precise mechanism of how JSAP1 protects cells from curcumin-induced cell death is unclear at present. Autolysosome accumulation has been associated with inefficient autophagic lysosome reformation, a lysosome recycling process at the terminal step of autophagy (32,33). As a result, the number of free lysosomes is reduced and eventually hinders autophagic clearance, leading to cell death during the peak of autophagy (34-36). Thus, the autolysosome accumulation with attenuated autophagic flux in JSAP1 knockout cells may increase sensitivity toward curcumin-induced cell death. However, we cannot rule out the possibility that other unknown functions of JSAP1 may be involved in curcumin-induced cell death.

Curcumin has been viewed as a potential chemotherapeutic agent for cancer. The findings of this study would contribute to the development of novel therapeutic approaches for cancer.

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