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Upregulation of Beclin-1 expression and phosphorylation of Bcl-2 and p53 are involved in the JNK-mediated autophagic cell death

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ABSTRACT

Though the activation of c-Jun NH2-terminal kinase (JNK) has been reported to be essential for autophagic cell death in response to various stressors, the molecular links between JNK activation and autophagic cell death signaling remain elusive. Here we report that, in the JNK-dependent autophagic cell death of HCT116 cells induced by an agonistic single chain variable fragment antibody, HW1, against human death receptor 5 (DR5), JNK activation upregulated Beclin-1 expression and induced Bcl-2 and p53 phosphorylation. Further, the p53-deficient HCT116 cells showed less susceptibility to the HW1-mediated autophagic cell death than the wild type cells, suggesting that JNK-mediated p53 phosphorylation promotes the autophagic cell death. Our results suggest that DR5-stimulated JNK activation and its consequent fluxes into the pro-autophagic signaling pathways contribute to the autophagic cell death in cancer cells.

Introduction

Autophagy, or cellular self-digestion, is a dynamic intracellular process for degrading cytoplasmic proteins and organelles in response to external stressors (reviewed in [1–3]). Paradoxically, although autophagy principally serves as a cellular survival pathway in response to nutrient deprivation and other stressors, recent studies suggested that autophagy can also contribute to another cell death pathway under certain circumstances, so called type II cell death ('autophagic cell death'), that is morphologically and biochemically distinct from the type I cell death of apoptosis [3,4]. Autophagic cell death has been involved in physiological cell death during development [5] and reported in cancer cells treated with chemicals or irradiation [1,6–8], where dying cells showed the typical autophagy features, such as autophagosomes and autolysosomes, but neither apoptotic nor necrotic morphological features.

A number of complicated signaling pathways are involved in the regulation of autophagy. Beclin-1 is one of the essential components that involved in autophagosome formation, the expression level of which usually increases during autophagy [1,2]. The pro-autophagic function of Beclin-1 can be inhibited by interaction with Bcl-2 family proteins, such as Bcl-2 and Bcl-xL, to form Beclin-1-Bcl-2/Bcl-xL complexes [2]. Bcl-2 phosphorylation disrupts the interactions between Beclin-1 and Bcl-2, releasing Beclin-1 to promote autophagy [9]. In the cytoprotective autophagy induced by external stimuli, the activation of c-Jun NH2-terminal kinase (JNK), also referred to as stress-activated kinases, upregulated Beclin-1 expression [10] and mediated Bcl-2 phosphorylation [9], thereby promoting cellular survival. In other cellular settings, JNK activation has also been essential for autophagic cell death induced by tumor necrosis factor-α (TNFα) [11,12], anti-cancer agents [10], and chemicals [6,8]. We also recently reported that an agonistic antibody (Ab), single chain variable fragment (scFv) HW1, which specifically bound to TNF-related apoptosis inducing ligand (TRAIL) receptor 2 (death receptor 5 (DR5)), triggered autophagic cell death of cancer cells dominantly through JNK pathway in a caspase-independent manner [13]. Blocking the HW1-mediated autophagy by the inhibitor of 3-methyladenine (3-MA) or depletion of Beclin-1 and Atg7 inhibited the autophagosome formation to increase cellular survival [13]. However, no detailed studies have been performed to determine the molecular links between JNK activation and autophagic cell death. Further, the roles of the JNK-mediated beclin-1 upregulation and Bcl-2 phosphorylation, which have been observed in the cytoprotective autophagy, have not been directly addressed in the autophagic cell death.

In this study we showed that DR5-stimulated JNK activation, which was essential for the autophagic cell death of HCT116 cells induced by anti-DR5 HW1 scFv, resulted in upregulation of Beclin-1 expression, Bcl-2 phosphorylation, and p53 phosphorylation,

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suggesting that these pro-autophagic signaling pathways are involved in the autophagic cell death.

Materials and methods

Cell lines and reagents. Human colon cancer cell lines, HCT116, and its p53-deficient ($p53^{-/-}$) cells were obtained from the Cell center of John's Hopkins University School of Medicine [14] and cultured in DMEM (WelGENE, Korea) supplemented with 10% fetal bovine serum (GIBCO, Invitrogen), 100 units/ml of penicillin, and 100 μg/ml of streptomycin (Life Technologies, Invitogen). The following rabbit Abs were used for Western blotting: anti-JNK, anti-phospho-JNK, and anti-β actin were from Cell signaling technology; anti-Beclin-1 and anti-LC3 were from Novous biotechnology; anti-Bcl-xL, anti-p53, anti-phospho-p53 (Ser392), anti-Bcl2, anti-phospho-Bcl2 (Ser87), and anti-phospho-p70S6 kinase (p70S6 K) (Thr389) were from Santa Cruz biotechnology; anti-DRAM were from Proscience. The plasmid pcDNA3-Flag-MKK7-JNK1 (constitutively active JNK1) and pcDNA3-Flag-MKK7-JNK1(APF) (phosphorylation-negative mutant JNK1 with Thr^{180}-Pro-Tyr^{182} replaced by Ala-Pro-Phe) were provided by Prof. Roger Davis (Univ. of Mass Medical School, MA, USA) [15]. The gene encoding anti-DR5 human scFv HW1 were subcloned in-frame from the bacterial expression plasmid of pKJ1-HW1 [13,16] into the yeast secretion plasmid of pRSGAL [17] using NheI/BamHI sites, resulting in pRSGAL-HW1. The plasmid was transformed by electroporation into the Saccharomyces cerevisiae strain YVH10 and HW1 was purified with purity more than 98% following the protocols described before [17]. The purified proteins were sterilized by filtration using a cellulose acetate membrane filter (0.2 μm) (Nalgene Co.) before usages in cell assays. The protein concentrations were determined using the Bio-Rad protein assay.

Cell viability assays. Cell viability was analyzed using a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)-based cell growth determination kit (Sigma), as described previously [13,18]. In brief, cells seeded at a density 1×10^4 cells/well in 96-well plates were cultured overnight and treated with HW1 (0.1–50 μg/ml) for 36 h, followed by MTT assay. In HCT116 cells transfected with 1 μM plasmid constitutively expressing active JNK1 or phosphorylation-negative mutant JNK1(APF) by electroporation in a 0.1-cm diameter capillary (Digital Biotech), the transfected cells were incubated for 48 h at a density of 5×10^4 cells/well in 96-well plate and then treated with HW1 (0.1–50 μg/ml) for 36 h in the absence and presence of 3-MA (Sigma) before MTT assay. We used 125 μM 3-MA, at which it did not show any significant cytotoxicity and completely inhibited the autophagy induced by HW1 treatments, as we reported previously [13].

Western blotting analyses and immunoprecipitations. Cells (3×10^5 cells/well) were seeded in six-well plates, grown overnight and then treated with 50 μg/ml HW1 for the indicated periods with or without the JNK inhibitor, SP600125 (10 μM) (Calbiochem). The standard procedures for Western blotting were then done as described previously [13]. For immunoprecipitations, cells (2×10^7 cells/time point) were treated with HW1 (50 μg/ml) for the indicated periods, collected, and lysed, as essentially described before [13]. The cleared cell lysates were then subjected to immunoprecipitations with anti-Beclin-1 rabbit Ab and then agarose-conjugated anti-rabbit Ab (Sigma), followed by western blotting analysis of coimmunoprecipitated Bcl-2 and Bcl-xL.

Results and discussion

Constitutive JNK activation enhanced HW1-mediated cell death

JNK activation was essential for the HW1-induced autophagic cell death of various types of tumor cells, including HCT116 cells [13]. Analyses of secondary signaling complex and upstream kinase(s) for JNK activation upon HW1 binding to DR5 revealed that JNK activation was most likely mediated by TRAF2-MKK4/MKK7-dependent signaling cascade (Supplementary Fig. S1). To examine whether the HW1-induced cell death can be modulated by the JNK activation level, HCT116 cells transiently transfected with the plasmid expressing constitutively active JNK1 or phosphorylation-negative mutant JNK1(APF) [15] were treated with various concentrations of HW1 for 36 h. Expression of constitutively active JNK1 resulted in significant augmentation in the HW1-mediated cell death, whereas expression of phosphorylation-negative mutant JNK1(APF) almost abrogated the HW1-mediated cell death, compared with the cells treated HW1 alone (Fig. 1A). The anchorage-dependent HCT116 cells dying by exposure to HW1 or HW1 plus constitutive active JNK1 expression became rounded and eventually detached from the culture plates to be floated, indicative of cellular demise but not growth inhibition (Supplementary Fig. S2). The presence of 3-MA, a known potent inhibitor of autophagy [1,13], substantially suppressed the HW1-induced cell death of HCT116 cells untransfected or transfected with the plasmid constitutively expressing active JNK1 (Fig. 1B and Supplementary Fig. S2), consistent with the previous observation that HW1 triggered JNK activation-dependent autophagic cell death [13]. Taken together, these data indicated that JNK activation critically regulated the HW1-induced autophagic cell death.

HW1-induced JNK activation mediated upregulation of Beclin-1 expression and Bcl-2 phosphorylation

In order to determine the molecular mechanism(s) leading to the autophagic cell death by HW1-stimulated JNK activation, the activations of autophagic signaling molecules known to be activated by JNK were monitored by western blotting in HCT116 cells treated with 50 μg/ml HW1 for the indicated periods in the absence and presence of the JNK inhibitor, SP600125 (10 μM). Compared with the cells left untreated or treated with HW1 plus the JNK inhibitor, the HW1-treated cells exhibited significant activations of JNK starting from 12–18 h (Fig. 2A), which were temporally matched with the activations of its upstream kinases MKK4 and MKK7 (Supplementary Fig. S1D). One of the hallmarks of

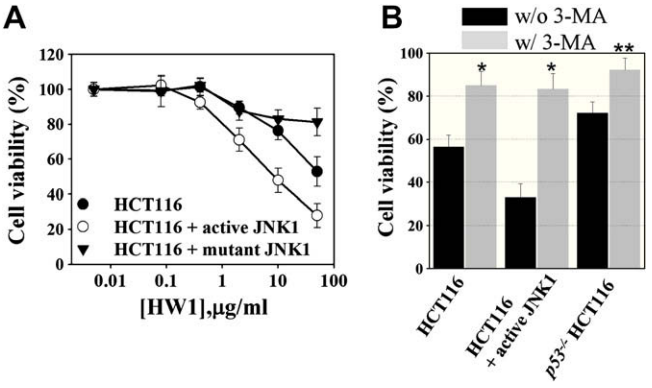

Fig. 1. Enhanced HW1-mediated cell death by constitutive JNK activation, which was significantly inhibited by the autophagic inhibitor 3-MA. (A) Cell viability of HCT116 cells untransfected or transiently transfected with the plasmid of pcDNA3-Flag-MKK7-JNK1 (expressing constitutively active JNK1) or pcDNA3-Flag-MKK7-JNK1(APF) (expressing phosphorylation-negative JNK1 mutant), which were treated with the indicated concentrations of HW1 for 36 h. (B) Effects of 3-MA on the cell viability of HCT116 cells untransfected or transiently transfected with the plasmid expressing constitutively active JNK1 and $p53^{-/-}$ HCT116 cells, which were pretreated for 1 h with 125 μM 3-MA and further incubated with 50 μg/ml HW1 for 36 h. Error bars indicate the standard deviations for at least triplicated experiments. Significance was determined using the Student's *t*-test versus control samples not pretreated with 3-MA ($^{*}P < 0.001$, $^{**}P < 0.05$).

autophagy is the conversion of cytosolic LC3-I into autophagosome-associated LC3-II, the amount of which reflects the abundance of the autophagosomes [1]. Western blotting analysis revealed the significant increase in the amount of LC3-II from 12 h of HW1 treatment by more than 2-fold in the HW1-treated cells, but not in the cells left untreated or treated with HW1 plus the JNK inhibitor (Fig. 2A). These results, together with 3-MA-mediated suppression of the HW1-induced cell death, demonstrated that HCT116 cells by exposure to HW1 undergo JNK activation-dependent autophagic cell death [13].

Only the HW1-treated cells, but not the cells treated with HW1 plus the JNK inhibitor, exhibited significantly increased levels of Beclin-1 expression and phosphorylated Bcl-2 after 12 h treatment, which closely paralleled the time-course JNK activations (Fig. 2A), indicating that JNK activation upregulated Beclin-1 expression and induced Bcl-2 phosphorylation. These data were in line with JNK-mediated upregulation of Beclin-1 and Bcl-2 phosphorylation in the autophagy induced by anti-cancer agents [10] and nutrient deprivation [9], respectively. JNK can directly phosphorylate Bcl-2 [9] and indirectly increase the expression level of Beclin-1 through c-Jun activation, its target transcriptional factor [10]. When the physical interactions of Beclin-1 with Bcl-2 and/or Bcl-xL were examined by immunoprecipitations [2,9], the amount of coimmunoprecipitated Bcl-2 with Beclin-1 significantly decreased after 12 h of HW1 treatment (Fig. 2B), coordinated temporally with the HW1-induced Bcl-2 phosphorylation (Fig. 2A). However, the level of coimmunoprecipitated Bcl-xL with Beclin-1 was only slightly affected by HW1 treatment for 12 h. Thus, it seems that JNK-mediated Bcl-2 phosphorylation facilitated the dissociation of Bcl-2 from the Bcl-2–Beclin-1 complex, releasing Beclin-1 to promote autophagy [2,9]. Taken altogether, HW1-induced JNK activation mediated transcriptional upregulation of Beclin-1 and Bcl-2 phosphorylation.

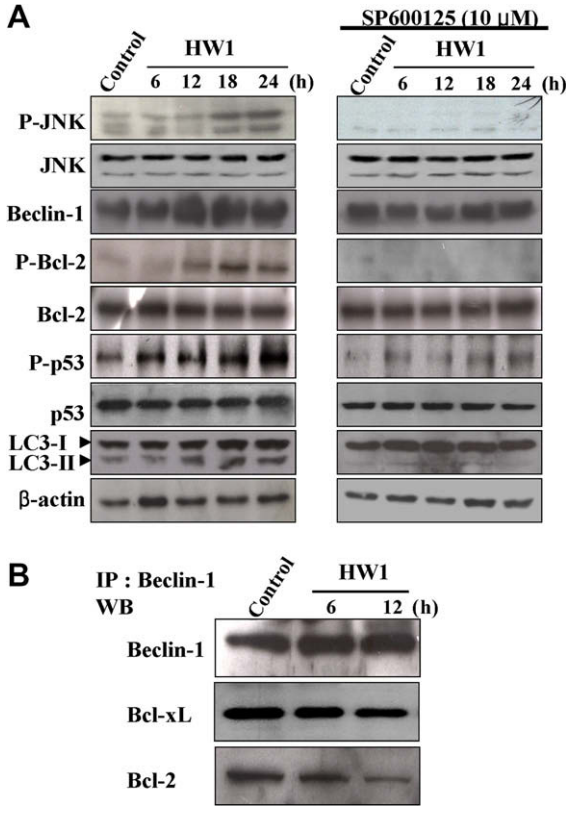

Fig. 2. HW1 treatment induced JNK activation, which in turn upregulated Beclin-1 expression and induced phosphorylation of Bcl-2 and p53. (A) Representative western blotting to analyze the expression of LC3 (LC3-I and LC3-II) and Beclin-1 and the phosphorylation of Bcl-2 (Ser87) and p53 (Ser392) in HCT116 cells, which were left untreated (*control*, 24 h) or treated with 50 μg/ml HW1 for the indicated periods, in the absence (left panel) and presence (right panel) of the JNK inhibitor, SP600125 (10 μM). The β-actin protein levels are included as a control for protein loading. (B) Coimmunoprecipitation of Bcl-2 and Bcl-xL with Beclin-1 in HCT116 cells remained untreated (*control*) or treated with 50 μg/ml HW1 for 6 or 12 h.

HW1-induced JNK activation phosphorylated p53, which in turn inhibited mTOR activity and upregulated DRAM expression

In addition to the pro-apoptosis function, the tumor suppressor protein p53 has recently been found to also regulate autophagy [1,2]. Given the reported ability of p53 activation to stimulate autophagy [19], we also examined whether HW1-mediated JNK activation can trigger p53 phosphorylation by Western blotting. HW1 treatment resulted in significant phosphorylation of p53 on Ser392, indicative of p53 activation, particularly after 18 h of treatment (Fig. 2A). The presence of the JNK inhibitor completely abrogated the p53 phosphorylation, indicating that the p53 activation was induced by JNK activation. A recent study also showed that TNFα-mediated JNK activation induced p53 phosphorylation to promote autophagy [12]. To further determine the role of p53 activation in HW1-induced autophagic cell death, we examined cell-death inducing activity of HW1 for the isogenic wild type and $p53^{-/-}$ HCT116 cells. The $p53^{-/-}$ HCT116 cells were much less susceptible to HW1 showing ~30% reduced cell death at the highest concentration of HW1 (50 μg/ml), compared with the wild type cells (Fig. 3A). 3-MA also inhibited the HW1-mediated cell death of $p53^{-/-}$ HCT116 cells (Fig. 1B). These data suggested that p53 partially involved in the HW1-mediated autophagic cell death.

The p53 stimulates autophagy by inhibiting anti-autophagic mTOR (mammalian target of rapamycin) pathway [19] and/or transcriptional upregulation of DRAM (damage-regulated autophagy modulator), a lysosomal protein stimulating the accumulation of autophagic vacuoles [20]. We investigated whether HW1-mediated p53 activation could regulate mTOR activity and transcriptional activation of DRAM by time-course HW1 treatments in

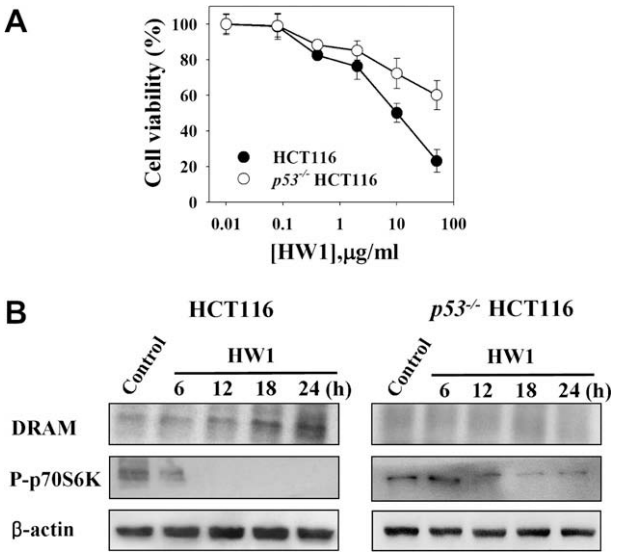

Fig. 3. p53 phosphorylation involved in the HW1-mediated cell death. (A) Cell viability of wild type and $p53^{-/-}$ HCT116 cells, which were treated with the indicated concentrations of HW1 for 36 h. Error bars indicate the standard deviations for at least triplicated experiments. (B) Representative Western blotting to analyze the expression level of DRAM and the phosphorylation of p70S6K (Thr389) in the wild type and $p53^{-/-}$ HCT116 cells, which were left untreated (*control*, 24 h) or treated with 50 μg/ml HW1 for the indicated periods.

wild type and $p53^{-/-}$ HCT116 cells. The activity of mTOR can be specifically assayed by monitoring the phosphorylation level of its downstream target p70S6K on Thr389 [19]. Compared with the cells left untreated, the HW1-treated cells did not exhibit detectable level of phosphorylated p70S6K from 12 h of treatment, suggesting that HW1-mediated p53 activation inhibited the mTOR signaling pathway (Fig. 3B). In $p53^{-/-}$ HCT116 cells, no significant differences in the level of phosphorylated p70S6K were observed between the HW1-treated and -untreated cells (Fig. 3B), which could be attributed to the lack of p53. The HW1-treated cells also showed significant upregulation of DRAM expression after 12 h of treatment, compared with the cells left untreated (Fig. 3B). On the other hand, DRAM was negligibly detected in $p53^{-/-}$ HCT116 cells, regardless of HW1 treatment, suggesting that p53 activation transcriptionally upregulated the expression of DRAM. Taken together, these results indicated that HW1-mediated p53 activation inhibited mTOR activity and upregulated DRAM expression.

A recent study showed that, in contrast to the pro-autophagic activity of phosphorylated p53, cytoplasmic p53 suppressed autophagy, the exact mechanism of which is not clear yet [21]. The less susceptibility of $p53^{-/-}$ HCT116 cells to HW1-mediated cell death than the wild type cells suggested that the inhibition of mTOR pathway and/or upregulation of DRAM mediated by phosphorylated p53 might be prerequisite for triggering the HW1-mediated autophagic cell death.

Conclusion

Here we showed that JNK activation stimulated by HW1 binding to DR5 sequentially upregulated Beclin-1 expression and induced the phosphorylation of Bcl-2 and p53, which seemed to contribute to the HW1-mediated autophagic cell death. The p53-deficient HCT116 cells showed less susceptibility to the HW1-mediated autophagic cell death than the wild type cells, suggesting that JNK-mediated p53 phosphorylation promotes the autophagic cell death, most likely by upregulation of DRAM expression and inhibition of mTOR activity, which has not been previously reported in the autophagic cell death. Even though the exact role of each signaling in the autophagic cell death should be determined further, these molecular links between JNK activation and the autophagic cell death might be the case for JNK activation-dependent autophagic cell death caused by other stressors [6,8,10,11].

The term of 'autophagic cell death' is now very controversial whether it can be defined as 'cell death by autophagy' or 'cell death with autophagy' (reviewed in [3,22]), partially because direct assays to distinguish them are not clearly established yet. However, many accumulating evidences [5–8,10–12], along with our results [13], support that autophagy is an active cell death-inducing mechanism at least under some circumstances because blocking autophagy by the specific inhibitors or depleting of autophagy-involved genes inhibited the cell death to increase cellular survival. Excess subcellular degradation by autophagy might surpass the threshold of cytoprotective autophagic function, resulting in irreversible cell death. Finally, our study suggests that DR5-stimulated JNK activation and its consequent fluxes into the pro-autophagic signaling pathways might be one of the strategies to increase the therapeutic potential of autophagy as anti-cancer therapy.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2009.03.095.

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