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Research Paper

# Vitamin K2 induces autophagy and apoptosis simultaneously in leukemia cells

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**Key words:** vitamin K2, autophagy, apoptosis, leukemia, acute myeloid leukemia, bcl-2

Vitamin K2 (menaquinone-4: VK2) is a potent inducer for apoptosis in leukemia cells in vitro. HL-60*bcl-2* cells, which are derived from a stable transfectant clone of the human *bcl-2* gene into the HL-60 leukemia cell line, show 5-fold greater expression of the Bcl-2 protein compared with HL-60*neo* cells, a control clone transfected with vector alone. VK2 induces apoptosis in HL-60*neo* cells, whereas HL-60*bcl-2* cells are resistant to apoptosis induction by VK2 but show inhibition of cell growth along with an increase of cytoplasmic vacuoles during exposure to VK2. Electron microscopy revealed formation of autophagosomes and autolysosomes in HL-60*bcl-2* cells after exposure to VK2. An increase of acid vesicular organelles (AVOs) detected by acridine orange staining for lysosomes as well as conversion of LC3B-I into LC3B-II by immunoblotting and an increased punctuated pattern of cytoplasmic LC3B by fluorescent immunostaining all supported induction of enhanced autophagy in response to VK2 in HL-60*bcl-2* cells. However, during shorter exposure to VK2, the formation of autophagosomes was also prominent in HL-60*neo* cells although nuclear chromatin condensations and nuclear fragments were also observed at the same time. These findings indicated the mixed morphologic features of apoptosis and autophagy. Inhibition of autophagy by either addition of 3-methyladenine, siRNA for Atg7, or Tet-off Atg5 system all resulted in attenuation of VK2-induced cell death, indicating autophagy-mediated cell death in response to VK2. These data demonstrate that autophagy and apoptosis can be simultaneously induced by VK2. However, autophagy becomes prominent when the cells are protected from rapid apoptotic death by a high expression level of Bcl-2.

## Introduction

The vitamin K family contains both natural and synthetic forms, the former including phytonadione (VK1) and the menaquinone series (VK2), and the latter including menadione (VK3). Recent studies have reported that VK2 can exert cell growth-inhibitory effects in various human cancer cells including leukemia, lung cancer and hepatocellular carcinoma (HCC) cells.<sup>1-5</sup> We previously reported that this cytotoxic effect of VK2 appears to be selective to leukemic blasts with almost no effects on normal hematopoietic progenitor cells.<sup>2,6</sup> This suggests a therapeutic advantage for using VK2 in therapy for leukemia. Several recent clinical trials demonstrated that oral administration of VK2 reduced leukemic blasts in acute myeloid leukemia (AML) and improvement of cytopenias in myelodysplastic syndromes (MDS), and also decreased the development and recurrence rates of HCC, thus improving the overall survival of patients with HCC.<sup>7-10</sup> Although the precise mechanism of how VK2 exerts its growth-inhibitory effects in cancer cells has not been determined, VK2 has been reported to be a potent inducer of apoptosis in various tumor cells including leukemic cells in vitro.<sup>2,3,11-15</sup>

Apoptosis is an evolutionally conserved, orchestrated cell-death process characterized by membrane-blebbing, DNA fragmentation, and the formation of distinct apoptotic bodies that contain components of the dead cell.<sup>16,17</sup> This process occurs without membrane breakdown and does not elicit an inflammatory response. Apoptotic bodies are eventually removed by phagocytic cells. Central to this apoptotic process are a group of caspases, which effect the destruction of the cell in an orderly fashion.<sup>17</sup> Extensive evidence suggests that therapeutic effects of various anti-cancer reagents and of radiation are mediated through apoptosis of cancer cells.<sup>18</sup> However, another type of caspase-independent cell death designated autophagic cell death has recently been suggested in some cancer cells in response to anticancer therapy.<sup>19-22</sup>

Autophagy is also an evolutionally conserved membrane trafficking process that leads to degradation of cytosolic proteins and organelles by lysosomes.<sup>23,24</sup> Cytosol and organelles such as mitochondria and endoplasmic reticulum are engulfed into double-membraned vesicles called autophagosomes. Fusion subsequently occurs between the

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autophagosomes and lysosomes to form autolysosomes in which the cargo of the autophagosome is degraded by lysosomal hydrolases. Autophagy is induced above basal levels in response to nutrient deprivation or trophic factor withdrawal, and it sustains metabolism through the targeted catabolism of long-lived proteins. Thus, autophagy acts as a self-limited survival mechanism.<sup>23,24</sup> A group of genes known as Atg genes, which are conserved from yeast to humans, have been found to regulate autophagy.<sup>25</sup> A number of studies have reported that autophagy is activated in cancer cells derived from breast, colon, prostate and brain in response to various anticancer therapies.<sup>21,22</sup> Since inhibition of autophagy results in suppression of cancer cell death by anti-cancer reagents, autophagy is suggested to be a potential contributor to non-apoptotic programmed cell death.<sup>19,21</sup> When cell death involves autophagy, it is now designated as type II programmed cell death (PCD) or autophagic cell death, in contrast to apoptosis, which is referred to as type I PCD.<sup>19,21</sup> The morphological and biochemical features of autophagic cell death and apoptosis are generally distinct. In autophagic cell death, unlike apoptotic cell death, caspases are not activated, and neither DNA degradation nor nuclear fragmentation is apparent. Instead, autophagic cell death is characterized by degradation of the Golgi apparatus, polyribosomes and endoplasmic reticulum before nuclear destruction, whereas these organelles are preserved in apoptosis.<sup>19,21</sup> However, the involvement of autophagy in programmed cell death is still controversial, probably because the molecule(s) which execute cell death in autophagy have not been identified.<sup>20-22</sup>

We have previously reported that overexpression of Bcl-2 in a leukemia cell line resulted in resistance against VK2-induced apoptosis but these cells still underwent differentiation via G<sub>1</sub> arrest.<sup>12</sup> In addition, downregulation of Bcl-2 was reported to cause autophagy of HL-60 cells in a caspase-independent manner.<sup>26</sup> It has also been reported that Bcl-2 negatively regulates Beclin 1-dependent autophagy and Beclin 1-dependent autophagic cell death.<sup>27</sup> All these data suggest the involvement of Bcl-2 in some autophagic signaling and also imply some direct or indirect interaction between mitochondria and lysosomes.<sup>28</sup>

In the present study, we investigated whether autophagy is involved in VK2-induced leukemic cell death using subclones of HL-60 cell line with higher and lower expression levels of the Bcl-2 protein. Our data demonstrated that both autophagy and apoptosis are simultaneously induced after VK2 treatment, but autophagy become more evident and detectable when the cells are protected from apoptosis.

## Results

**Bcl-2 abolished VK2-induced apoptosis but had little effect on VK2-induced growth inhibition in HL-60 cells.** We have reported that treating the primary cultured leukemic cells and leukemic cell lines with 1–10  $\mu$ M of VK2 for 48 to 96 hr induces apoptosis in vitro, and that the expression levels of Bcl-2 determine whether the leukemic cells to undergo apoptosis or differentiation.<sup>2,12</sup>

Immunoblotting with anti-Bcl-2 mAb revealed that HL-60*bcl-2* cells, which are stably transfected with human *bcl-2*, express Bcl-2 at a five-fold higher level than HL-60*neo* control cells (Fig. 1A). There was no difference in growth rate, morphology, and antigen expressions among the parental HL-60, HL-60*neo*, and HL-60*bcl-2* cells (data not shown). Thereafter, cell growth inhibition was assessed after 96-hr exposure to VK2 (menaquinone-4) at various

concentrations. As shown in Figure 1B, VK2 inhibited HL-60 cell growth in a dose-dependent manner. HL-60*bcl-2* cells were less sensitive as compared with HL-60*neo* cells; the concentrations for the 50% growth inhibition (IC<sub>50</sub>) were 6  $\mu$ M for HL-60*neo* and 14  $\mu$ M for HL-60*bcl-2* cells, respectively.<sup>12</sup> As previously reported in other leukemic cell lines and primary cultured leukemic cells,<sup>2,11,13</sup> treatment with 10  $\mu$ M of VK2 for 72 hr potently induced apoptosis of HL-60*neo* cells as assessed by morphology, depolarization of mitochondrial membrane potential, and annexin V staining (Fig. 1C–E). In contrast, apoptosis induction by VK2 treatment was significantly suppressed in HL-60*bcl-2* cells (Fig. 1D and E), although the inhibition of cell growth was still detectable, and extended exposure to VK2 induced cell death in HL-60*bcl-2* cells (Fig. 1B). We therefore investigated whether the autophagy or non-apoptotic cell death occurred in HL-60*bcl-2* cells after treatment with VK2.

**Induction of autophagy in HL-60*neo/bcl-2* cells by VK2.** Since cytoplasmic vesicles became evident in HL-60*bcl-2* cells after treatment with VK2 (Fig. 1C), we first performed electron microscopy to determine whether VK2 induces autophagy in HL-60*bcl-2* cells and compared the results with those obtained with HL-60*neo* cells. As shown in Figure 2A (upper right), the nuclear degeneration and multiple chromatin bodies, which are characteristic features for the cells undergoing apoptosis, were observed in HL-60*neo* cells treated with 10  $\mu$ M of VK2 for 72 hr. In contrast, numerous autophagic vacuoles and empty vacuoles were observed in HL-60*bcl-2* cells under the same conditions (Fig. 2B; upper right and lower). Most of the autophagosomes contained lamellar structures with residual digested materials. These results indicated that HL-60*bcl-2* cells treated with VK2 undergo autophagy.

It was previously reported that during amino acid starvation, LC3 becomes localized to form isolated membrane following formation of autophagosome membranes.<sup>34</sup> Therefore detection of the punctuated pattern of cytosolic LC3 indicates the involvement of LC3 for autophagosome formation. This phenomenon has been used as method for monitoring autophagy.<sup>35</sup> To further confirm the involvement of LC3 in VK2-induced autophagy, we performed fluorescent immunocytochemical staining of HL-60*neo/bcl-2* cells with anti-LC3B Ab and subsequent counter staining with DAPI for the detection of nuclei. As shown in Figure 3A and B, untreated HL-60*neo* and HL-60*bcl-2* cells both showed diffuse distribution of green fluorescence and no fragmented nuclei, whereas treatment with VK2 increased the punctuated pattern of LC3B in HL-60*bcl-2* cells, representing autophagic vacuoles. HL-60*neo* cells with fragmented nuclei showed no punctuated pattern of LC3B. However, in a minor population of HL-60*neo* cells without fragmented nuclei, the punctuated pattern of LC3B staining was detected. Among the cells without nuclear fragments during 72 hr-treatment with 10  $\mu$ M of VK2, the percentage showing the punctuated pattern of LC3B increased in HL-60*neo* cells as well as HL-60*bcl-2* cells (Fig. 3B). This indicates that autophagy can be detected not only HL-60*bcl-2* cells but also HL-60*neo* cells. Within 48 hrs of exposure to VK2, autophagy induction was rather prominent in HL-60*neo* cells as compared with HL-60*bcl-2* cells.

**Simultaneous induction of both apoptosis and autophagy in HL-60 cells after treatment with VK2.** The data shown above suggested that both autophagy and apoptosis appear to be induced in response to VK2 in HL-60*neo* cells. For semi-quantitative assessment of the induction of autophagy, we examined the development of acid

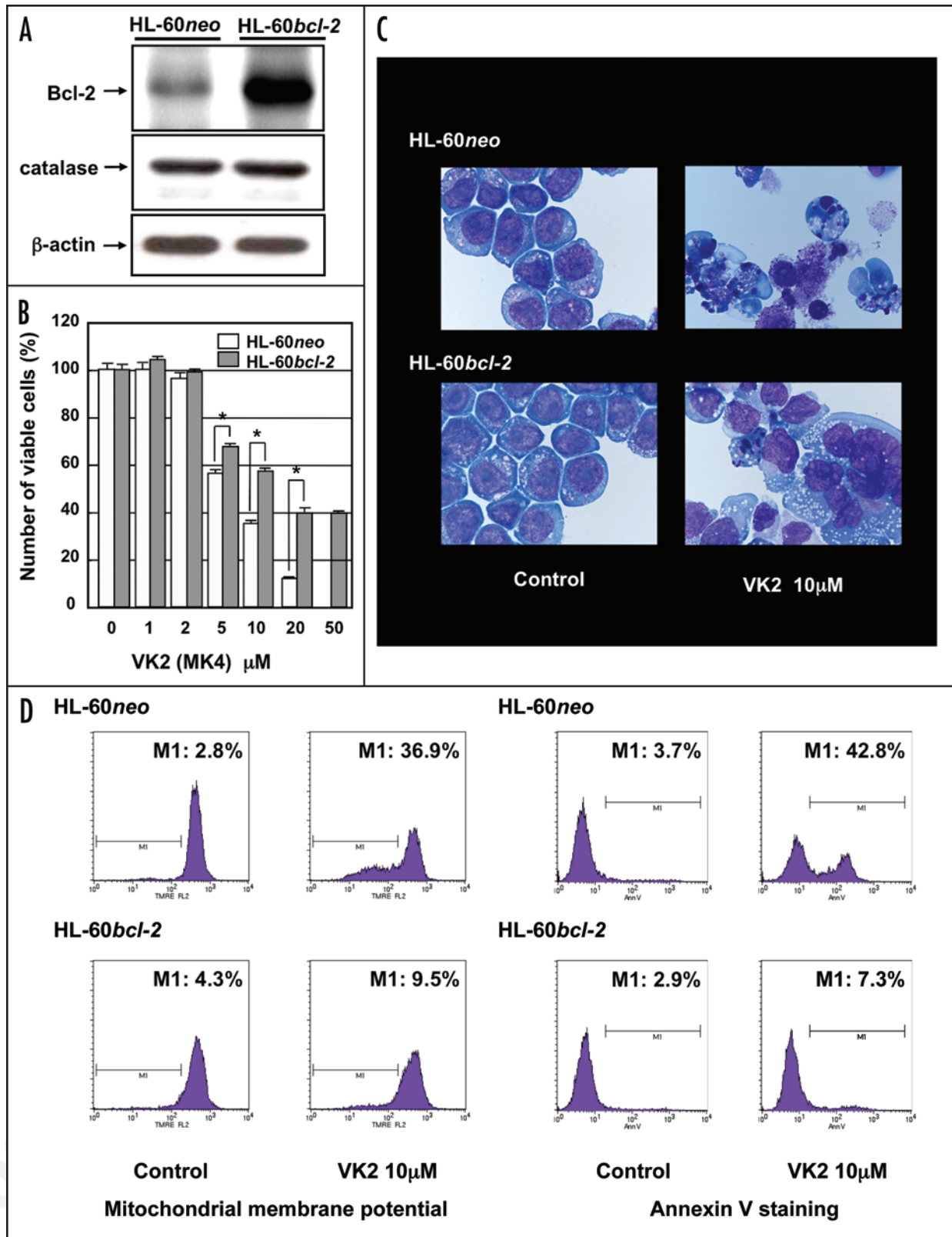


Figure 1. Inhibition of cell growth and induction of apoptosis after treatment with VK2 in HL-60 cells. (A) Expression of Bcl-2 and catalase in HL-60neo and HL-60bcl-2 cells: Cellular proteins were lysed and separated by 15% SDS-PAGE for Bcl-2 and 11.25% SDS-PAGE for catalase and β-actin, and immunoblotted with anti-human Bcl-2 mAb, anti-catalase rabbit mAb, or anti-β-actin Ab, respectively. (B) Cell growth inhibition in response to VK2 in HL-60neo/HL-60bcl-2 cells: Cells were cultured in the presence of various concentrations of VK2 (0.1–50 µM) for 72 hr. The number of cells was assessed with the WST cell counting kit as described in Materials and Methods. Cell growth is expressed as a ratio to the untreated control cells. \*p < 0.001. After 72 hr exposure to 10 µM of VK2, HL-60neo and HL-60bcl-2 cells were processed for (C) May-Grünwald-Giemsa staining for assessment of morphologic changes (original magnification x1,000), and flow cytometry for (D) mitochondrial membrane potential using TMRE, and (E) Annexin V staining as described in Materials and Methods.

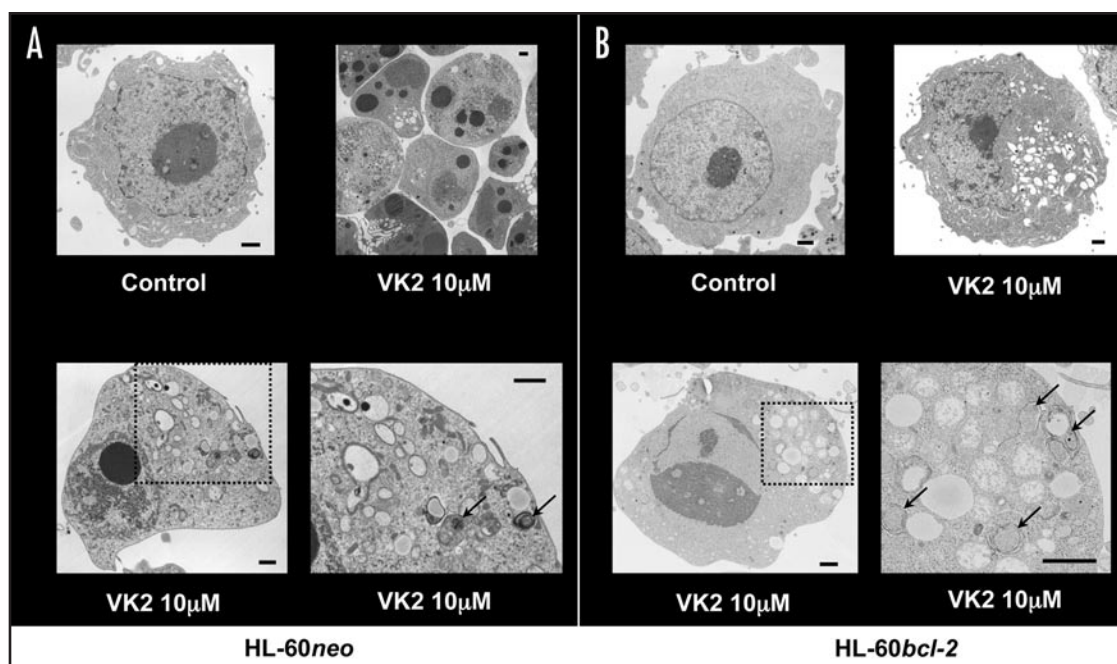


Figure 2. Ultrastructural features of HL-60neo/HL-60bcl-2 cells after exposure to VK2. HL-60neo/HL-60bcl-2 cells treated with or without 10  $\mu$ M of VK2 for 72 hr. Then the cells were fixed, and the electron microscopy was performed. (A) HL-60neo cells: upper left; untreated control cells, upper right and lower; VK2-treated cells. (B) HL-60bcl-2 cells: upper left; untreated cells, upper right and lower; VK2-treated cells. Arrows indicate autolysosomes.

vesicular organelles (AVOs) with acridine orange staining followed by flow cytometry as previously reported.<sup>29</sup> Figure 3C demonstrates that treatment with 10  $\mu$ M VK2 increased the bright red fluorescence intensity (y-axis) from 4.8% to 46.8% in HL-60neo cells and that from 4.7% to 47.3% in HL-60bcl-2 cells, respectively. These data further support the conclusion that autophagy is induced as well as apoptosis in HL-60neo cells. By increasing the concentration of VK2 at 20  $\mu$ M, the intensity of AVO decreased to 31.5% in HL-60neo cells, indicating suppression of autophagy. Under these conditions apoptosis masked autophagy by inducing rapid cell death.

Additionally, LC3 protein is known to exist in two cellular forms such as LC3-I and LC3-II. LC3-I is converted to LC3-II by conjugation to phosphatidylethanolamine, and the amount of LC3-II is a good early marker for the formation of autophagosomes.<sup>36,37</sup> During 96 hr exposure to VK2, we analyzed the amount of LC3-II and also assessed the cleaved forms of caspase-3 as marker for apoptosis in HL-60neo/bcl-2 cells. In HL-60neo cells, the amount of LC3B-II increased within 48 hrs of exposure to VK2. In HL-60 bcl-2 cells the amount of LC3B-II was detected after 72–96 hr exposure to VK2 (Fig. 3D). In addition, recent evidence suggests that the accumulation of LC3-II is more accurately represented in autophagic flux in the presence of lysosomal inhibitors.<sup>38</sup> In the presence of E-64-d and pepstatin A, endogenous LC3B-II increased after treatment with VK2 in both HL-60neo and HL-60bcl-2 cells (Fig. 3E). Immunoblotting with anti-caspase-3 mAb revealed that the cleaved form of caspase-3 was significantly increased after treatment with VK2 for 48–96 hr in HL-60neo cells, whereas the cleaved caspase-3 was almost undetectable in HL-60bcl-2 cells during the 96 hr-exposure to VK2 (Fig. 3D). These results demonstrated that both autophagy and apoptosis occurred at the same time in HL-60neo cells. In addition, autophagy was induced earlier in HL-60neo cells than in HL-60bcl-2 cells during exposure to VK2. This was further confirmed by electron microscopy

of HL-60neo cells without nuclear fragments but with chromatin condensation after treatment with VK2, which may represent the earlier phase of apoptosis. These cells showed autophagosomes and autolysosomes in the cytoplasm (Fig. 2A; lower). Interestingly, some HL-60bcl-2 cells with chromatin condensation also showed autophagosome formation (Fig. 2B; lower).

All data shown above indicated that autophagy can be induced both in HL-60neo and HL-60bcl-2 cells in response to VK2, but it becomes more prominent in HL-60bcl-2 cells because the cells are protected from rapid apoptotic cell death after 96 hr-treatment with VK2. In addition, HL-60neo cells undergo both autophagy and apoptosis. To confirm this evidence, we further examined the effect of Z-DEVD-FMK, a caspase-3 inhibitor, in HL-60neo cells. HL-60neo cells were cultured with Z-DEVD-FMK at various concentrations with/without 10  $\mu$ M VK2. As shown in Figure 4A, cell growth inhibition by VK2 was attenuated in the presence of 10  $\mu$ M and 100  $\mu$ M of Z-DEVD-FMK. Z-DEVD-FMK alone at these concentrations did not show any cytotoxic effect during 96 hr-exposure. Then, HL-60neo cells were treated with VK2 with/without 100  $\mu$ M of Z-DEVD-FMK for the assessment of autophagy induction. Since many cells were lost via apoptosis during 96 hr-exposure to VK2 without Z-DEVD-FMK, cellular proteins were lysed and protein contents were equalized for immunoblottings. Z-DEVD-FMK showed significant inhibition of VK2-induced caspase-3 activation as assessed by caspase-3 cleavage. Notably, autophagy induction assessed by LC3B-II formation was significantly enhanced in the presence of Z-DEVD-FMK after 96 hr exposure to VK2 (Fig. 4B). These data also supported that autophagy becomes more prominent by blocking apoptotic cell death.

**Mixed morphologic feature of autophagy and apoptosis in HL-60bcl-2 cells.** The data shown above led us to further examine the morphologic features of HL-60bcl-2 and HL-60neo cells in detail after exposure to VK2.

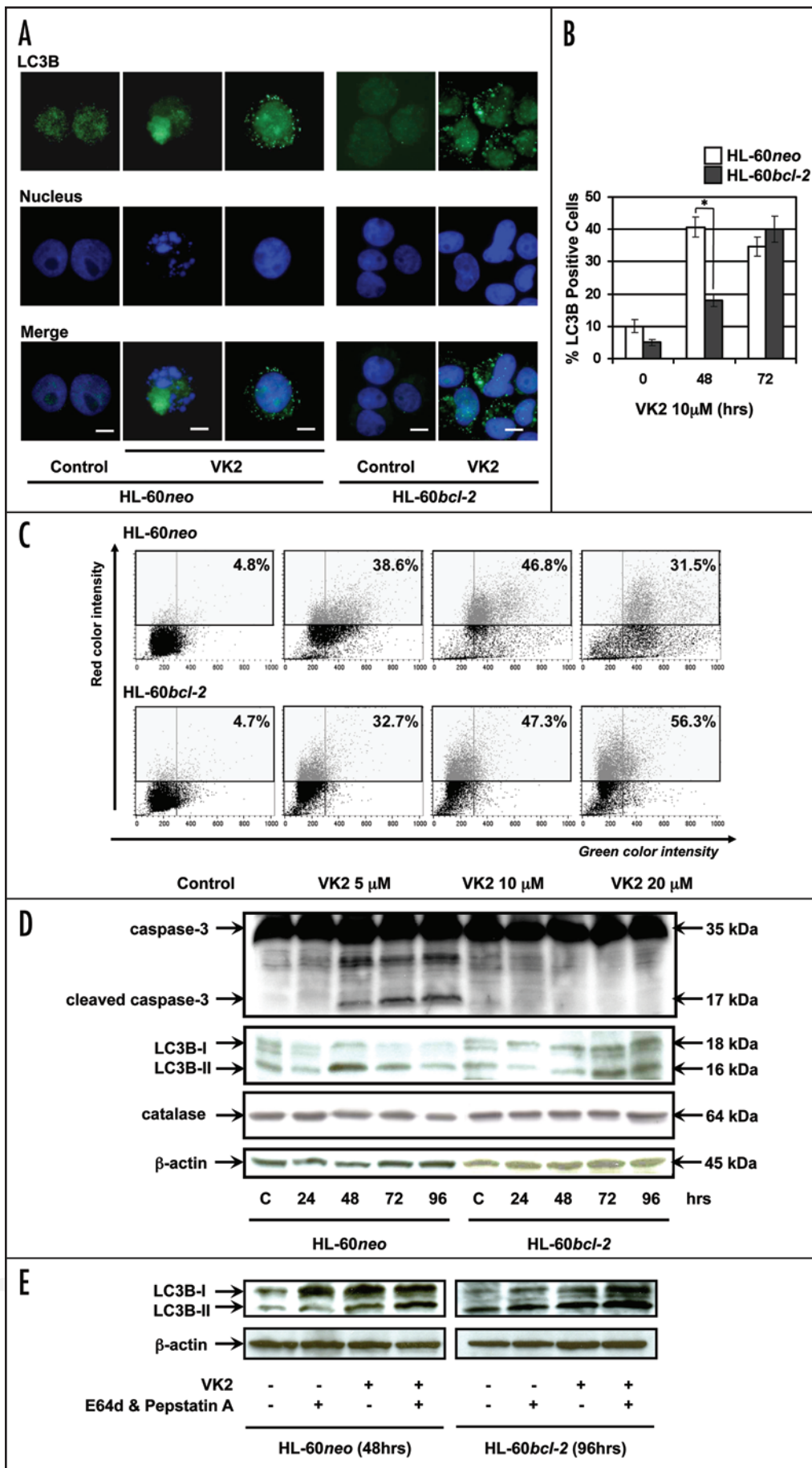


Figure 3. Induction of autophagy in response to VK2 in HL-60neo/HL-60bcl-2 cells. *Involvement of LC3B in VK2-induced autophagy:* After exposure to 10  $\mu$ M VK2 for 48 and 72 hr, the cells were processed for fluorescent immunocytochemistry with anti-LC3B Ab and counterstaining with DAPI for nucleus as described in Materials and Methods. (A) HL-60neo/bcl-2 cells (original magnification  $\times$  1,000). (B) Quantification of the cells showing the punctuated pattern of LC3B staining, which marks cells with autophagosome formation. One hundred cells were assessed and ratios for the cells showing the punctuated pattern of LC3B staining were expressed. Results shown are the means  $\pm$  SD from the results of three independent experiments. \* $p < 0.001$ . *Development of acidic vesicular organelles (AVOs) after VK2 treatment:* (C) After staining the cells with acridine orange, AVOs were quantified using flowcytometer in HL-60neo (upper) and HL-60bcl-2 (lower) treated with or without VK2 (5  $\mu$ M, 10  $\mu$ M or 20  $\mu$ M) for 72 hr. X-axis, green color intensity; Y-axis, red color intensity. *Expression of isoforms of LC3B and caspase-3:* (D) Cellular proteins were lysed at the indicated time after incubation with or without 10  $\mu$ M of VK2. Proteins were separated by either 11.25% or 15% SDS-PAGE. Aliquots of 40  $\mu$ g of protein extracts were used for immunoblotting using anti-caspase-3 and anti-LC3B Abs, respectively. The anti- $\beta$ -actin mAb was used for protein-loading equivalence. *Expression of isoforms of LC3B in the presence and absence of protease inhibitors:* (E) After treatment with 10  $\mu$ M of VK2 for 44 hrs in HL-60neo and for 92 hrs in HL-60bcl-2 cells, cells were further cultured with/without protease inhibitors, E-64-d (10  $\mu$ g/ml) and pepstatin A (10  $\mu$ g/ml) in the presence of VK2 for 4 hrs. Cellular proteins were lysed and immunoblotted with anti-LC3B Ab.

After exposure to VK2 for 96 hr, HL-60bcl-2 cells showed an increased percentage of fragmented nuclei compared to 72 hr treatment. Intriguingly, the morphologic findings in HL-60bcl-2 cells undergoing apoptosis were different from those in HL-60neo cells; each nuclear fragment appeared to be encapsulated by vesicles resulting in the formation of a “sunny side-up” appearance (Fig. 5A). A “sunny side-up” appearance was also observed in HL-60neo cells treated with VK2 in the presence of a caspase-3 inhibitor, Z-DEVD-FMK. Extended exposure to VK2 up to 6 days resulted in the disappearance

of this characteristic feature and the appearance of the typical apoptotic bodies like in HL-60neo cells treated with 72 hr exposure to VK2 (Fig. 5B). Therefore, this morphologic change in HL-60neo cells seems to be an early phase of cells undergoing apoptosis. To investigate whether the “sunny side-up” feature is macroautophagy of nuclear fragments, we examined the VK2 treated HL-60neo cells by electron microscopy. Fragmented nuclei with condensed chromatin appeared to be surrounded by double-membrane (Fig. 5C). However, fluorescent immunocytochemical staining with anti-LC3B Ab showed that nuclear fragments were not surrounded by punctuated LC3B. Therefore, “sunny side-up” feature does not appear to represent the macroautophagy of nuclear fragments.

**The biological roles of VK2-induced autophagy in HL-60neo/bcl-2 cells.** Since autophagy was initially recognized as a cellular survival process under amino acid starvation,<sup>23,24</sup> the concept of autophagic cell death as type II PCD and the significance of cancer treatment-induced autophagy is a subject of debate. Autophagy may function as an anti-cancer effect or a cytoprotective reaction against the cytotoxic reagents.<sup>20-22</sup> To assess the role of VK2-induced autophagy in HL-60 cells, we attempted to block the autophagic process in the presence or absence of VK2 using 3-MA, an inhibitor of class III phosphatidylinositol-3 kinase (PI3K),<sup>31</sup> and siRNA against Atg7, respectively. Treatment with 3-MA suppressed the PI-staining positive cells in HL-60neo/bcl-2 cells treated with VK2. 3-MA alone showed no effect on HL-60 cell growth. In addition, the decreased viability of HL-60neo and HL-60bcl-2 cells after VK2 treatment was reversed in the presence of 3-MA (Fig. 6A and B). Furthermore, 3-MA suppressed the induction of AVOs in HL-60neo/bcl-2 cells treated with VK2 (Fig. 6C). siRNA effectively suppressed Atg7 expression in HL-60neo/bcl-2 cells. Suppression of Atg7 resulted in significant attenuation of cell growth inhibition by VK2 in both cell lines (Fig. 6D). All these data support the conclusion that autophagic cell death was induced after treatment with VK2. To further confirm this interpretation, we used the Tet-off system with an Atg5<sup>-/-</sup> mouse embryonic fibroblast (MEF) cell line.<sup>33</sup> Pretreatment of Tet-off Atg5<sup>-/-</sup> MEF cells with 10 ng/ml doxycycline hydrochloride (Dox) for 120 hrs induced complete suppression of Atg5 expression. Thereafter, the cells were further cultured in the presence or absence of VK2 for 72 hrs. As shown in Figure 7, the VK2-induced cell growth inhibition at 10  $\mu$ M was significantly attenuated in Dox treated-MEF cells along with suppression of the induction of LC3B-II. These data also support the occurrence of autophagic cell death in response to VK2.

## Discussion

In the present study, we demonstrated that VK2 induces autophagy as well as apoptosis in HL-60 cells. In addition, the mixed

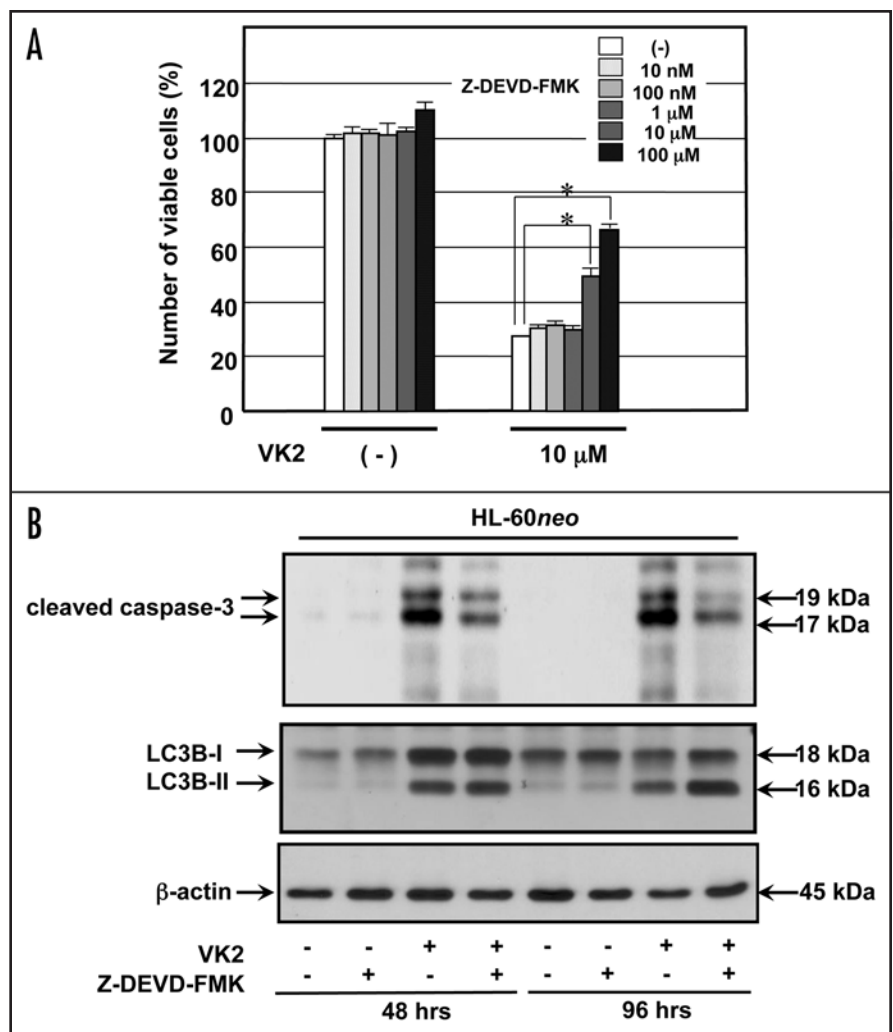


Figure 4. Effects of a caspase-3 inhibitor on VK2-induced autophagy in HL-60neo cells. (A) HL-60neo cells were cultured with Z-DEVD-FMK at various concentrations with/without 10  $\mu$ M of VK2 for 96 hr. The number of cells was assessed with the WST cell counting kit as described in Materials and Methods. \* $p < 0.001$ . (B) HL-60neo cells were treated with 10  $\mu$ M VK2 with/without 100  $\mu$ M of Z-DEVD-FMK for 48 hr and 96 hr, respectively. Cellular proteins were lysed and separated by either 11.25% or 15% SDS-PAGE. Aliquots of 40  $\mu$ g of protein extracts were used for immunoblotting using anti-cleaved caspase-3 Ab, anti-LC3B Ab, and anti- $\beta$ -actin mAb, respectively.

morphologic features of apoptosis and autophagy such as chromatin condensation and fragmented nuclei for apoptosis and autophagosomes and autolysosomes for autophagy were detected in the same cells (Fig. 2A and B). This suggests that the cellular processes for apoptosis and autophagy are simultaneously induced in response to VK2 as well as some previous reports in *Drosophila* and sympathetic neurons.<sup>39,40</sup> Immunoblottings for LC3B and caspase-3, which were performed for assessment of autophagy and apoptosis induction, also support this evidence (Fig. 3D). During extended exposure to VK2 in cells with higher expression of Bcl-2, autophagy became more evident, because cells with lower expression of Bcl-2 underwent apoptotic cell death and were eliminated from culture system. This indicates that the cellular expression levels of Bcl-2 appear to determine the phenotype of cell death. Induction of autophagy in cancer cells in response to various anti-cancer reagents has been reported: Temozolomide (TMZ), a DNA alkylating agent, and arsenic trioxide

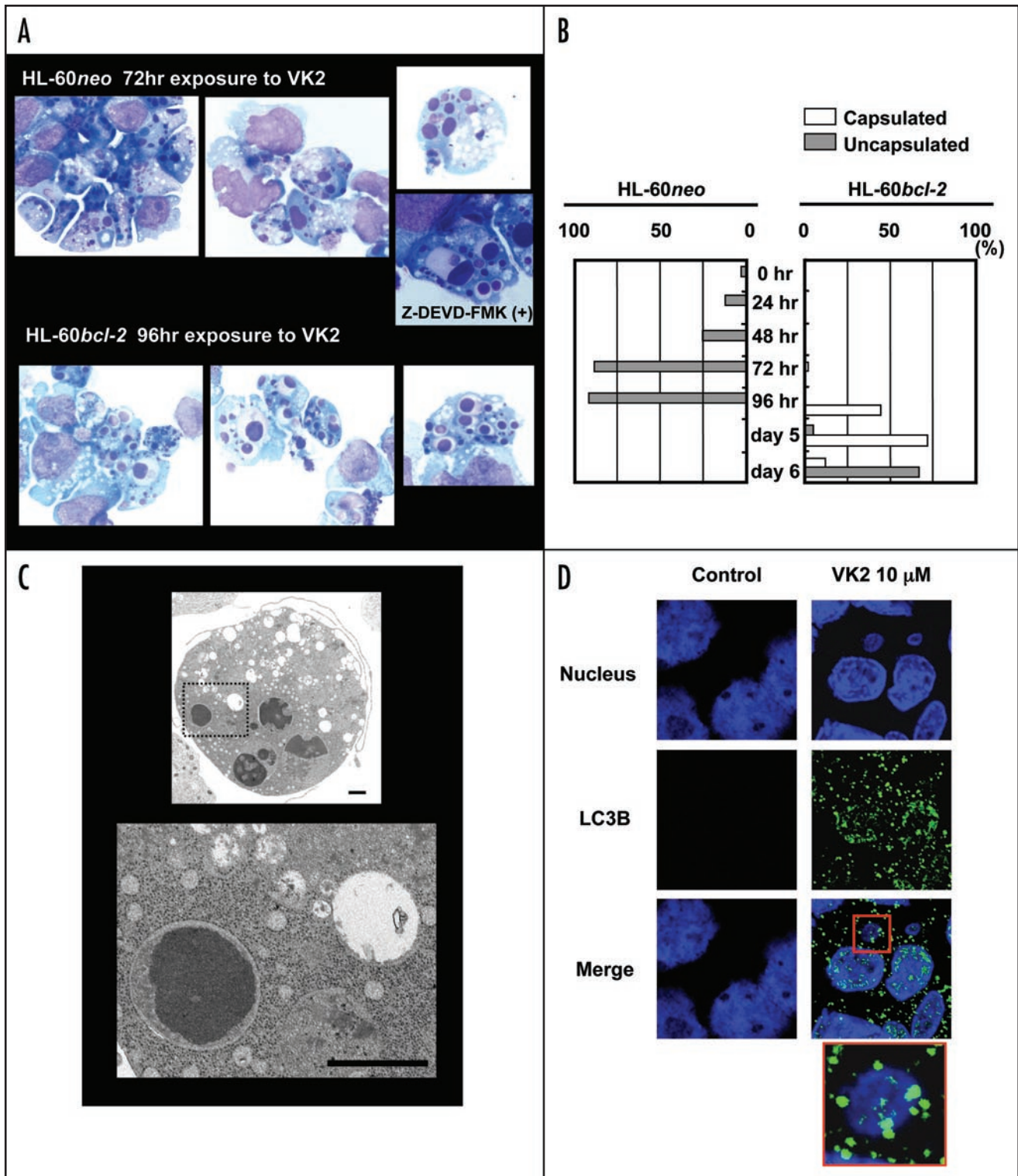
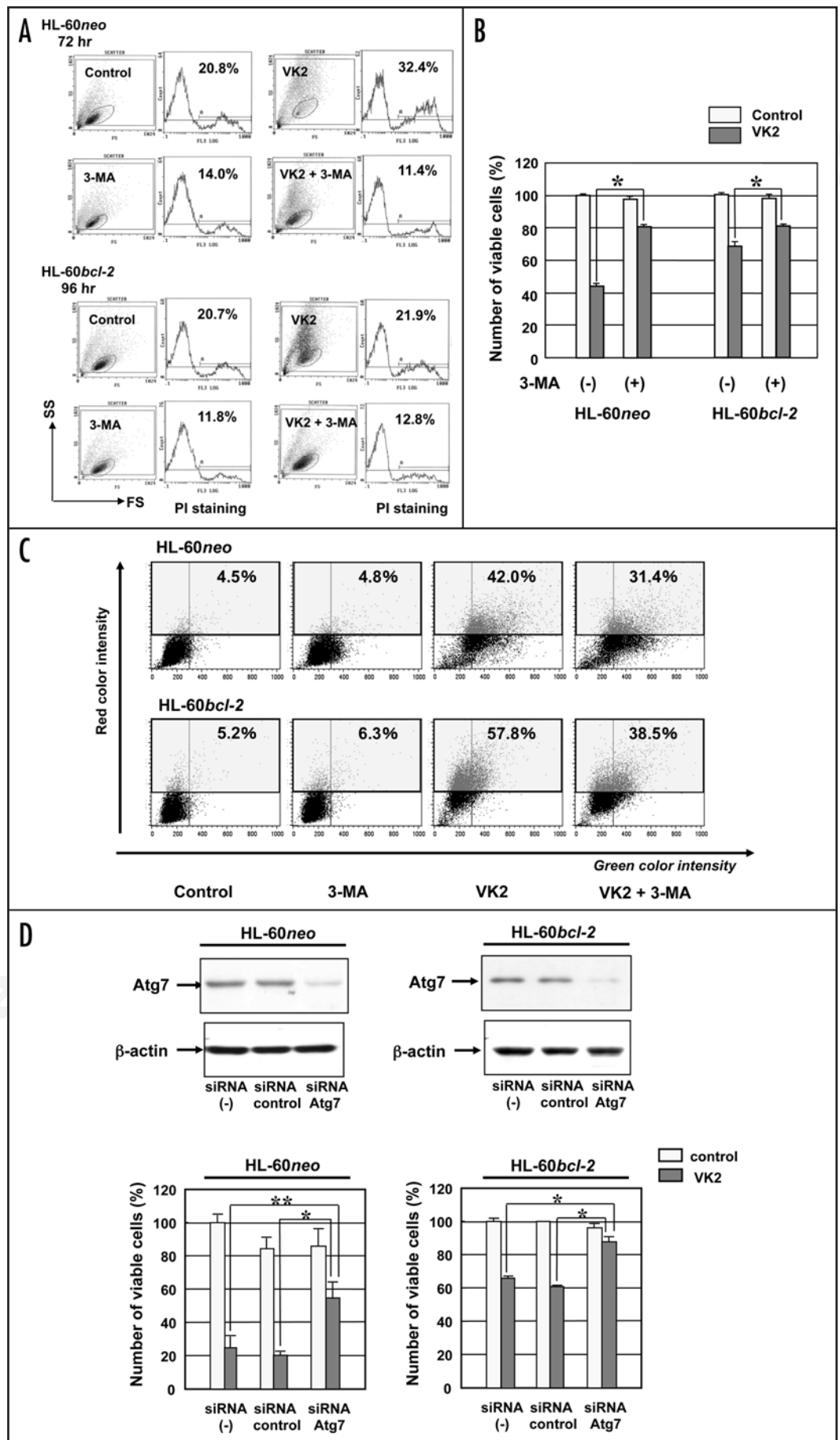


Figure 5. Capsulated fragmented nuclei in HL-60bcl-2 cells after treatment with VK2. (A) Morphological features of HL-60neo after 72 hr-treatment with VK2 (10  $\mu$ M) with/without Z-DEVD-FMK (100  $\mu$ M) and of HL-60bcl-2 cells after 96 hr-treatment with VK2. May-Grünwald-Giemsa staining, original magnification  $\times$  1,000. (B) Percentages of HL-60 cells with apoptotic bodies containing either capsulated nuclear fragments or uncapsulated nuclear. After treatment HL-60neo/bcl-2 cells with 10  $\mu$ M of VK2 for various length of time, morphologic changes were assessed in 100 cells after May-Grünwald Giemsa stain as well as Figure 4A. (This is one of representative result from 3 separate experiments.) (C) Electron microscopy of HL-60bcl-2 cells after treatment with 10  $\mu$ M VK2 for 96 hr. (D) Fluorescent immunocytochemical staining with anti-LC3B Ab in HL-60bcl-2 cells after 96 hr-treatment with 10  $\mu$ M VK2. Fluorescent immunocytochemical staining with anti-LC3B Ab (original magnification  $\times$  1,000) was performed as described in Figure 3A and C, respectively. The slides stained with anti-LC3B Ab were monitored using Zeiss LSM510 confocal microscope (Original magnification  $\times$  600).



Figure 6. Effects of inhibition of VK2-induced autophagy in HL-60neo/*bcl-2* cells. (A and B) To inhibit autophagy, 2 mM 3-MA was added to HL-60 cell cultures for 72 hr with or without VK2. Viable cell numbers were assessed using a flow cytometer as described in Materials and Methods. \**p* < 0.001 (C) Treatment with 3-MA suppressed the induction of acidic vesicular organelles (AVOs) in HL-60neo/*bcl-2* cells treated with 10 μM of VK2. AVOs were quantified with fluorescence-activated cell sorter in HL-60neo (upper)/HL-60*bcl-2* (bottom). x-axis, green color intensity; y-axis, red color intensity. (D) Inhibition of Atg7 protein expression by siRNA transfection: HL-60neo/*bcl-2* cells were transfected with random siRNA or siRNA for Atg7 for 72 hrs. Relative expression levels of Atg7 proteins were assessed by western blotting. (upper). After treatment with/without random siRNA or siRNA for ATG7, the cells were further treated with VK2 for 72 hrs. Thereafter, cell growth inhibition was assessed by WST cell counting kit (lower). \**p* < 0.001, \*\**p* < 0.05.

(As<sub>2</sub>O<sub>3</sub>) induce autophagy of malignant glioma cells, which are usually refractory to various anti-cancer therapies.<sup>30,41</sup> The histone deacetylase inhibitors sodium butyrates and suberoylanilide hydroxamic acid (SAHA) induce autophagic cell death in HeLa cells which overexpress the anti-apoptotic protein Bcl-X<sub>L</sub>, but they induce apoptosis in parental HeLa cells.<sup>42</sup> Murine L929 fibroblastic cells, murine RAW 264.7 macrophages, and human U937 leukemia cells underwent autophagy after treatment with *z*-VAD-fmk, a pancaspase inhibitor.<sup>43</sup> Etoposide induces autophagic cell death in Bax/Bak-double knockout MEF, and these cells also undergo protective autophagy under conditions of nutrition depletion.<sup>44</sup> These conditions for induction of autophagy by cytotoxic stimuli include cellular resistance against apoptosis or protection from apoptosis. Our observations showing autophagy in HL-60*bcl-2* rather than HL-60neo cells meet these conditions. However, our data also demonstrated that apoptotic and



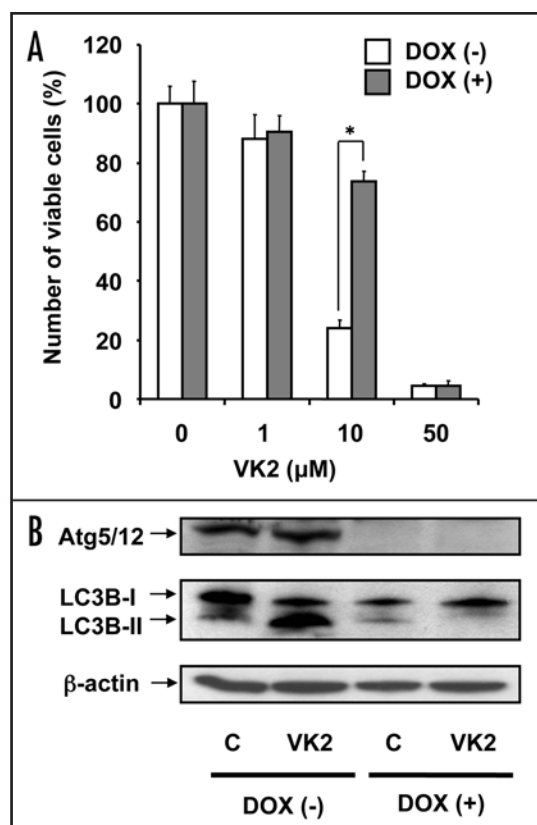


Figure 7. Effects of inhibition of VK2-inducing autophagy in an *Atg5*<sup>-/-</sup> mouse embryonic fibroblast (MEF) cell line with the *Atg5* Tet-off system. Tet-off *Atg5* MEF cells were incubated with 10 ng/ml doxycycline hydrochloride (Dox) for 120 hrs, and thereafter cultured in the presence or absence of 1 to 50 μM of VK2 for 72 hrs. (A) Cell growth inhibition in response to VK2 was assessed using WST assay kit. (B) MEF cells treated with or without Dox were cultured in the presence or absence of 10 μM of VK2 for 72 hr. Then, cellular proteins were separated by 11.25% SDS-PAGE and immunoblotted with either anti-*Atg5*, anti-LC3B, or anti-β-actin Abs.

autophagic processes can be simultaneously induced by VK2 (Figs. 2 and 3).<sup>39,40</sup> Therefore, cell death induced by cytotoxic reagents cannot be simply characterized in stereotyped manner such as type I PCD versus type II PCD.

Although autophagy became evident in HL-60*bcl-2* cells, previous reports have demonstrated that Bcl-2 negatively regulates induction of autophagy. Bcl-2 localized on endoplasmic reticulum was reported to interact with Beclin 1 and to inhibit Beclin 1-dependent autophagy.<sup>27</sup> This anti-autophagy function of Bcl-2 was suggested to help maintain autophagy at the levels that are compatible with cell survival, rather than cell death.<sup>27</sup> In addition, downregulation of Bcl-2 by the conditional expression of the full length *bcl-2* antisense message was reported to induce autophagic cell death in HL-60 cells,<sup>26</sup> and Bcl-2/Bcl-X<sub>L</sub> antagonist HA14-1 also induces autophagy and apoptosis in L1210 murine leukemia cells,<sup>45</sup> suggesting anti-autophagic function of Bcl-2. In our data, comparing autophagy induction between HL-60*neo* and HL-60*bcl-2* by the formation of punctuated LC3B, autophagy was rather enhanced in HL-60*neo* cells within 72 hrs of exposure to VK2 (Fig. 3B). Delayed induction of autophagy in HL-60*bcl-2* as assessed by conversion of LC3B type I to type II was also observed (Fig. 3D). These data agree with previous reports that Bcl-2 has a negative effect on the induction

of autophagy. However, since *bcl-2* knockout mice are viable with almost intact bone marrow functions,<sup>46</sup> other regulatory pathway(s) might be involved for autophagy, especially when the cells are exposed to cytotoxic reagents. It has been reported that intracellular calcium mediates the induction of autophagy in a Bcl-2 regulated fashion.<sup>47</sup> These findings raise the question as to how a cell decides to undergo either apoptosis or autophagy in response to calcium signals.

It was intriguing that some morphologic features of the same death pathway differed between HL-60*neo* and HL-60*bcl-2* cells. As shown in Figure 5A and B, nuclear fragments were capsulated in HL-60*bcl-2* cells but not in HL-60*neo* cells. Longer exposure to VK2 resulted in disappearance of this “sunny side-up” feature which seems to detect an early phase of apoptosis. These encapsulated nuclear fragments should be designated as “apoptotic bodies” as in previous reports.<sup>48</sup> The formation of “sunny side-up” may be only detectable under conditions for autophagy inductions along with a higher threshold for apoptosis such as overexpression of anti-apoptotic Bcl-2 proteins or in the presence of a caspase-3 inhibitor. However, we could not prove the macroautophagy formation of fragmented nuclei by LC3B fluorescence immunocytostaining (Fig. 5D) Biological significance of this distinct phenomenon remains to be cleared.

Although “autophagic cell death” has recently been clearly demonstrated in salivary gland cells during development of *Drosophila*,<sup>49</sup> the concept of autophagy as type II PCD is still controversial, especially the cell death induced by anti-cancer reagents.<sup>20-22</sup> In the presence of 3-MA, an inhibitor of type III PI3K, VK2-induced cell death was significantly suppressed not only in HL-60*bcl-2* but also in HL-60*neo* cells (Fig. 6A–C). This suggests that the VK2-induced autophagy induces cell death. However, 3-MA is not a specific inhibitor for autophagy and it may interact with other signaling pathways for cell death.<sup>22,37</sup> Knockdown of *Atg7* with siRNA prevented VK2-induced cell death (Fig. 6D). Tet-off *Atg5* MEF system also demonstrated an attenuation of VK2-induced cell growth inhibition along with inhibition of autophagy (Fig. 7). Therefore, the enhanced autophagy in response to VK2 is correlated with induction for cell death in our system. These lines of evidence strongly suggest “autophagic cell death” or “autophagy mediated cell death” in leukemia cells. However, the executor(s) for induction of cell death still remains to be cleared. Yu Li et al., reported that caspase inhibition leading to cell death by means of autophagy involves accumulation of reactive oxygen species (ROS), membrane lipid oxidation, and loss of plasma membrane integrity in L929 cells.<sup>50</sup> The accumulation of abnormal ROS was caused by the selective autophagic degradation of the catalase, which is the major enzymatic ROS scavenger.<sup>50</sup> The accumulation of ROS following degradation of catalase might be one of the executors of autophagic cell death when caspases are inhibited. However, as shown in Figures 1A and 3D, catalase expression was not different between HL-60*neo* and HL-60*bcl-2* cells, and no significant change of catalase expression during 96 hr exposure to VK2. Therefore, our observation cannot be explained by degradation of catalase. In fat body cells and wing discs of *Drosophila*, overexpression of *Atg1* itself was sufficient to induce autophagy along with apoptosis in a caspase-dependent manner.<sup>40</sup> This suggests that autophagy represents an alternative induction of apoptosis rather than a distinct form of cell death. In contrast, autophagy is activated by apoptotic signaling in sympathetic neurons.<sup>39</sup> In this system, the same apoptotic signal induces

mitochondrial dysfunction and also activates autophagy. Once it was activated, autophagy was suggested to mediate caspase-independent neuronal death.<sup>39</sup> It is noteworthy that knockdown of Atg7 using siRNA as well as 3-MA treatment showed attenuation of growth inhibition by VK2 in HL-60neo cells (Fig. 6D). Since apoptotic cell death should be dominant in HL-60neo cells, autophagy may function as an alternative inducer for apoptosis. This may be further supported by the distinct morphologic change in some but not all HL-60bcl-2 cells after longer exposure to VK2, which showed apoptotic bodies with “sunny side-up” feature (Fig. 4A and B).

Qu X et al., demonstrated that autophagy contributes to the clearance of dead cell by a mechanism that likely involves the generation of energy-dependent engulfment signals.<sup>51</sup> This suggests an extended role for autophagy in the disposal of dead cells by a phagocytic system in vivo. Although this novel function of autophagy for PCD was demonstrated in ES cells during embryonic development, it may also extend to cancer cells. Autophagy induction in response to VK2 may explain the selective clearance of a leukemic clone via the phagocytic system in vivo.<sup>2,6,11</sup> Autophagy induction by VK2 shown in this study may give us novel therapeutic tools in the treatment of leukemia.

## Materials and Methods

**Cell lines and reagents.** HL-60 cells obtained from the American Type Culture Collection (Rockville, MD) were maintained in continuous culture in RPMI 1640 medium (GIBCO, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT), 2 mM L-glutamine, penicillin (50 U/ml), and streptomycin (100 µg/ml). A clone stably transfected with the human *bcl-2* gene, HL-60bcl-2, was established by electroporation using a recombinant pdDNA3-plasmid-containing the human *bcl-2a* gene.<sup>26</sup> HL-60neo is a control cell line transfected with pdDNA3-plasmid alone.<sup>26</sup> VK2 (menaquinon-4) was supplied by Eisai Co., Ltd. (Tokyo, Japan). E-64-d and Pepstatin A, which are inhibitors for lysosomal proteases, were purchased from BIOMOL International L.P. (Plymouth Meeting, PA). Z-DEVD-FMK for a caspase-3 inhibitor was purchased from R & D Systems Inc. (Minneapolis, MN).

**Assessment of viable cell count.** HL-60 cells treated with or without VK2 were stained with a solution containing 1% (v/v) propidium iodide (PI) (Sigma-Aldrich, St. Louis, MO) for 30 min at 4°C. First, the gating area of cytogram for detecting viable HL-60 cells was established according to both the PI staining-negative area (indicating viable cells) and the forward- and side- scatter intensities.<sup>2</sup> Then the cell cultures were pipetted gently to obtain uniform cell suspension, and were introduced to a flow cytometer. The number of cells in gating area for viable HL-60 cells was assessed for 60 sec. The number relative to the cells treated with control medium revealed to be well-correlated with the results obtained from a WST Cell Counting Kit (Dojin East, Tokyo, Japan), with absorption measurements at 450 nm.<sup>2</sup> In some experiments viable cell counts were assessed by trypan blue dye exclusion.

**Quantitative detection of acid vesicular organelles (AVOs) with acridine orange staining.** Autophagy is the process of sequestering cytoplasmic proteins and organelles into the lysosomal component and characterized by development of AVOs. To detect and quantify AVOs in VK2-treated cells, we performed vital staining with acridine orange (Polysciences, Warrington, PA) as described previously.<sup>29,30</sup>

To inhibit autophagy 2 mM 3-methyladenine (3-MA) (Polysciences Inc, Warrington, PA), an inhibitor of the phosphatidylinositol 3-kinase (PI3K),<sup>31</sup> was added to the cultures for 72 hr with/without VK2. HL-60 cells were stained with 1.0 µg/ml acridine orange for 15 min at room temperature and processed for flow cytometry using the FACScan Cytometer (Becton Dickinson, San Jose, CA) and analyzed with CellQuest software (Becton Dickinson).

**Immunoblotting.** Cells were lysed in Lysis Buffer (10 mM Tris-HCL PH 7.8, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 0.15 U/ml aprotinin, 10 mg/ml leupeptine, 100 mM sodium fluoride, 2 mM sodium orthovanadate ), and cellular proteins were quantified using the Protein Assay kit of Bio-Rad (Richmond, CA). Equal amounts of proteins were loaded, separated by SDS-PAGE and transferred onto Hybond-P membranes (Amersham Biosciences Corp., Piscataway, NJ). The membranes were probed with antibodies (Abs) such as anti-human Bcl-2 monoclonal (m) Ab (BD Biosciences Pharmingen, San Jose, CA), anti-microtubule-associated protein 1 light chain 3B (LC3B) Ab, anti-caspase-3 Ab (Cell Signaling Technology, Danvers, MA), anti-cleaved caspase-3 Ab (Cell Signaling Technology), anti-Atg5 Ab (Abgent, San Diego, CA), anti-Atg7 Ab (ProSci, Poway, CA), anti-human catalase rabbit mAb (Epitomics Inc., Burlingame, CA), and anti-β-actin Ab (Sigma-Aldrich), respectively. Anti-LC3B Ab was generated as previously described.<sup>32</sup> Immunoreactive proteins were detected by horseradish peroxidase-conjugated second Ab and an enhanced chemiluminescence reagent (ECL) (Amersham Biosciences Corp.).<sup>3</sup>

**Assessment of apoptosis.** Apoptosis was detected by morphology, flow cytometry using Annexin V-florescein isothiocyanate (FITC) apoptosis detection kit I (BD Biosciences, San Diego, CA), and by immunoblotting with either anti-caspase-3 or anti-cleaved caspase-3 Abs as described above. For flow cytometry, HL-60 cells treated with or without VK2 were washed twice with ice cold phosphate-buffered saline and then resuspended in 1 X binding buffer (1 x 10<sup>6</sup> cells/ml). 100 µl of cell suspension (1 x 10<sup>5</sup> cells) was then transferred to a 5 ml tube, and 5 µl of Annexin V-PE and 5 µl of 7-amino-actinomycin were added. After 15 min of incubation at room temperature in the dark, 400 µl of 1 X binding buffer was added and the tubes were analyzed in a FACScan instrument with CellQuest software (Becton Dickinson). For morphologic assessment for apoptosis, the cell suspension was sedimented in a Shandon Cytospin II (Shandon, Pittsburgh, PA), and preparations were stained with May-Grünwald-Giemsa and with 4'-6-diamidino-2-phenylindole (DAPI, Vector laboratories, Inc., Burlingame, CA).

**Measurement of mitochondrial membrane potential.** Mitochondrial membrane potential was measured with tetramethyl rhodamine ethyl ester perchlorate (TMRE: Molecular Probe Inc., Eugene, OR) using flow cytometry as described previously.<sup>30</sup> Cells were incubated with 150 nM TMRE at 37°C for 20 min in the dark and analyzed by FACScan using CellQuest software. The fluorescent dye TMRE is accumulated by mitochondria as a result of the mitochondrial membrane potential. Shift to the left in the emission spectrum indicates the depolarization of mitochondrial membrane potential in the apoptotic cells.

**Electron microscopy.** HL-60 cells were treated with/without VK2 and then fixed with a solution containing 3% glutaraldehyde plus 2% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.3) for

1 hr. The samples were further post-fixed in 1% OsO<sub>4</sub> in the same buffer for 1 hr, and subject to the electron microscopic analysis using an electron microscope H-7000 (Hitachi, Tokyo, Japan) as described previously.<sup>30</sup> Representative areas were chosen for ultra-thin sectioning and viewed with an electron microscope.

**Fluorescent immunocytostaining.** Cells were fixed on glass slide by Shandon Cytospin II followed by further fixation with 4% paraformaldehyde for 20 min. After fixation, the cells on the slides were incubated with anti-LC3B Ab (1:5,000 dilution) in 0.5% Triton X-100-phosphate-buffered saline (PBS) overnight at 4°C. The slides were then washed with PBS and incubated with Alexa Fluor 488-labeled secondary goat anti-rabbit Ab (1:200 dilution) for 1 hr at room temperature. The slides were mounted with DAPI and monitored under an Axioskop 40 (Carl Zeiss MicroImaging, Inc., Thornwood, NY) or LSM 510 (Carl Zeiss MicroImaging).

**Small interfering RNA (siRNA) experiments.** siRNA oligonucleotides for human Atg7 (accession # NM-006395, catalog # L-020112), and siCONTROL non-targeting siRNA (catalog # D-001220) were purchased from GE Healthcare UK Ltd. (Buckinghamshire, England), and resuspended in RNase-free H<sub>2</sub>O at 20 μM. HL-60*bcl-2* cells were washed twice in serum-free media and resuspended to 1 × 10<sup>7</sup> cells per 500 μl of cold, serum-free OPTI-MEM (GIBCO), and transferred to pre-chilled 0.4 cm-gap electroporation cassettes (Bio-Rad). Cells were mixed with/without siRNA at 20 nM (final concentration) on ice for 5 min, thereafter, cells were pulsed once at 250 mV, 960 μF by using a Bio-Rad electroporator. The cell suspensions were then transferred to the incubator in 5% CO<sub>2</sub> at 37°C. Five hr after electroporation, the same volume of RPMI 1640 medium containing 20% FBS was added. Forty-eight hr after electroporation, protein knockdown was determined by immunoblotting, and the cells were treated with the indicated concentration of VK2 for 72 to 96 hr, and viable cells were assessed as previously described.

**Tet-off system with an Atg5<sup>-/-</sup> mouse embryonic fibroblasts (MEFs).** The Atg5 Tet-off MEF cell line was a kind gift from Dr Noboru Mizushima (Tokyo Medical and Dental University School of Health Science, Tokyo, Japan).<sup>33</sup> Atg 5 expression can be completely suppressed when this cell line is cultured in the presence of doxycycline. The culture condition is precisely described in elsewhere.<sup>33</sup>

**Statistics.** All data are given as the mean ± SD. Statistical analysis was performed by using Mann-Whitney's U test (two-tailed). The criterion for statistical significance was taken as p < 0.05.

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