Biochemical and Biophysical Research Communications 430 (2013) 876–882

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Honokiol induces caspase-independent paraptosis via reactive oxygen species production that is accompanied by apoptosis in leukemia cells

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ARTICLE INFO

Article history:
Received 25 November 2012
Available online 21 December 2012

Keywords:
Honokiol
Cytoplasmic vacuolization
Programmed cell death
Paraptosis
Apoptosis

ABSTRACT

Our previous report has shown that honokiol (HNK), a constituent of *Magnolia officinalis*, induces a novel form of non-apoptotic programmed cell death in human leukemia NB4 and K562 cells. In this study, we further explored the relationship between the cell death pathway and cytoplasmic vacuolization and studied the underlying mechanism of leukemia cell death mediated by honokiol. The results showed that low concentrations of honokiol activated an novel alternative cell death fitted the criteria of paraptosis, such as cytoplasmic vacuolization derived from endoplasmic reticulum swelling, lack of caspase activation, and lack of apoptotic morphology. Results further indicated that the cell death was time- and concentration-dependent. In addition, honokiol-induced paraptosis did not involve membrane blebbing, chromatin condensation and phosphatidylserine exposure at the outer of the plasma membrane. The mechanism of the cell death may be associated, at least in part, with the increased generation of reactive oxygen species. Further analysis showed that honokiol induces cell death predominantly via paraptosis and to a certain extent via apoptosis in NB4 cells, and predominantly via apoptosis and to a certain extent via paraptosis in K562 cells. These observations suggest that cell death occurs via more than one pathway in leukemia cells and targeting paraptosis may be an alternative and promising avenue for honokiol in leukemia therapy.

1. Introduction

Several compounds have been reported to induce the formation of vacuoles that can be visualized by light microscopy in various types of cultured cells. Taxol, artesunate and procaine induced numerous small and large cytoplasmic vacuoles in cancer cells in vitro [1–3].

Cell death has been classified into two main types: programmed cell death (PCD) and passive (necrotic) cell death. PCD is morphologically classified into three main types [4], including apoptosis, autophagy and non-lysosomal vacuolated degeneration, the latter of which includes oncosis and paraptosis. Paraptosis is a new type of PCD, characterized by cytoplasmic vacuolization derived from endoplasmic reticulum and/or mitochondria swelling, caspase-independent, and lack of apoptotic morphology. This type of cell death is also insensitive to apoptotic and autophagic inhibitor, lack of DNA fragment and poly (ADP-ribose) polymerase (PARP) cleavage, mediated by mitogen-activated protein kinases and inhibited by AIP/Alix [4,5]. Inhibitors of the MEK and p38 pathways prevented the appearance of cytoplasmic vacuolization [6].

Honokiol (HNK) is a pure compound isolated from *Magnolia officinalis* Rehd. et Wils. Extract. HNK-induced apoptosis may be either caspase-dependent or -independent [7,8]. Our previous studies have shown that low concentrations of HNK induces paraptosis-like cell death characterized by cytoplasmic vacuolization in human leukemia NB4 and K562 cells [9]. In the current study, we aimed to further investigate the origin of the cytoplasmic vacuoles and their relationship to various potential cell death pathways, and the underlying mechanism of leukemia cell death following the induction of cell death by HNK in the NB4 and K562 cell lines. These observations enhance our understanding of the mechanism of action of HNK on leukemia cells and will enable further investigations into the development of new therapies for leukemia.

2. Materials and methods

2.1. Reagents

HNK with a purity of up to 99.5%, kindly provided by Dr. Youfu Luo (State Key Laboratory of Biotherapy, Western China Hospital, Sichuan University, China), was dissolved in 40% dimethylsulfoxide

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http://dx.doi.org/10.1016/j.bbrc.2012.12.063

(DMSO) to make a stock solution of 20 mM. Imatinib mesylate (98.00%), purchased from 3B Pharmachem. International Co., Ltd. (Wuhan, China), was dissolved in 50% DMSO to make a stock solution of 10 mM. The two solutions were diluted at least 1000-fold in the growth medium such that the final concentration of DMSO had no effect on the differentiation and proliferation of cells. Doxorubicin (DOX, Wanle Pharmaceutical Company, Shenzhen, China), was dissolved at 20 mg/L in RPMI 1640 medium. Trypan blue, Hoechst 33342, 4′,6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and *N*-acetyl-L-cysteine (NAC) were purchased from Sigma (St. Louis, Mo, USA). The pan-caspase inhibitor, Z-VAD-fmk, was purchased from Beyotime Institute of Biotechnology (Jiangsu, China). All stock solutions of drugs were further diluted to appropriate concentrations with cell culture medium immediately before use. DMSO (0.1%) was used as vehicle control for all assays.

2.2. Cell lines

NB4 and K562 cells were provided by Key Lab of Hematology of Sichuan Province (West China Hospital, Sichuan University) and cultured in RPMI 1640 medium (Hyclone, UT, USA) supplemented with 10% fetal bovine serum (FBS), streptomycin (100 μg/ml) and penicillin (100 U/ml) at 37 °C in a humidified atmosphere of 5% CO_2. Exponentially growing cells were used for all experiments at a concentration of 0.2–1.0 × 10^6 cells/ml.

2.3. Detection of cytoplasmic vacuolization

NB4 and K562 cells were grown on 24-well plates and treated with the indicated concentrations of HNK for 24, 48 or 72 h. At these time points, the cells were visualized by light microscopy and photographed. Both light and fluorescent microscopic images were taken from representative fields in one of the quadruplicate wells for each experimental time point, and all experiments were performed independently at least three times. All experimental images were processed in the same manner.

2.4. Detection of membrane permeability by PI and trypan blue staining

NB4 and K562 cells were grown on 24-well plates and exposed to 15 or 20 μM HNK for 48 h (NB4 cells), or 40 or 50 μM HNK for 24 h (K562 cells), and the cells were then stained with trypan blue (0.4% in phosphate buffered saline) and analyzed by light microscopy. For PI staining, cells were stained with PI (10 μg/ml) and analyzed by light and fluorescence microscopy in the same field of view.

2.5. Detection of caspase activation and signs of apoptosis in cells with cytoplasmic vacuolization

To determine whether cytoplasmic vacuolization was due to a caspase-dependent pathway, NB4 cells were grown on 24-well plates and treated with HNK at 20 μM for 24 h or a combination of both 50 μM Z-VAD-fmk and 20 μM HNK for 24 h and analyzed by light microscopy. As a positive control, NB4 cells were treated with an increased concentration of 35 μM HNK for 24 h or a combination of both 50 μM Z-VAD-fmk and 35 μM HNK for 24 h, and the cells were then stained with 1 μg/ml DAPI for 30 min, washed with cell culture medium and imaged by fluorescence microscopy.

To determine whether cytoplasmic vacuolization was associated with apoptotic markers, NB4 cells were treated with 30 μM HNK for 24 h and K562 cells were treated with 40 μM HNK for 48 h. Cells were then stained with 1 μg/ml DAPI for 30 min and imaged by light and fluorescence microscopy in the same field of view.

2.6. Washout assay

In order to test whether the extensive cytoplasmic vacuolization was reversible, a washout assay was performed. NB4 cells were treated with 20 μM HNK and analyzed by light microscopy at various time points (4, 6, 10, 18, 24, 36, 44, 48, 60, 72 and 80 h). At each time point, the cells were collected, washed twice with PBS, re-suspended in complete medium, and returned to the incubator for a further 12 h prior to analysis by light microscopy.

2.7. Analysis of the role of reactive oxygen species (ROS) in HNK-induced cytoplasmic vacuolization

NB4 cells were pretreated with NAC (10 mM) for 1 h, following which 15 or 20 μM HNK was added and the cells were incubated for an additional 18 h and analyzed by light microscopy.

2.8. Detection of phosphatidylserine (PS) externalization in the cells undergoing cytoplasmic vacuolization and apoptosis

PS externalization analysis was performed as described previously [10]. FITC-conjugated annexin V was added according to the instructions provided with the annexin V-FITC/PI apoptosis detection kit (Nanjing Keygene, China). The samples were analyzed by light microcopy and fluorescence microscopy in the same field of view. Cells that were annexin V (−) and PI (−) were considered viable cells, cells that were annexin V (+) and PI (−) were considered early-stage apoptotic cells, and cells that were annexin V (+) and PI (+) were considered late-stage apoptotic or necrotic cells [11].

2.9. Transmission electron microscopic (TEM) analysis of cytoplasmic vacuolization

NB4 cells were treated with 20 μM HNK and K562 cells were treated with 40 μM HNK for 20 h, respectively. Ultrastructure of the cells was examined using a transmission electron microscope (H-600IV; Hitachi Co., Tokyo, Japan) as described previously [9].

3. Results

3.1. HNK induced cytoplasmic vacuolization and which was time- and concentration-dependent

HNK-treated NB4 and K562 cells exhibited dramatic cytological changes, including altered size, shape and overall appearance compared with untreated viable cells, which remained compact and uniformly round (Fig. 1). The largest vacuoles were induced by a HNK concentration of 20 μM for 24 or 48 h in NB4 cells. On increasing the concentration to 25 μM, the vacuoles in each cell became smaller and sieve-like in appearance. Cell death occurred in NB4 cells following treatment with 25 or 30 μM HNK for 72 h. However, vacuolization was induced in K562 cells following treatment with 40 μM HNK for 24 h, 30 μM HNK for 48 h, and 40 μM HNK for 72 h. Cytoplasmic vacuolization and cell death appeared at the same time when K562 cells were treated with 50 μM HNK for 72 h. Used as controls, imatinib mesylate, a potent and specific inhibitor of receptor tyrosine kinases, and doxorubicin (DOX) induced different morphological effects on K562 cells and NB4 cells, which fragmented into small particles.

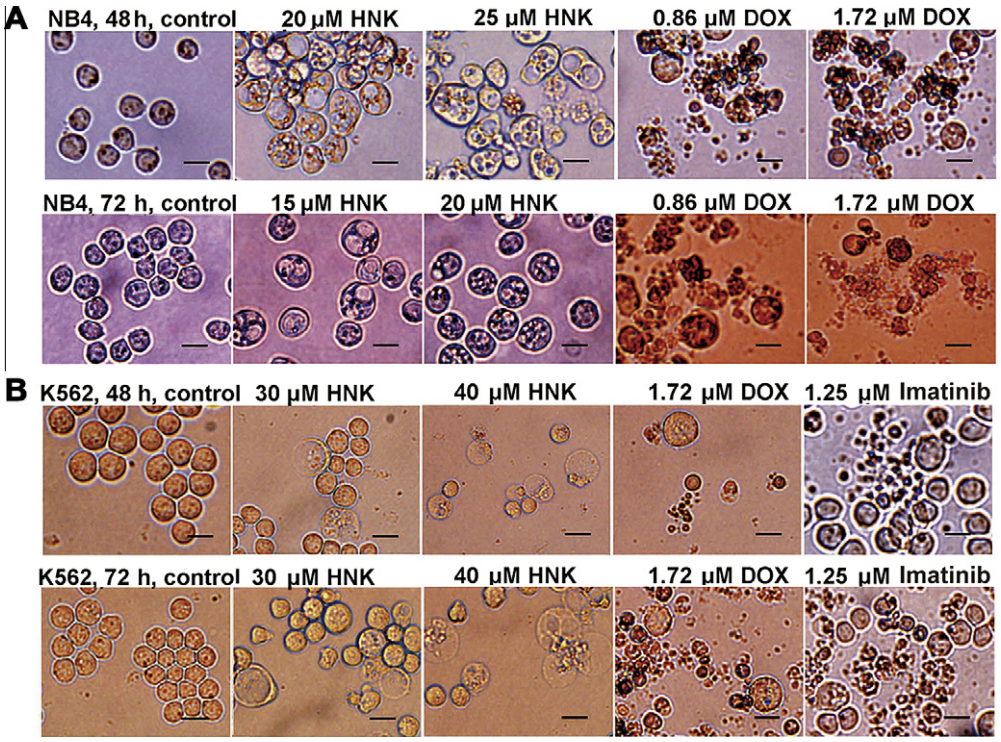

Fig. 1. Phase-contrast micrographs of extensive cytoplasmic vacuolization in honokiol-induced NB4 and K562 cells. Doxorubicin- or imatinib mesylate-induced morphological changes in cells are shown in comparison with honokiol (HNK). Observations were replicated on at least four different experimental days. Magnification: 400×, scale bar: 10 μm.

3.2. The plasma membranes of NB4 and K562 cells with cytoplasmic vacuolization were intact

PI and trypan blue staining were used to verify the permeabilization of cell membranes. These studies indicated that cytoplasmic vacuolization induced in cells by HNK did not result in entry of trypan blue or PI, i.e., the plasma membranes were intact in NB4 and K562 cells with cytoplasmic vacuolization (Fig. 2A and B).

3.3. Cytoplasmic vacuolization was not due to a caspase-dependent pathway and was not associated with apoptotic markers

To demonstrate that cytoplasmic vacuolization was not due to a caspase-dependent pathway, caspase activation experiments were performed. In order to prove that 50 μM Z-VAD-fmk was sufficient to inhibit the caspase-activated pathway in NB4 cells, the concentration of HNK was increased to 35 and 50 μM Z-VAD-fmk was added to inhibit the caspase-activated pathway, following which the cells were stained with DAPI (a DNA-specific dye) to observe nuclear morphological changes. This experiment was performed as a positive control for the activity of Z-VAD-fmk (Fig. 2C, panel (c) and (d)).

Although 50 μM Z-VAD-fmk did not inhibit cytoplasmic vacuolization (Fig. 2C, panel (a) and (b)), it did inhibit HNK-induced apoptosis in NB4 cells almost completely (characteristic morphological changes such as chromatin condensation and nuclear fragmentation were observed) when the concentration of HNK was increased to 35 μM (Fig. 2C, panel (c) and (d)), i.e., 50 μM Z-VAD-fmk almost completely inhibited HNK-induced caspase activation. These results demonstrated that caspase inhibition had no effect on the appearance of vacuoles in the presence of 50 μM Z-VAD-fmk in NB4 cells (Fig. 2C, panel (a) and (b)), suggesting that HNK-induced cytoplasmic vacuolization was caspase-independent.

Further investigations were performed to determine whether apoptotic markers (chromatin condensation and nuclear fragmentation) were present in the induced cells with vacuolization by DAPI staining. The results demonstrated that at 24 or 48 h post-induction, the nuclei in both NB4 and K562 cells with extensive vacuolization exhibited morphologically normal nuclei and no evidence of bright staining with DAPI or chromatin condensation (Fig. 2D).

3.4. HNK-induced cytoplasmic vacuolization was a time-dependent process and eventually led to cellular apoptosis or necrosis

We investigated whether HNK-induced cytoplasmic vacuolization could be reversed by a washout assay. By extending the time of HNK treatment, the degree of cytoplasmic vacuolization was enhanced when NB4 cells were treated with HNK for 48, 60, 72 or 80 h (Fig. 3A). The degree of vacuolization in most cells returned to normal, but a small amount of damage began to appear in some cells at the 48 h time point. At the 60 h time point, vacuolization returned to normal in some cells, but necrosis was apparent in other cells. DAPI staining showed that apoptotic cells and necrotic cells continued to increase at the 72 h time point, revealing that cellular vacuolization was time-dependent process and eventually led to cellular apoptosis or necrosis.

3.5. Cytoplasmic vacuolization was induced by HNK partially through the generation of reactive oxygen species (ROS)

To explore the role of ROS in HNK-induced cytoplasmic vacuolization, the general antioxidant, *N*-acetyl-L-cysteine (NAC), which serves as both a reduction–oxidation buffer and a reactive oxygen intermediate scavenger [12], was used. Light microscopic imaging results showed that HNK-induced morphological changes were partially abolished by treatment with NAC (Fig. 3B).

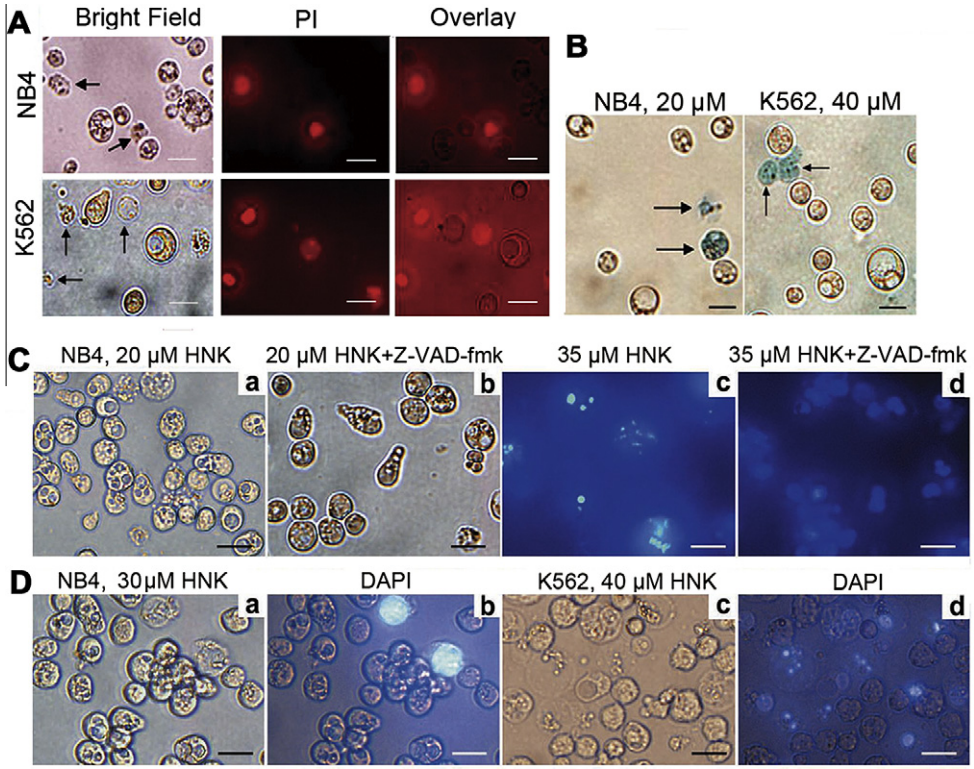

Fig. 2. Cells with cytoplasmic vacuolization possess an intact plasma membrane. Membrane permeability was analyzed by PI staining (A) and trypan blue staining (B). The black arrows indicate positive cells. Scale bar: 15 μm (A) and 10 μm (B), respectively. (C) Inhibition of caspase activation does not block cytoplasmic vacuolization. HNK-induced apoptosis (c) was completely blocked by Z-VAD-fmk (d), which served as a positive control (c and d). HNK-induced cytoplasmic vacuolization (a) was not affected by treatment with Z-VAD-fmk (b). (D) Panel (a) and (c) shows light microscopic images of representative NB4 and K562 cells, respectively. Panel (b) and (d) shows fluorescent microscopic images of the same NB4 and K562 cells stained with DAPI, respectively. Microscopic analysis of DAPI stained cells does not reveal brightly stained nuclei in vacuolized cells. Magnification: 400×. Scale bar: 10 μm.

3.6. HNK induced apoptosis and cytoplasmic vacuolization in cells in the absence of phosphatidylserine (PS) externalization

It is well-accepted that the appearance of phosphatidylserine (PS) on the surface of the plasma membrane is a universal phenomenon that occurs in cells undergoing apoptosis [13], Exposure of PS on the surface of cells can be identified easily by analysis with fluorescently-labeled annexin V, which specifically binds to PS. Plasma membrane integrity was simultaneously assessed by PI exclusion staining. As shown in Fig. 3C, vacuolized cells were not stained by annexin V-FITC or by PI, which further demonstrated that the cells with cytolasmic vacuolization were not undergoing necrosis or apoptosis. In addition, cells with cytoplasmic vacuolization, early apoptotic cells, late apoptotic cells and necrotic cells were all observed.

3.7. Ultrastructural analysis confirmed that HNK induced cytoplasmic vacuolization through swelling of the endoplasmic reticulum (ER)

The control cells exhibited an intact cellular morphology with ultrastructurally normal nuclei and organelles (Fig. 4(a), (b), (e) and (f)). In contrast, HNK-treated NB4 or K562 cells exhibited numerous small and large cytoplasmic vacuoles. However, mitochondria remained unaffected (Fig. 4(d) and (h)). High magnification electron micrographs of HNK-treated cells revealed the presence of ribosomes along the edges of the vacuoles, confirming that the small and large cytoplasmic vacuoles were derived from the swollen ER. In addition, HNK-induced cytoplasmic vacuoles were not observed to contain organelles or cytoplasm, i.e., no ultrastructural evidence of autophagy was observed.

4. Discussion

Extensive cytoplasmic vacuolization was thought to be associated with necrosis and necroapoptosis [14,15]. Li et al. have reported that HNK induces necrotic cell death in HL60 cells, MCF-7 cells and HEK293 cells [16]. In this study, we found that the HNK-induced cytoplasmic vacuolization was time- and concentration-dependent, and that the amount and size of vacuoles observed in leukemia cells were correlated with the cell type.

Damage to the cell membrane is one of the characteristics of oncosis [15,17]. Oncosis is defined as a form of cell death accompanied by cytoplasmic vacuolization, cellular swelling, membrane blebbing and increased membrane permeability [18,19]. The loss of structural integrity of the plasma membrane is also a hallmark of necrosis. The plasma membranes of NB4 and K562 cells with cytoplasmic vacuolization were impermeable to PI and trypan blue, suggesting that the vacuolization did not alter plasma membrane integrity. Our results therefore indicated that HNK-induced cytoplasmic vacuolization was neither necrosis nor oncosis.

The use of the pan-caspase inhibitor, Z-VAD-fmk, failed to prevent cytoplasmic vacuolization in NB4 cells, suggesting that it occurs through a non-caspase pathway, which is consistent with previous reports [5]. To further characterize the apoptosis–vacuolization association, the nuclei of highly vacuolated cells were examined by DAPI staining. This study indicated that none of the vacuolized cells contained apoptotic bodies or condensed chromatin in their nuclei. These data were also in agreement with our electron microscopic observations, suggesting that cellular vacuolization was not involving apoptosis.

The results of washout assay showed that cytoplasmic vacuolization returned to normal at the early time points, but eventually

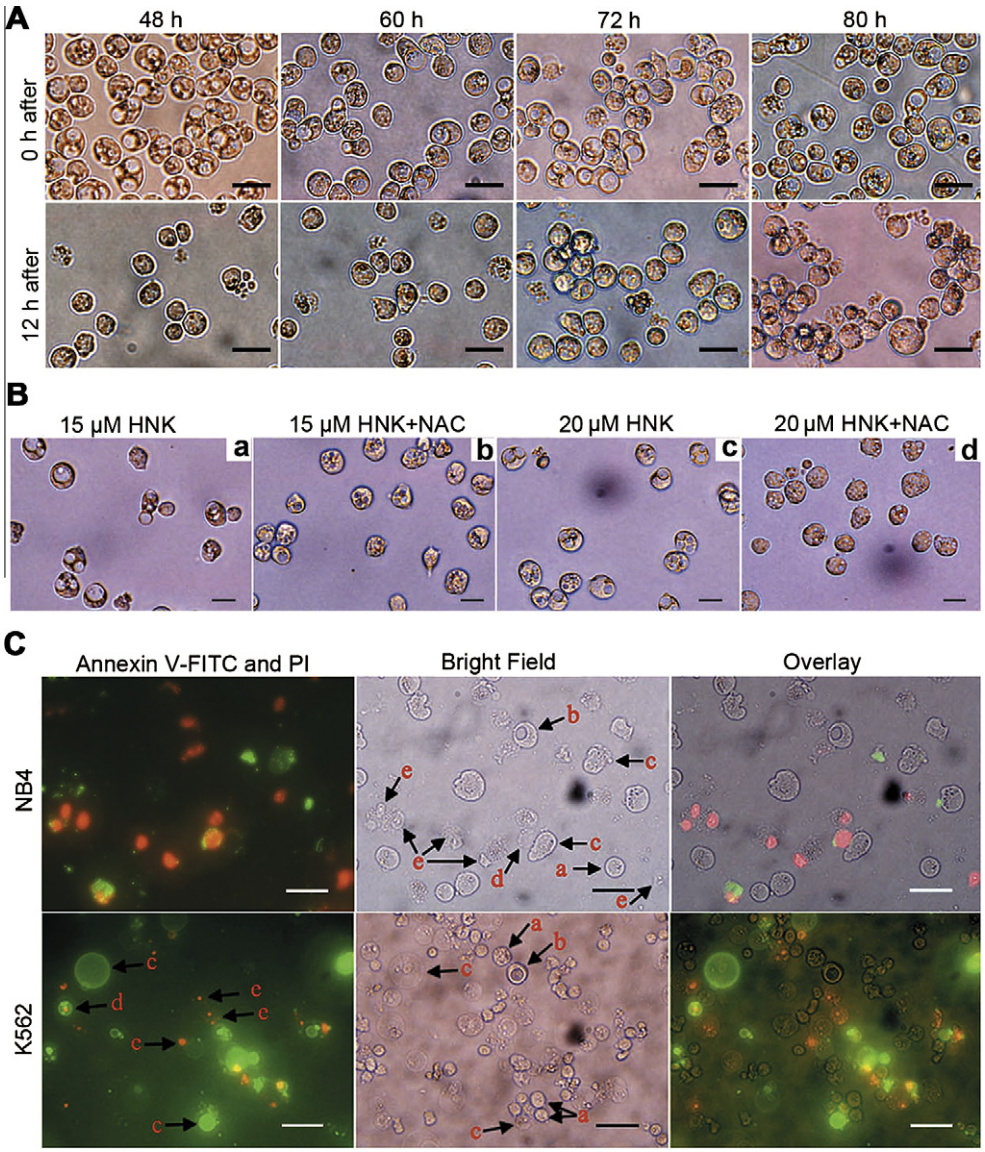

Fig. 3. Cytoplasmic vacuolization is a form program of cell death (A). NB4 cells were analyzed by a washout assay using phase contrast microscopy at the indicated time points (upper panel, 0 h after washout; lower panel, 12 h after washout). (B) Cytoplasmic vacuolization is partly abolished by treatment with *N*-acetyl-L-cysteine (NAC). (Magnification: 400×, scale bar: 10 μm.) (C) HNK induces cytoplasmic vacuolization without phosphatidyl serine externalization. Arrows: (a) normal cells; (b) cells with cytoplasmic vacuolization; (c) early apoptotic cells; (d) late apoptotic cells; (e) necrotic cells. Magnification: 400×, scale bar: 30 μm.

led to cellular apoptosis or necrosis at the late time points. This indicated that HNK induced cytoplasmic vacuolization in a time-dependent manner and that the process was a form of program cell death.

Ultrastructural studies of HNK-induced cytoplasmic vacuolization revealed the presence of ribosomes along the edges of the vacuoles, which confirmed that small and large cytoplasmic vacuoles were derived from the swollen endoplasmic reticulum. Furthermore, double-membraned autophagosomes were not observed under the transmission electron microscope, demonstrating that the cell death was not due to autophagy, the typical hallmark of which is the formation of phagocytic vacuoles called autophagosomes containing cytoplasm and organelles [20]. Results that these vacuoles are mainly derived from swelling of the endoplasmic reticulum are consistent with the morphologic criteria of paraptosis [5], which is characterized by cytoplasmic vacuolization and begins with progressive swelling of the mitochondria and/or endoplasmic reticulum.

However, mitochondria in HNK-induced cells with cytoplasmic vacuolization appear to be normal by ultrastructural examination, unlike the mitochondrial abnormalities such as swelling that are observed during necrosis [14]. Overproduction of ROS resulting from dysfunction of the mitochondrial respiratory chain may also lead to caspase-independent cell death [21], and it was reported that HNK-treated cells generated ROS [12,16]. Thus, although mitochondrial swelling did not occur in HNK-induced cells with cytoplasmic vacuolization, mitochondrial function might be impaired. Another possible explanation is that mitochondrial swelling did not occur in cells with cytoplasmic vacuolization because of the relatively low concentrations of HNK used and the effects of short-term exposure to HNK.

Paraptosis and apoptosis have been reported to share many common features, including phosphatidylserine exposure on the plasma membrane and a decline in the mitochondrial transmembrane potential [10]. By annexin V-FITC/PI staining, the externalization of phosphatidylserine on the plasma membrane was not

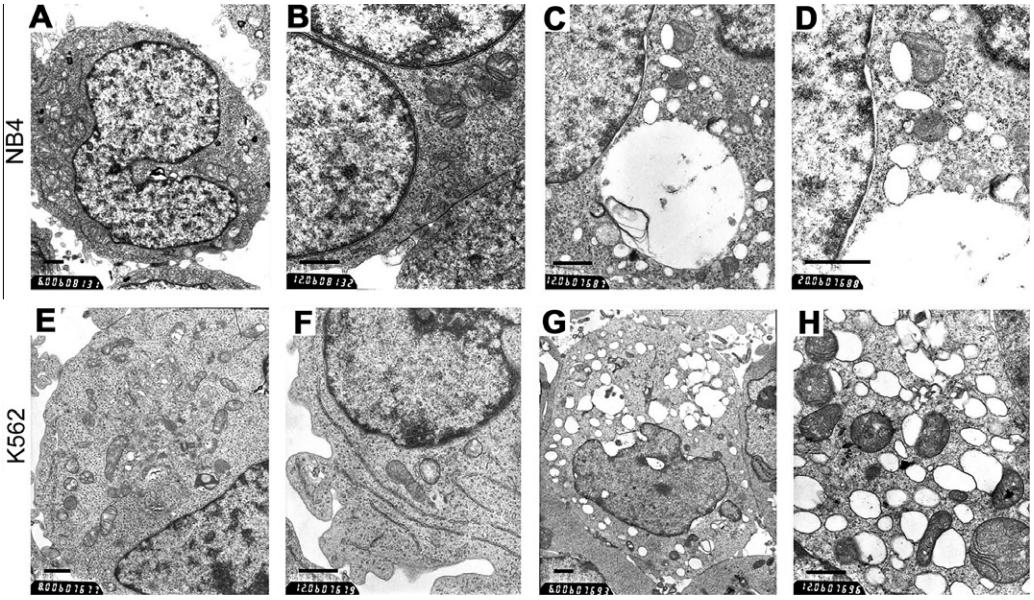

Fig. 4. HNK induces cytoplasmic vacuolization through swelling of the endoplasmic reticulum (ER). NB4 cells were treated with 20 μM HNK for 20 h (c) and (d) and K562 cells were treated with 40 μM HNK for 20 h (g and h). Control NB4 cells (a) and (b) cells and in K562 cells (e) and (f) show intact, ultrastructurally-normal nuclei and cellular organelles. HNK-treated cells (c), (d), (g) and (h) show extensive cytoplasmic vacuolization, unaffected mitochondria and swelling of the ER, and ribosomes can be observed along the edges of the vacuoles. Original magnification: (a) 6000×; (b–h) 12,000×; (d) 20,000×; (e) 8000×; (g) 6000×. Scale bar: 1 μm.

observed in cells with vacuoles in their cytoplasm. However, at a high concentration, DAPI and annexin V-FITC/PI staining demonstrated that HNK induced apoptosis and necrosis, suggesting that extensive cytoplasmic vacuolization is the earliest morphological change in response to HNK. These results supported the earlier viewpoint that crosstalk exists between apoptosis and nonapoptotic PCD [5].

Apoptosis and cell cycle arrest induced by many naturally occurring anticancer agents are correlated with ROS generation [22,23]. Cytoplasmic vacuolization in rat T9 glioma cells can be initiated through a big potassium (BK) channel-dependent process induced by ROS [24]. It was also reported that cells treated with HNK generated ROS [12,16]. Thus, it was hypothesized that HNK-mediated ROS generation was possibly involved in the process of cytoplasmic vacuolization. Light microscopic imaging showed that addition of NAC, a total ROS scavenger, could suppress this vacuolization in NB4 cells, indicating that ROS may be a promoter of HNK-induced cytoplasmic vacuolization, and that the mechanism of cytoplasmic vacuolization was partially associated with an increase in ROS generation.

In conclusion, HNK induced a novel form of cell death at low concentration in NB4 and K562 cells. This form of cell death was morphologically different from oncosis, autophagy, apoptosis and necrosis, but it was consistent with the characteristics of paraptosis, such as cytoplasmic vacuolization derived from endoplasmic reticulum swelling, caspase-independent, retention of membrane integrity, and lack of apoptotic body formation. HNK induced cell death in a time- and concentration -dependent manner and the new form of cell death was correlated with the cell type. Furthermore, it was not involve membrane blebbing, chromatin condensation and phosphatidylserine exposure at the outer of the plasma membrane. The mechanism of the cell death might be associated, at least in part, with the increased generation of ROS. In addition, HNK mainly induced paraptosis at lower concentration in NB4 cells, whereas apoptosis at higher concentration in K562 cells. Thus, paraptosis and apoptosis might be complementary cell death programs in leukemia cells.

The results potentially imply that cell death occurs via more than one pathway in leukemia cells. The ability of HNK to induce cell death through both paraptosis and apoptosis makes HNK a more versatile "killer" of cancer cells. Further studies should be carried out to achieve a deeper understanding of this important biological process, and to develop new therapies for leukemia.

Acknowledgments

We thank Dr. Youfu Luo for kindly providing HNK and Dr. Jiang Wu for helpful comments. This work was supported by the National 985 Key Project of Sichuan University of the Education Ministry of China.

References

[1] W.J. Guo, T.S. Chen, X.P. Wang, R. Chen, Taxol induces concentration-dependent apoptotic and paraptosis-like cell death in human lung adenocarcinoma (ASTC-a-1) cells, J. X-ray Sci. Technol. 18 (2010) 293–308.
[2] J.H. Du, H.D. Zhang, Z.J. Ma, K.M. Ji, Artesunate induces oncosis-like cell death in vitro and has antitumor activity against pancreatic cancer xenografts in vivo, Cancer Chemother. Pharmacol. 65 (2010) 895–902.
[3] M. Michalik, M. Pierzchalska, A. Pabianczyk-Kulka, W. Korohoda, Procaine-induced enhancement of fluid-phase endocytosis and inhibition of exocytosis in human skin fibroblasts, Eur. J. Pharmacol. 475 (2003) 1–10.
[4] S. Sperandio, K. Poksay, I. de Belle, M.J. Lafuente, B. Liu, J. Nasir, D.E. Bredesen, Paraptosis: mediation by MAP kinases and inhibition by AIP-1/Alix, Cell. Death Differ. 11 (2004) 1066–1075.
[5] S. Sperandio, I. de Belle, D.E. Bredesen, An alternative, nonapoptotic form of programmed cell death, Proc. Natl. Acad. Sci. USA 97 (2000) 14376–14381.
[6] W.B. Wang, L.X. Feng, Q.X. Yue, W.Y. Wu, S.H. Guan, B.H. Jiang, M. Yang, X. Liu, D.A. Guo, Paraptosis accompanied by autophagy and apoptosis was induced by celastrol, a natural compound with influence on proteasome, ER stress and Hsp90, J. Cell. Physiol. 227 (2011) 2196–2206.
[7] T.E. Battle, J. Arbiser, D.A. Frank, The natural product honokiol induces caspase-dependent apoptosis in B-cell chronic lymphocytic leukemia (B-CLL) cells, Blood 106 (2005) 690–697.
[8] K. Ishitsuka, T. Hideshima, M. Hamasaki, N. Raje, S. Kumar, H. Hideshima, N. Shiraishi, H. Yasui, A.M. Roccaro, P. Richardson, K. Podar, S. Le Gouill, D. Chauhan, K. Tamura, J. Arbiser, K.C. Anderson, Honokiol overcomes conventional drug resistance in human multiple myeloma by induction of caspase-dependent and -independent apoptosis, Blood 106 (2005) 1794–1800.
[9] Y. Wang, Z. Yang, X. Zhao, Honokiol induces paraptosis and apoptosis and exhibits schedule-dependent synergy in combination with imatinib in human leukemia cells, Toxicol. Mech. Methods 20 (2010) 234–241.
[10] Y. Wang, X. Li, L. Wang, P. Ding, Y. Zhang, W. Han, D. Ma, An alternative form of paraptosis-like cell death, triggered by TAJ/TROY and enhanced by PDCD5 over expression, J. Cell. Sci. 117 (2004) 1525–1532.

[11] T. Nishikawa, T. Nakajima, M. Moriguchi, M. Jo, S. Sekoguchi, M. Ishii, H. Takashima, T. Katagishi, H. Kimura, M. Minami, Y. Itoh, K. Kagawa, T. Okanoue, A green tea polyphenol, epigalocatechin-3-gallate, induces apoptosis of human hepatocellular carcinoma, possibly through inhibition of Bcl-2 family proteins, J. Hepatol. 44 (2006) 1074–1082.
[12] E.R. Hahm, S.V. Singh, Honokiol causes G0–G1 phase cell cycle arrest in human prostate cancer cells in association with suppression of retinoblastoma protein level/phosphorylation and inhibition of E2F1 transcriptional activity, Mol. Cancer Ther. 6 (2007) 2686–2695.
[13] V.A. Fadok, D.L. Bratton, S.C. Frasch, M.L. Warner, P.M. Henson, The role of phosphatidylserine in recognition of apoptotic cells by phagocytes, Cell Death Differ. 5 (1998) 551–562.
[14] A. Degterev, Z. Huang, M. Boyce, Y. Li, P. Jagtap, N. Mizushima, G.D. Cuny, T.J. Mitchison, M.A. Moskowitz, J. Yuan, Chemical inhibitor of nonapoptotic cell death with therapeutic potential for ischemic brain injury, Nat. Chem. Biol. 1 (2005) 112–119.
[15] G. Kroemer, W.S. El-Deiry, P. Golstein, M.E. Peter, D. Vaux, P. Vandenabeele, B. Zhivotovsky, M.V. Blagosklonny, W. Malorni, R.A. Knight, M. Piacentini, S. Nagata, G. Melino, Classification of cell death: recommendations of the Nomenclature Committee on Cell Death, Cell. Death Differ. 12 (Suppl 2) (2005) 1463–1467.
[16] L. Li, W. Han, Y. Gu, S. Qiu, Q. Lu, J. Jin, J. Luo, X. Hu, Honokiol induces a necrotic cell death through the mitochondrial permeability transition pore, Cancer Res. 67 (2007) 4894–4903.
[17] X. Liu, J.J. Rainey, J.F. Harriman, R.G. Schnellmann, Calpains mediate acute renal cell death: role of autolysis and translocation, Am. J. Physiol. Renal Physiol. 281 (2001) F728–738.
[18] G. Majno, I. Joris, Apoptosis, oncosis, and necrosis: an overview of cell death, Am. J. Pathol. 146 (1995) 3–15.
[19] B.F. Trump, I.K. Berezesky, S.H. Chang, P.C. Phelps, The pathways of cell death: oncosis, apoptosis, and necrosis, Toxicol. Pathol. 25 (1997) 82–88.
[20] A.M. Cuervo, Autophagy: many paths to the same end, Mol. Cell. Biochem. 263 (2004) 55–72.
[21] C. Fleury, B. Mignotte, J.L. Vayssiere, Mitochondrial reactive oxygen species in cell death signaling, Biochimie. 84 (2002) 131–141.
[22] S.V. Singh, S.K. Srivastava, S. Choi, K.L. Lew, J. Antosiewicz, D. Xiao, Y. Zeng, S.C. Watkins, C.S. Johnson, D.L. Trump, Y.J. Lee, H. Xiao, A. Herman-Antosiewicz, Sulforaphane-induced cell death in human prostate cancer cells is initiated by reactive oxygen species, J. Biol. Chem. 280 (2005) 19911–19924.
[23] D.C. Han, M.Y. Lee, K.D. Shin, S.B. Jeon, J.M. Kim, K.H. Son, H.C. Kim, H.M. Kim, B.M. Kwon, 2'-Benzoyloxycinnamaldehyde induces apoptosis in human carcinoma via reactive oxygen species, J. Biol. Chem. 279 (2004) 6911–6920.
[24] N. Hoa, M.P. Myers, T.G. Douglass, J.G. Zhang, C. Delgado, L. Driggers, L.L. Callahan, G. VanDeusen, J.T. Pham, N. Bhakta, L. Ge, M.R. Jadus, Molecular mechanisms of paraptosis induction: implications for a non-genetically modified tumor vaccine, PLoS One 4 (2009) e4631.