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Wortmannin Enhances CPP32-like Activity during Neuronal Differentiation of P19 Embryonal Carcinoma Cells Induced by Retinoic Acid

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P19EC cells undergoes apoptosis during neuronal differentiation induced by retinoic acid. Two CPP32-like proteases, CPP32 and Mch-3, are expressed in untreated and retinoic acid-treated P19 EC cells. CPP32-like activity is remarkably increased in apoptosis during neuronal differentiation of P19 EC cells. Inhibition of CPP32-like proteases prevents apoptosis, suggesting that activation of CPP32-like proteases play central roles in the apoptosis during neuronal differentiation of P19 EC cells. Wortmannin, PI-3K inhibitor, enhances the CPP32-like activity of the retinoic acid-treated P19 EC cells. PI-3K may be involved in the apoptosis during neuronal differentiation as negative regulator. © 1997 Academic Press

Ced-3/ICE proteases are components of the mammalian cell death pathway (1,2). Expression of cowpox virus *crmA,* an inhibitor of ICE, inhibits apoptosis initiated by either Fas or the TNF receptor (3-5). Thus, *crmA*-inhibitable ICE-like protease is important in the cell death pathway. CPP32/apopain (CPP32), one of the *crmA*-inhibitable *Ced-3*/ICE-like proteases, is the mammalian enzyme most closely related to CED-3 (6). Poly (ADP-ribose) polymerase (PARP), an enzyme involved in DNA repair and genome surveillance and integrity, is one potential substrate for CPP32 during apoptosis (7, 8). Inhibition of CPP32-mediated PARP cleavage by *crmA* or the specific inhibitor Ac-DEVD-CHO attenuates apoptosis, demonstrating the importance of this protease in the apoptosis of mammalian cells.

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Considerable evidence suggests that *Ced-3*/ICE proteases are components of the apoptosis of vertebrate nervous system. *CrmA* prevents the cell death of DRG neurons induced by NGF-deprivation (9). The overexpression of Bcl-2 delays the apoptosis in sympathetic neurons and sensory neurons in response to NGF deprivation (10,11). Recently, we demonstrated that CPP32 was specifically expressed in sensory neurons including DRGs of mouse 11.5-day embryos and that CPP32-like activity was increased in the apoptosis of DRGs induced by NGF-deprivation (12). Moreover, brain development in CPP32-deficient mice is profoundly affected by decreased apoptosis, resulting in a variety of hyperplasias and disorganized cell deployment (13). These results suggest that CPP32 plays a critical role in the neuronal cell death during development.

P19 EC cells, which differentiate into neuronal cells by all *trans*-retinoic acid (RA), die with a hallmark of apoptotic cell death such as cytoplasmic contraction and DNA fragmentation during neuronal differentiation (14, 15). The apoptosis of P19 EC cells induced by RA can be inhibited by *bcl-2,* a homologue of *Ced-9* in *C. elegance.* (14). Recently, we demonstrated that ICE-like activity increased during neuronal differentiation of P19 EC cells (16).

In the present study, we show that CPP32-like proteases are involved in the apoptosis during neuronal differentiation of P19 EC cells induced by RA and that wortmannin, an inhibitor for PI-3K, enhances CPP32-like activity and the apoptosis of RA-treated P19 EC cells.

MATERIALS AND METHODS

Cell culture of P19 EC cells and detection of cell death. P19 EC cells, which were originally established by McBurney *et al.*

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(17) were kindly donated by Dr. H. Hamada (Osaka University, Osaka, Japan). P19 EC cells were cultured in α-minimum essential medium (Sigma, St Louis, MO). For the neuronal differentiation, P19 EC cells were cultured in the aggregate form in the presence of 1 μM RA (Sigma, St Louis, MO.) for 3 days and then further cultured in the non-aggregate form in the absence of RA for 2 days. Cell viability was determined by trypan blue exclusion.

Isolation of fragmented DNA. DNA isolation was performed according to Pringent *et al.* (18) with some modification. Untreated and the RA-treated P19 EC cells were lysed with 400 μl of lysis buffer (20 mM Tris-HCl, pH 7.4, 20 mM EDTA and 1 % Triton X-100). Lysis was allowed to proceed on ice for 10 min and the mixture centrifuged for 10 min at 15,000 rpm. The supernatant digested with 50 μg/ml RNase A at 37°C for 1h. This was followed by a proteinase K digestion at 50 μg/ml, at 37°C for 1h. After phenol-chloroform extraction, the DNA was then precipitated by adding 2.5 vol. of 100 % ethanol and CH_3COONa (0.3M at final concentration). DNA ladders could be visualized by running on 1.8 % Nusieve agarose (TaKaRa, Co., Kyoto) gel.

Cleavage of PARP. P19 EC cells were cultured with RA (1 μM) in the aggregated forms for one to 3 days. After cells were harvested and washed two times with PBS, the cell pellets were lysed with the sample buffer (50 mM Tris-HCl pH7.5, 10% glycerol, 1% SDS) and sonicated. After centrifugation at 10,000 $\times$ g for 10 min, the cell extracts (100 μg protein) were subjected to SDS gel (10%) electrophoresis. Proteins of the gels were electrophoretically transferred to the nitrocellulose filters. After filters were incubated with monoclonal anti-PARP antibody, the reactivities on the filters were detected by alkaline phosphatase-conjugated goat anti-mouse immunoglobulin (Promega, Madison, WI) and nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl-1-phosphate.

CPP32-like activity. After P19 EC cells were cultured with RA (1 μM) for indicated periods, cells were washed two times with PBS, and the cell pellets were lysed in PBS containing 0.2% Triton X-100 on ice for 10 min. After centrifugation at 10,000 $\times$ g for 5 min, the cell extracts (50 μg protein) were incubated with 10 μM Ac-DEVD-MCA (Peptide Institute, Osaka), a synthetic peptide of PARP, in the incubation buffer (50 mM Tris-HCl pH7.5, 1 mM DDT) for 20 min in order to measure CPP32-like activity. The reactions were halted by the addition of 10% SDS. Fluorescence was measured at 380 nm for excitation and at 460 nm for emission.

The expression of two CPP32-like proteases and bcl-2. The expression of *bcl-2* during neuronal differentiation of P19 EC cells was examined by quantitative RT-PCR as described previously (15): 1 cycle at 95°C for 2 min, 30 cycles at 95°C for 1 min and 60°C for 1 min. 5′-AAGAATTCCGGGAGAACAGGGTATG-3′ and 5′-AAGGATCCGGTAGCGACGAGAGAAG-3′ were used as *bcl-2* primers. As a internal control, we used G3PDH primer; 5′-CTCATGCCACAGTCCATGC-3′ and 5′-CTCTTGCTCAGTGTCCTTGC-3′. The expression of CPP32 and Mch-3 during the differentiation of P19 EC cells was examined by RT-PCR using consensus primers: 5′-ATTCAGGC(C/T)TGCCG(T/A)GG(T/G)AC-3′ and 5′-AGTTC(C/T)TT(G/T)GTGAGCATGGA-3′. 1 cycle at 95°C for 2 min, 27 cycles at 95°C for 1 min and 60°C for 1 min.

Assay for PI-3K. PI-3K activity was assayed according to the procedure as described (19). P19EC cells were incubated with wortmannin (Sigma, St Louis, MO) at various concentrations (1-1000 nM) and were lysed with lysis buffer (20 mM Tris-HCl pH7.5, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 137 mM NaCl, 1 mM Na_3VO_4, 1% NP-40, 10% glycerol, 1 mM PMSF, 10 μg/ml leupeptin). After centrifugation at 13,000 $\times$ g for 10 min, supernatant was incubated with anti-PI-3K (p85) (MBL, Nagoya) at 4°C overnight. Immuno-complex was precipitated with a mixture of protein G and A, and suspended with 20 μl of Hepes buffer (pH 7.4). The immuno-complex was incubated with in the reaction buffer (20 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 μM ATP, 200 μg/ml phosphatidylinositol, 200 μg/ml phosphatidylserine, 1 μCi γ-[^{32}P]-ATP) at 25°C for 10min and then subjected to thin layer chromatography. Thin layer plate (Merck, Darmstadt, Germany) was developed with chloroform/ methanol/25% ammonia/H_2O (86: 76: 10: 14). After development, thin layer plate was exposed to Fuji image analyzer.

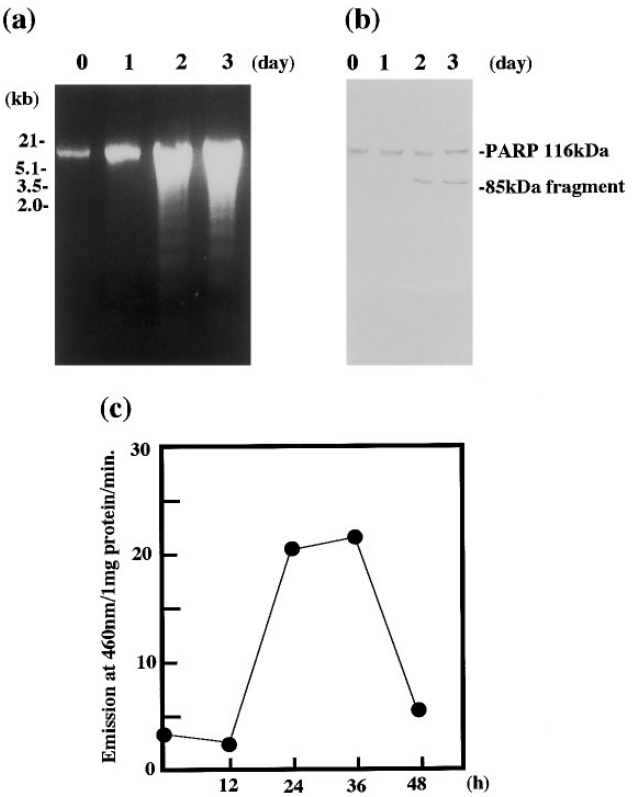

FIG. 1. Activation of CPP32-like protease in apoptosis during neuronal differentiation of P19 EC cells induced by RA. (a) DNA fragmentation, (b) Immunoblot analysis of PARP cleavage, (c) CPP32-like activity during neuronal differentiation.

RESULTS

Many of P19 EC cells die with DNA fragmentation during neuronal differentiation induced by RA (14). DNA fragmentation was observed at day 2 (Figure 1a). PARP cleavage activity was coincident with appearance of DNA fragmentation (Figure 1b). A 85 kDa fragment of PARP was also detected at day 2. These results suggest that CPP32-like proteases are activated during apoptosis of P19 EC cells induced by RA. To confirm the activation of CPP32-like proteases, the CPP32-like activity was measured by using Ac-DEVD-MCA, a synthetic peptide of PARP, as a substrate. The CPP32-like activity was transiently increased during apoptosis of P19 EC cells induced

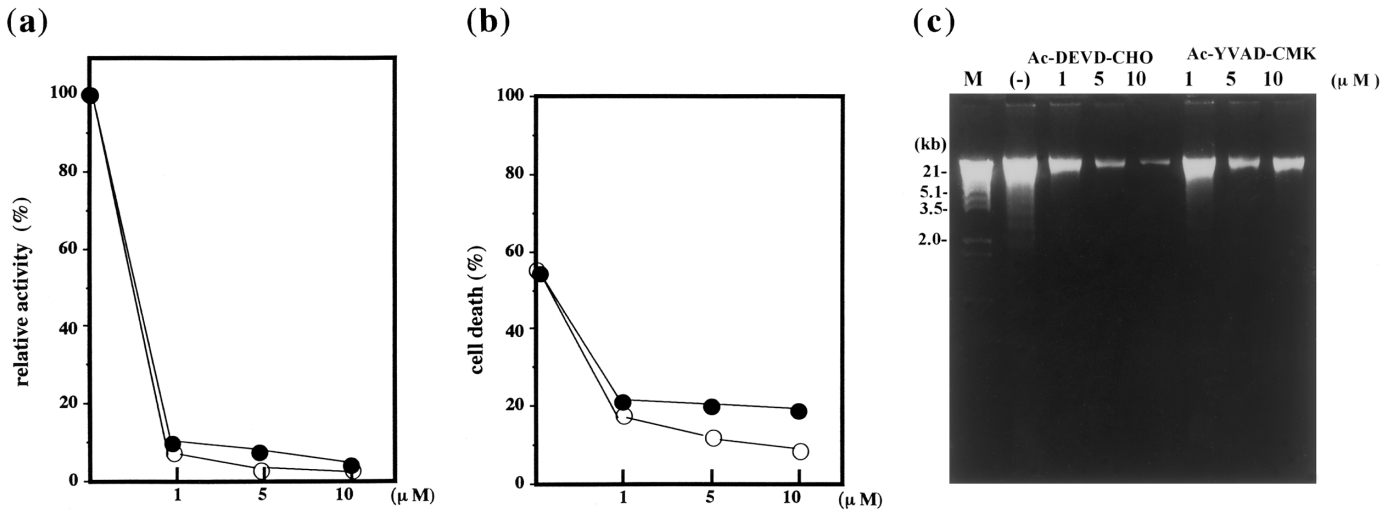

FIG. 2. Inhibition of cell death and DNA fragmentation of RA-treated P19 EC cells by Ac-DEVD-CHO or Ac-YVAD-CMK. (a) Inhibition of CPP32-like activity of RA-treated P19 EC cells by Ac-DEVD-CHO (open circles) or Ac-YVAD-CMK (closed circles). (b) Inhibition of cell death of RA-treated P19 EC cells by Ac-DEVD-CHO (open circles) or Ac-YVAD-CMK (closed circles). (c) Inhibition of DNA fragmentation of RA-treated P19 EC cells by Ac-DEVD-CHO or Ac-YVAD-CMK. M; λDNA digested with EcoRI and HindIII.

by RA (Figure 1c). The CPP32-like activity was increased before cleavage of PARP and achieved maximum level between 24-36 h after RA treatment, and then decreased.

The CPP32-like activity was dose-dependently inhibited by Ac-DEVD-CHO (1-10 μM), an inhibitor of CPP32-like protease or by Ac-YVAD-CMK (1-10 μM), an inhibitor of ICE-like protease (Figure 2a). Corresponding to the inhibition of CPP32-like activity, the cell death and DNA fragmentation were prevented by Ac-DEVD-CHO or Ac-YVAD-CMK (Figures 2b and c).

Wortmannin, an inhibitor for PI-3K, weakly inhibited PI-3K of P19 EC cells in the range of the concentration (1-10 nM). At more than 100 nM, PI-3K was prominently inhibited by wortmannin (Figure 3a). Corresponding to the inhibition of PI-3K activity, wortmannin enhanced the CPP32-like activity of the RA-treated P19 EC cells and stimulated the apoptosis with DNA fragmentation at more than 100 nM (Figure 3b and c). However, wortmannin (1-1000 nM) alone did not have any effect on the CPP32-like activity and the DNA fragmentation of untreated P19 EC cells.

Two CPP32-like protease, CPP32 and Mch-3, were expressed in P19 EC cells. The expression of CPP32 and Mch-3 was not changed during neuronal differentiation of P19 EC cells, while the expression of *bcl-2* was gradually decreased by RA treatment (Figure 4a). However, wortmannin did not affect either the expression of these two CPP32-like proteases nor that of *bcl-2* (Figure 4b).

DISCUSSION

ICE protease has an ability to cleave CPP32 into an active form (5). ICE-like activity was shown to increase before the activation of CPP32 during Fas-dependent apoptosis (20). ICE-like and CPP32-like proteases are sequentially activated during Fas-dependent apoptosis.

In the present study, we showed that CPP32-like activity was remarkably and transiently increased during apoptosis of RA-treated P19 EC cells (Figure 1). Inhibition of CPP32-like activity as well as that of ICE-like activity prevented apoptosis of P19 EC cells during neuronal differentiation (Figure 2). Thus transient activation of CPP32-like proteases as well as activation of ICE-like proteases seems to be essentially involved in the apoptosis of P19 EC cells during neuronal differentiation. The apoptosis of RA-treated P19 EC cells may be induced by sequential activation of ICE- and CPP32-like proteases.

The expression of two CPP32-like proteases, CPP32 and Mch-3, was not changed during neuronal differentiation of P19 EC cells induced by RA (Figure 4). Since CPP32-like activity was increased corresponding to the down-regulation of *bcl-2* (Figure 1 and 4) (15) and overexpression of *bcl-2* inhibits the activation of CPP32-like proteases (21), it is likely that RA regulates the activation of CPP32-like protease through down-regulation of *bcl-2*. Thus apoptosis of P19EC cells during neuronal differentiation as well as that of *C. elegance* may be regulated by *Ced-3* and *Ced-9* homologue.

The ability of NGF preventing the apoptosis of

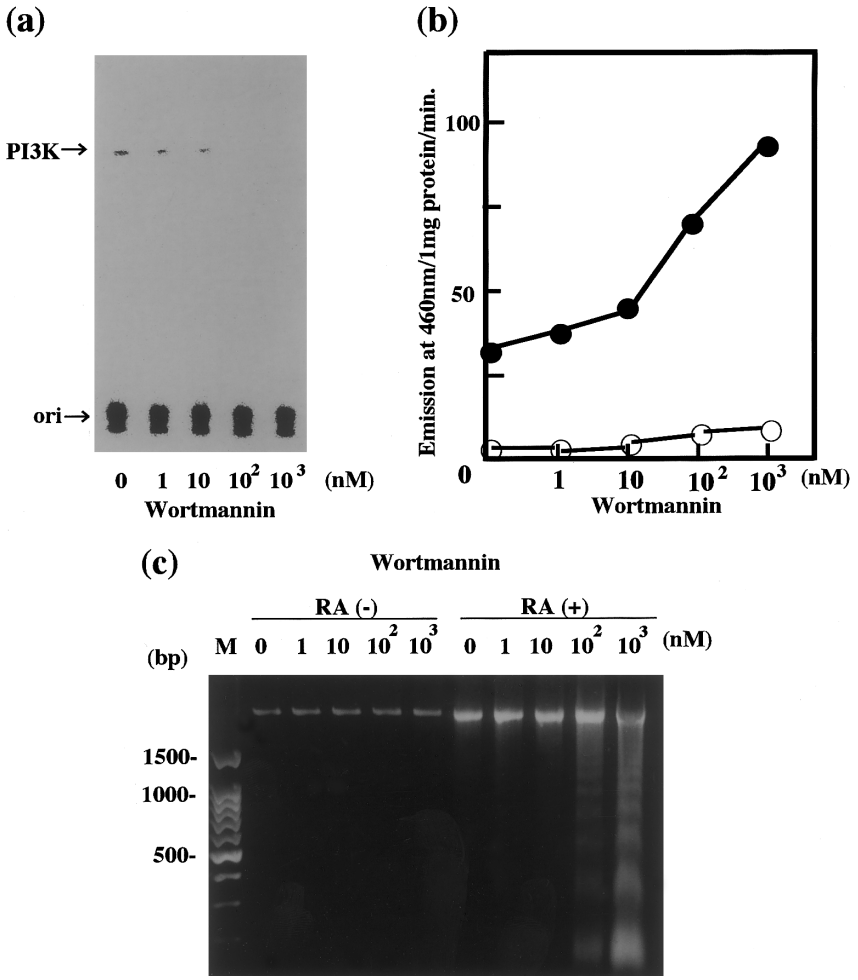

FIG. 3. Enhancement of DNA fragmentation and CPP32-like activity by wortmannin. (a) Dose-dependent inhibition of PI-3K activity of P19 EC cells by wortmannin. (b) CPP32-like activity of untreated (open circle) and RA-treated P19 EC cells (closed circles) stimulated by wortmannin. (c) DNA fragmentation of the untreated and the RA-treated P19 EC cells stimulated by wortmannin.

PC12 cells induced by serum deprivation was inhibited by wortmannin, an inhibitor for PI-3K, suggesting that PI-3K is involved in the signal pathway of survival factors of neurons (22). CPP32-like proteases are activated in the apoptosis induced by withdrawal of NGF (12). In the present study, we showed that wortmannin enhanced the CPP32-like activity of RA-treated P19 EC cells corresponding to the inhibition of PI-3K (Figure 3). These observation suggest that survival factors prevent apoptosis of neurons by inhibiting CPP32-like proteases through activation of PI-3K signal pathway.

Wortmannin did not have any effect on the transcription of two CPP32-like proteases and *bcl-2* in untreated or the RA-treated P19 EC cells (Figure 4b). Moreover, wortmannin alone did not induce the CPP32-like activity in the untreated P19 EC cells (Figure 3b), suggesting that PI-3K is not directly involved in the processing of CPP32-like proteases. At present, it is not clear about the molecular mechanism, by which wortmannin enhances the activation of CPP32-like proteases in the RA-treated P19 EC cells. One of the possibility is that wortmannin regulates phosphorylation of regulatory proteins for the activation of CPP32-like proteases.

BAD, a member of Bcl-2 family, exerts its death-promoting effects by heterodimerizing with complex with Bcl-2 or BCL-XL death antagonists (23). Recently, it has been shown that survival factor IL3 or Raf-1 phosphorylates BAD at serine residue and that only nonphosphorylated BAD is heterodimerized with BCL-XL (24,25). PI-3K can induce the protein phosphorylation at serine residue through activation of PKC (26). Thus wortmannin may activate CPP32-like proteases by indirectly regulating the phosphorylation of Bcl-2 family.

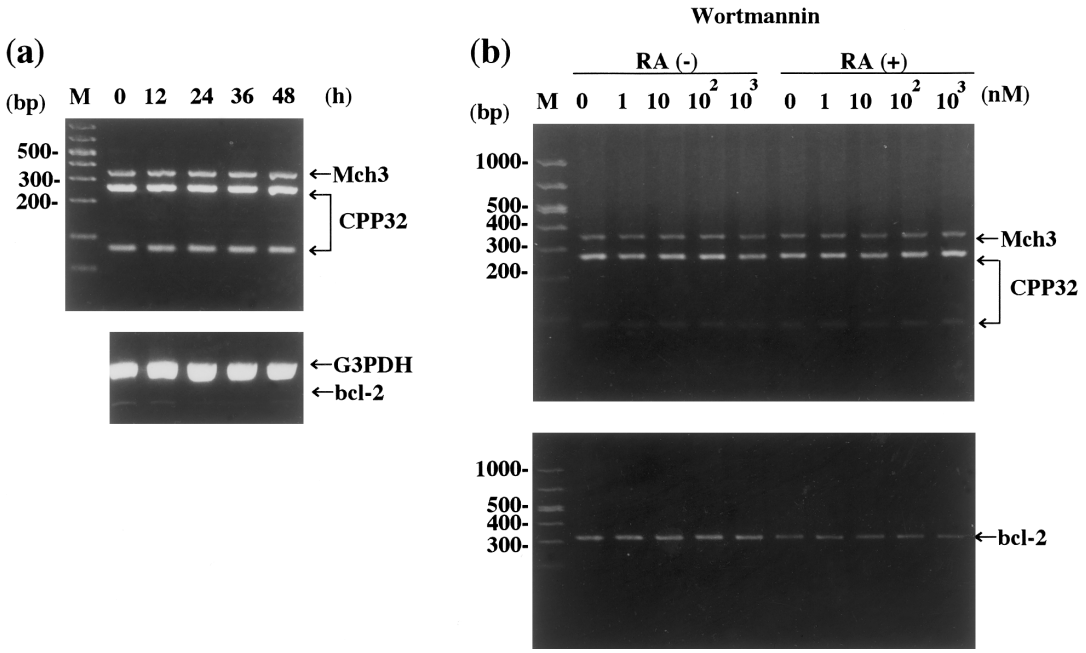

FIG. 4. Quantitative RT-PCR of two CPP32-like proteases and bcl-2 of P19 EC cells treated with RA or wortmannin. (a) The expression of CPP32, Mch-3 and bcl-2 during neuronal differentiation of P19 EC cells induced by RA. Since the fragments of RT-PCR products of CPP32 (341 bp) and Mch-3 (349 bp) had similar nucleotide length, CPP32 was identified by cleavage of the EcoRI site. (b) No effect of wortmannin (1-1000 nM) on the expression of CPP32, Mch-3 and bcl-2 of untreated and RA-treated P19 EC cells. P19 EC cells were incubated with wortmannin (1-1000 nM) in the presence or absence of RA (1 μM) for 24 h.

Further study on the relationship between phosphorylation of Bcl-2 family and PI-3K remain to be studied.

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