Acta Med. Okayama, 2005
Vol. 59, No. 6, pp. 253–260

Acta Medica Okayama
http://www.lib.okayama-u.ac.jp/www/acta/

Original Article

The Augmentation of TNFα-induced Cell Death in Murine L929 Fibrosarcoma by the Pan-caspase Inhibitor Z-VAD-fmk through Pre-mitochondrial and MAPK-dependent Pathways

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We investigated the mechanism of the pan-caspase inhibitor z-VAD-fmk's augmentation of TNFα-induced L929 cell death and found this mechanism differs from that of TNFα-induced L929 cell death. In the presence of 20 ng/ml TNFα, z-VAD-fmk initiated apoptosis and necrosis in the majority of L929 cells as measured by an agarose gel electrophoresis and lactate dehydrogenase (LDH) activity-based assay. Mitochondrial permeability transition (MPT) inhibitor (cyclosporine A) effectively inhibited z-VAD-fmk-augmented cell death. In addition, z-VAD-fmk plus TNFα increased Bax expression without affecting Bcl-2 and cytochrome *c* expression. Western-blot analysis showed that z-VAD-fmk plus TNFα caused persistent JNK activation and ERK inactivation. Poly (ADP-ribose) polymerase (PARP) inhibitor (DPQ) effectively reversed the cell death which was augmented by z-VAD-fmk, and z-VAD-fmk plus TNFα also caused PARP cleavage to an 85 KDa fragment. These results indicate that in the presence of TNFα, z-VAD-fmk further augments cell death which requires the mitochondrial permeability transition and the JNK activation. However, we did not detect the changes in cytochrome *c* expression and the participation of caspase-9 in this process, suggesting that there might exist an unknown signal pathway(s) from the mitochondria to the downstream protein PARP, which is cleaved in a caspase-independent manner.

Key words: TNFα, caspase, Bax/Bcl-2, MAPK, PARP

Tumor necrosis factor-α (TNFα) is an important mediator in many immunological and inflammatory responses, as well as in a number of pathological conditions. *In vitro*, TNFα is able to induce cell death, the activation of transcription factors and proliferation. Caspases are a family of cysteases that play key roles in mediating Fas or TNFα-induced apoptosis and are divided into 2 classes based on the lengths of their N-terminal prodomains: up-stream caspases such as caspase-8 and -10, and downstream caspases such as -3, -6 and -7 [1]. The activation of caspases is believed to be required for apoptosis. However, some researchers have reported that the pan-caspase inhibitor z-VAD-fmk augments TNFα-induced cell death in L929 cells and in RAW246.7 macrophages. However, unlike L929 cell death as induced by TNFα, a necrosis characterized by swelling which eventually leads to disruption of the plasma membrane, RAW246.7 cells manifest features of

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apoptosis such as chromatin condensation and nuclear fragmentation [2-4]. For L929 cells, some researchers have put forward a hypothetical model: TNFα-induced cell death is due to an increase in the accumulation of oxygen radicals in the mitochondria; caspases interfere with the death signal pathway to the mitochondria from activated receptors, acting as a negative regulator of pre-mitochondrial signals or the mitochondrial production of reactive oxygen intermediate (ROI). Alternatively, damage to the mitochondria by ROI could impair normal functioning, resulting in an even higher production of radicals [5].

The aims of this study were to further investigate the effecter mechanisms in z-VAD-fmk-augmented L929 cell death. We demonstrated that TNFα induced both apoptosis and necrosis, that z-VAD-fmk augmented this cell death, and that this augmented cell death was associated with pre-mitochondrial signaling, the activation of JNK, and the downstream PARP protein cleavage.

Materials and Methods

Regents. TNFα was prepared from PMAL-C_2-TNFα/JM109 (*E. coli.*). Pan-caspase inhibitor (z-VAD-fmk), caspase-3 inhibitor (z-DEVD-fmk), caspase-9 inhibitor (z-LEHD-fmk) and caspase-8 inhibitor (z-IETD-fmk) were purchased from Enzyme Systems (Livermore, CA, USA). Poly (ADP-ribose) polymerase (PARP) inhibitor (DPQ), cyclosporin A, ERK inhibitor (PD98059), p38 inhibitor (SB203580), and JNK inhibitor (SP600125) were obtained from Calbiochem (La Jalla, CA, USA). Polyclonal antibodies against p38, phospho-p38, ERK1/2, phospho-ERK, JNK, phospho-JNK, PARP, β-actin, and horseradish peroxidase-conjuated secondary antibodies (goat-anti-rabbit and goat-anti-mouse) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-Bcl-2, and Bax were from Oncogene Research Products (Boston, MA, USA).

Cell Culture. The murine fibrosarcoma cells (L929#CRL-2148) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (GIBCO, NYC, NY, USA) supplemented with 10% FBS (Shengma Yuanheng, Beijing, China), 100 mg/l streptomycin, 100 IU/ml penicillin, and 0.03% L-glutamine and maintained at 37 °C with 5% CO_2 in a humidified atmosphere.

Cytotoxity Assay. L929 cells were incubated at 1×10^5 cells/well in 96-well plates (NUNC, Roskilde Denmark). The cells were incubated with z-VAD-fmk, z-IETD-fmk, z-DEVD-fmk, z-LEHD-fmk, cyclosporine A, PD98059, SB203580, or SP600125 at given concentrations for 1 h, and then treated with TNFα for different time periods. Cell growth was measured by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay. The percentage of cell growth inhibition was calculated as follows:

$$\text{Cell death } (\%) = [A_{570}(\text{control}) - A_{570}(\text{oridonin})] / A_{570}(\text{control}) \times 100$$

DNA fragmentation assay. L929 cells (1×10^6) were collected by centrifugation at 150 × g for 5 min and washed with PBS. Then, they were pelleted and suspended in 10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0), 0.5% Triton X-100 and kept at 4 °C for 10 min. The supernatant was incubated with 20 mg/ml RNase A (2 μl) and 20 mg/ml proteinase K (2 μl) at 37 °C for 1hrs, then kept in 0.5 M NaCl (20 μl) and isopropanal (120 μl) at − 2 °C overnight, and centrifuged at 15000 × g for 15 min. DNA was dissolved in TE buffer [10 mM Tris (pH 7.4), 10 mM EDTA (pH 8.0)] and subjected to a 2% agarose gel electophorsis at 50 V for 40 min and stained with ethidium bromide.

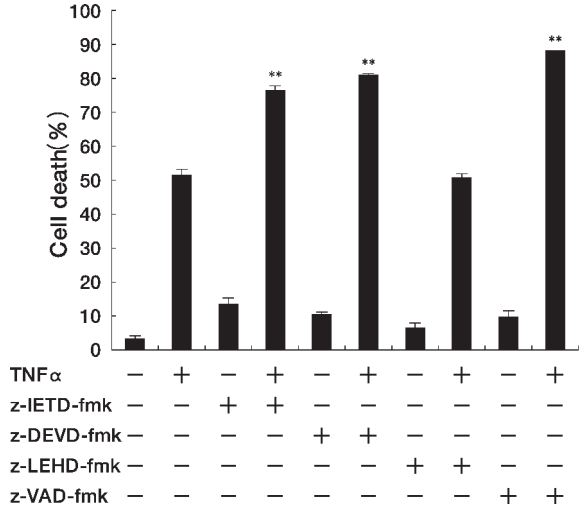
LDH Activity-Based Cytotoxicity Assays. LDH (lactate dehydrogenase) activity was assessed using a standardized kinetic determination (Zhongsheng LDH kit, Beijing, China), and measured in both floating dead cells and viable adherent cells. Floating cells were collected from culture medium by centrifugation (240 × g) at 4 °C for 5 min, and the LDH content in the pellets was used as an index of apoptotic cell death (LDHp). The LDH released in the culture medium (extracellular LDH or LDHe) was used as an index of necrotic death, and the LDH present in the adherent viable cells was used to determine intracellular LDH (LDHi). The percentage of apoptotic and necrotic cell death was calculated as follows:

$$\text{Apoptosis } (\%) = \text{LDHp}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

$$\text{Necrosis } (\%) = \text{LDHe}/(\text{LDHp} + \text{LDHi} + \text{LDHe}) \times 100$$

Western Blot Analysis. L929 cells were treated with TNFα (100 ng/ml) for different time periods. Both adherent and floating cells were collected and subjected to Western-blot analysis as previously

described with some modification. Briefly, the cell pellets were resuspended in lysis buffer consisting of Hepes 50 mmol/l pH 7.4, Triton-X 100 1%, sodium orthovanada 2 mmol/l, sodium fluoride 100 mmol/l, edetic acid 1 mmol/l, PMSF 1 mmol/l, aprotinin (Sigma, MO, USA) 10 mg/l and leupeptin (Sigma) 10 mg/l and lysed at 4 °C for 60 min. After 13000 × g centrifugation for 15 min, the protein content of the supernatant was determined using a protein assay reagent (Bio-Rad, Hercules, CA, USA). The protein lysates were separated by electrophoresis in 12% SDS polyacrylamide gel and blotted onto a nitrocellulose membrane. Proteins were detected using polyclonal antibody and visualized using anti-rabbit IgG conjugated with peroxidase (HRP), using 3,3-diaminobenzidine tetrahydrochloride (DAB) as the HRP substrate.



A

		Activity (fold)	
		12	24 (h)
Caspase-3	TNFα	6.2 ± 1.4	16.1 ± 1.4
	TNFα + z-DEVD-fmk	3.5 ± 1.4	6.8 ± 0.9
	TNFα + z-VAD-fmk	4.7 ± 2.1	9.7 ± 1.4
Caspase-8	TNFα	9.6 ± 0.7	4.5 ± 1.5
	TNFα + z-IETD-fmk	3.9 ± 1.2	2.1 ± 2.8
	TNFα + z-VAD-fmk	5.3 ± 2.1	2.8 ± 2.2
Caspase-9	TNFα	1.1 ± 2.3	1.2 ± 2.1
	TNFα + z-LEHD-fmk	1.2 ± 1.3	1.0 ± 1.6
	TNFα + z-VAD-fmk	1.3 ± 1.5	1.4 ± 3.1

B

Fig. 1 Effects of caspase inhibitors on TNFα-induced L929 cell death. Cells were treated with TNFα 20 ng/ml for 24 h in the presence of z-IETD-fmk 5 μM (caspase-8), z-DEVD-fmk 5 μM (caspase-3), z-LEHD-fmk 5 μM (caspase-9) or z-VAD-fmk 5 μM (pan-caspase), all of which were introduced 60 min prior to the administration of TNFα. Cell death rate was measured by MTT assay (**A**), caspase-3, -8 and -9 activities were assayed (**B**). n = 3, Mean ± S.D.**, $P < 0.01$

Statistical Analysis of the Data. The data are expressed as mean ± S.D. Statistical comparisons were made by student's *t*-test, $p < 0.05$ was considered significant.

Results

Z-VAD-fmk augmented TNFα-induced L929 cell death. TNFα produced a dose-dependent cytotoxic effect in L929 cells at 24 h (data not shown), and 20 ng/ml TNFα induced approximately 51.2% of the cell death. The inhibitor of caspase-3 z-DEVD-fmk, and the inhibitor of caspase-8 z-IETD-fmk, augmented cell death and the pan-caspase inhibitor z-VAD-fmk rendered the cells even more sensitive to TNFα; however, the caspase-9 inhibitor z-LEHD-fmk had almost no effect on the cell death. The cytotoxic affect of caspase inhibitors alone on L929 cells was minimal (Fig. 1A). We also assayed the caspase-3, -8 and -9 activities. Here, results showed that caspase inhibitors partially suppressed caspase-3 and -8 activities (Fig. 1B). To investigate the cytotoxic effects of z-VAD-fmk on L929 cells, the 20 ng/ml TNFα-treated cells were cultured in the presence of z-VAD-fmk at several doses for 24 h. We found that, in the presence of 20 ng/ml TNFα, z-VAD-fmk augmented L929 cell death in a dose-dependent manner, and z-VAD-fmk 0.5–20 μM exerted potent cytotoxic effects on L929 cells (Fig. 2).

Z-VAD-fmk augmented apoptotic and necrotic cell death in L929 cells. Apoptosis is a well-studied pathway of programmed cell death, and DNA fragmentation is a hallmark of typical apoptosis [3]. In our study, DNA fragmentation was observed in both the TNFα-treated and TNFα plus z-VAD-fmk-treated groups by agarose gel electrophoresis. However, when the L929 cells were cultured with 5 μM z-VAD-fmk alone, the DNA ladders disappeared (Fig. 3).

In order to further investigate whether z-VAD-fmk also *initiated* necrosis in L929 cells, the ratio of LDH (measured as described above) released from viable cells, floating dead cells, and the culture medium were mea-

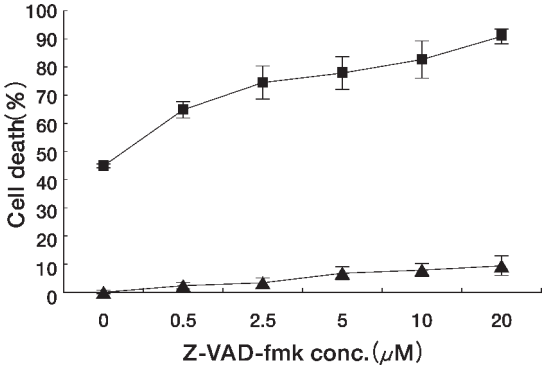


Fig. 2 Cytotoxic effects of z-VAD-fmk plus 20 ng/ml TNFα on L929 cells. The cells were treated with z-VAD-fmk with various doses in the presence (■) or absence (▲) of 20 ng/ml TNFα for 24 h. The cell death rate was measured by MTT assay. n = 3, Mean ± S.D.

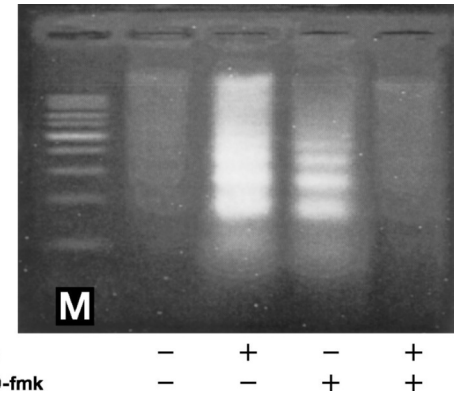


Fig. 3 Oligonucleosomal DNA fragmentation analysis in L929 cells. For 18 h, cells were treated with 5 μM z-VAD-fmk or 20 ng/ml TNFα or z-VAD-fmk (5 μM) plus 20 ng/ml TNFα. DNA was separated on 2% agarose gel. M: molecular weight marker.

sured. After treatment with 0.5–20 μM z-VAD-fmk in the presence of 20 ng/ml TNFα for 18 h, the number of apoptotic cells increased from 28.1% to 42.5%, and that of necrotic cells also increased from 27.3% to 36.7%. When the L929 cells were treated with 20 ng/ml TNFα alone for 18 h, the number of apoptotic cells was 23.1%, and that of necrotic cells was 16.9% (Fig. 4A). DNA ladders were observed in the floating dead cells, but the DNA band intensity was not as high as that in the floating dead cells from the culture treated with TNFα plus z-VAD-fmk (Fig. 4B). All these results suggested that z-VAD-fmk augmented L929 cell death through at least 2 different ways: apoptosis and necrosis, and that it promoted more necrosis than apoptosis.

Z-VAD-fmk augmented cell death through a pre-mitochondrial signaling pathway. The mitochondrial permeability transition (MPT), the opening of a nonspecific pore in the inner mitochondrial membrane, is a well known alteration implicated as a mechanism of cell injury. Cyclosporine A, the inhibitor against MPT, reduces this cell injury and prevents TNFα-induced L929 cell death in the presence of actinomycin D [6–8]. To assess the role of MPT in the process of z-VAD-fmk-augmented L929 cell death, cyclosporin A was applied. Cyclosporin A partially inhibited z-VAD-fmk-augmented L929 cell death, but it had no effect on TNFα-induced L929 cell death (Fig. 5).

It was reported that in mitochondria the over-expression of Bcl-2 family protein Bax induced MPT and initiated post-mitochondrial signal transduction [9]. In order to further confirm that the mitochondria is involved

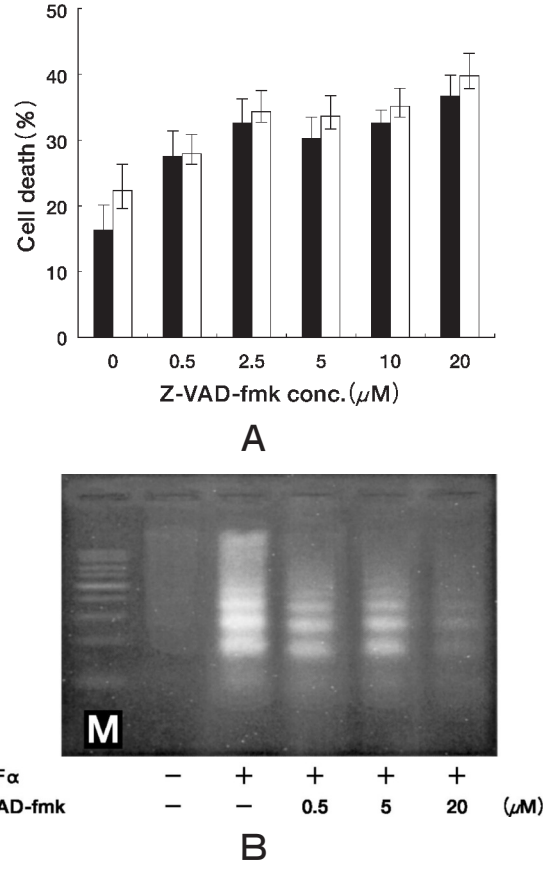


Fig. 4 Characterization of cell death augmented by z-VAD-fmk in L929 cells. Cells were treated with z-VAD-fmk of various doses plus 20 ng/ml TNFα for 18 h. The cell death rate was measured by LDH activity-based assay (■, necrosis; □, apoptosis. (**A**)). n = 3, mean ± S.D. In the floating cells, the DNA was separated on 2% agarose gel. M: molecular weight marker (**B**).

in z-VAD-fmk's augmentation of TNFα-induced L929 cell death, we examined the expression of Bcl-2, Bax and cytochrome *c* by Western-blot analysis. The results showed that the expression level of Bax was increased, but this was not the case in cells treated with TNFα alone. Bcl-2 and cytochrome c were not changed in the z-VAD-fmk-augmented cell death (Fig. 6).

MAPK was involved in z-VAD-fmk-augmented L929 cell death. MAPK signal pathways have been implicated in cell growth and cell death [10]. To determine whether the MAPK cascades are involved in z-VAD-fmk-augmented L929 cell death, specific inhibitors for p38 (SB203580), JNK (SP600125) and ERK (PD98059) were applied to evaluate the functions of MAPKs in the cell death. Pre-incubation with 5 μM SP600125 effectively reversed the augmented cell death, 5 μM PD98059 slightly increased the augmented cell death and 5 μM SB 203580 had almost no effect (Fig. 7A), all of which indicates that MAPKs were involved in

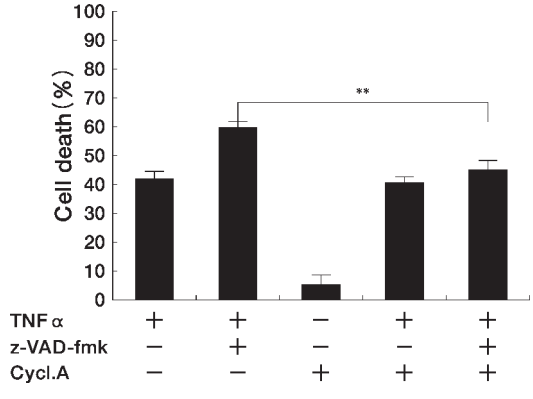


Fig. 5 Effects of cyclosporine A on z-VAD-fmk-augmented L929 cell death. For 18 h, cells were treated with TNFα 20 ng/ml or z-VAD-fmk 5 μM plus TNFα 20 ng/ml in the absence or presence of cyclosporine A (Cycl. A) 5 μM. Cell death was measured by MTT assay. n = 3, Mean ± S.D., $^{**}p < 0.01$ *vs* control group (z-VAD-fmk plus TNFα).

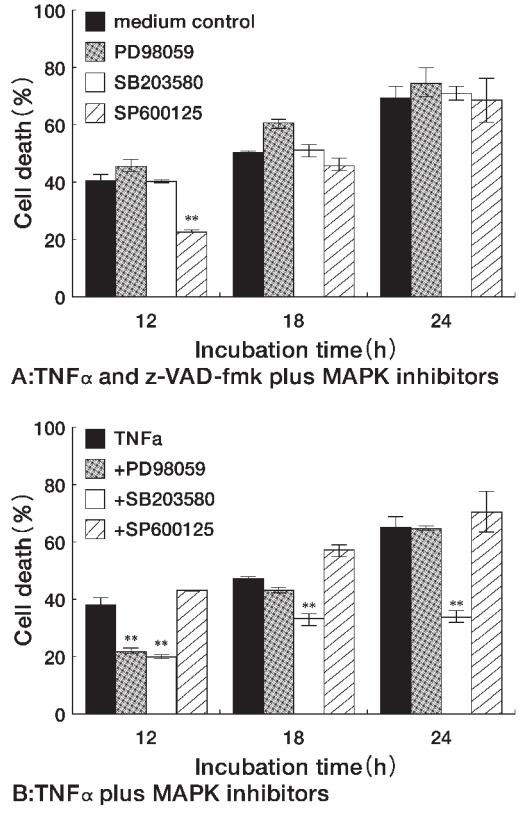


Fig. 7 Effects of MAPK inhibitors on z-VAD-fmk-augmented L929 cell death. Cells were treated with z-VAD-fmk 5 μM, TNFα 20 ng/ml and MAPK inhibitors (**A**) or TNFα 20 ng/ml and MAPK inhibitors (**B**) including 5 μM PD98059 (ERK), 5 μM SB203580 (p38) and 5 μM SP600125 (JNK) for varied time periods. Cell death was measured by MTT assay. n = 3, Mean ± S.D., $^{**}p < 0.01$ vs TNFα or medium control (z-VAD-fmk plus TNFα).

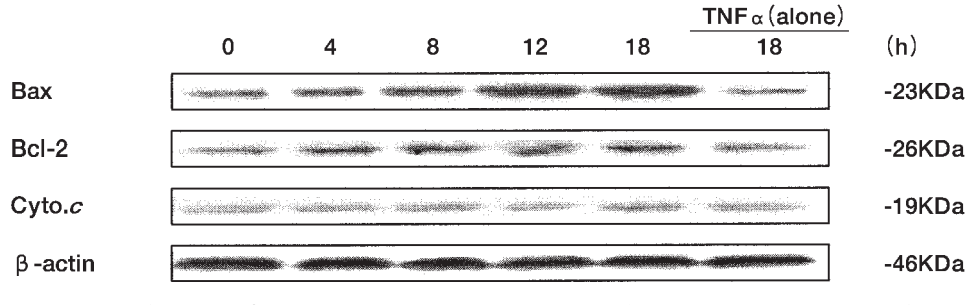


Fig. 6 Western-blot analysis of Bax, Bcl-2 and cytochrome c protein expression. Cells were treated with z-VAD-fmk 5 μM plus TNFα 20 ng/ml for varyed time periods. Cell lysates were separated by 12 % SDS-PAGE electrophoresis, and protein expression was detected by Western-blot analysis.

z-VAD-fmk's augmentation of TNFα-induced L929 cell death. However, 5 μM SB 203580 and 5 μM PD98059 markedly reduced TNFα-induced L929 cell death (Fig. 7B).

The effects of z-VAD-fmk and 20 ng/ml TNFα on the JNK and ERK phosphorylation were also examined. The expression of JNK and phosphorylated JNK was markedly up-regulated. ERK protein expression was not altered, but ERK phosphorylation was down-regulated. In TNFα alone case, however, ERK phosphorylation was increased (Fig. 8).

PARP participated in z-VAD-fmk-augmented L929 cell death. In order to examine whether poly (ADP-ribose) polymerase (PARP), as a downstream regulator, participated in z-VAD-fmk-augmented L929 cell death, the PARP inhibitor DPQ was applied to assess the function of PARP in the cell death process. It was found that a high dose (20 μM) of DPQ reduced cell death from 60.1 to 50.2% at 18 h, but

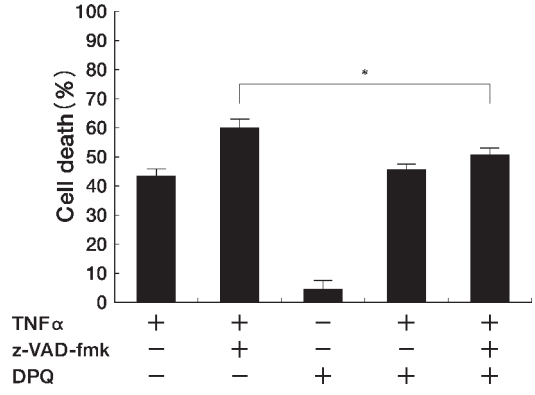


Fig. 9 Effects of PARP inhibitor on z-VAD-fmk-augmented L929 cell death. Cells were treated with TNFα 20 ng/ml or z-VAD-fmk 5 μM plus TNFα 20 ng/ml in the presence of various doses of PARP inhibitor DPQ for 18 h. Cell death was measured by MTT assay. n = 3, Mean ± S.D., $*p < 0.05$ *vs* z-VAD-fmk plus TNFα group.

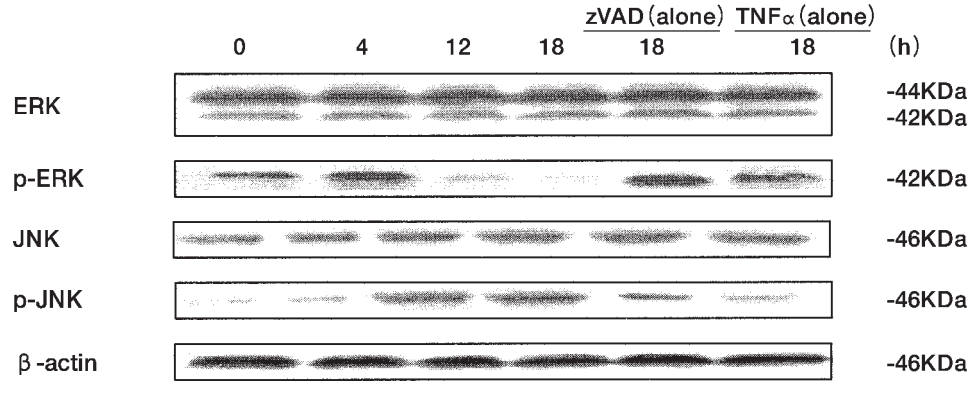


Fig. 8 Western-blot analysis of ERK and JNK protein expression. L929 cells were treated with z-VAD-fmk 5 μM plus TNFα 20 ng/ml for varied time periods. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the expression of protein was detected by Western-blot analysis.

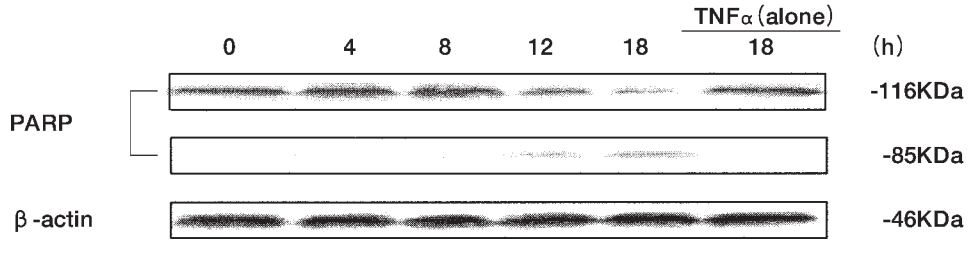


Fig. 10 Western-blot analysis of PARP protein expression. L929 cells were treated with z-VAD-fmk 5 μM plus TNFα 20 ng/ml for various time periods. Cell lysates were separated by 12% SDS-PAGE electrophoresis, and the expression of protein was detected by Western-blot analysis.

had no effect on TNFα-induced L929 cell death (Fig. 9); however, neither 10 μM nor 5 μM DPQ influenced the cell death (data not shown). In order to further confirm this, we analyzed the PARP expression by Western-blot analysis. The results showed that after treatment with z-VAD-fmk and 20 ng/ml TNFα for 12 h, the PARP protein was cleaved to an 85KDa fragment, but this was not the case in L929 cells treated with only TNFα (Fig. 10). These results suggest that z-VAD-fmk-augmented L929 cell death required PARP degradation in a caspase-independent manner.

Discussion

It has been reported that TNFα-induced L929 cell death in the presence of actinomycin D is the consequence of MPT in mitochondria and Bid translocation to the mitochondria which are not attributed to activation of caspase-8 [6-8]. TNFα induced the activation of p38, and the influence of p38 function on TNFα-induced apoptosis or necrosis has been a subject of controversy [11]. In this study, the inhibitor of caspase-3, z-DEVD-fmk, and the inhibitor of caspase-8, z-IETD-fmk, promoted the cell death. The pan-caspase inhibitor, z-VAD-fmk, partially inhibited the activities of caspases-3 and -8 and showed similar effects on TNFα-treated L929 cell; therefore, we only further investigated the effecter mechanisms of z-VAD-fmk-augmented L929 cell death. We found that z-VAD-fmk augments TNFα-induced L929 cell death through a different mechanism than that of TNFα-induced cell death. We also determined that z-VAD-fmk augments this cell death in a dose-dependent manner; however, z-VAD-fmk (0.5-20 μM) alone has almost no cytotoxic effects against L929 cells. After treatment with z-VAD-fmk and 20 ng/ml TNFα, the majority of the cells underwent apoptosis and necrosis, as measured by agarose gel electrophoresis and LDH assay.

It is well known that the majority of the cell death pass through one of the 2 distinct ways, necrosis and apoptosis, which has also been reported to share some common signal transduction pathways, such as death receptors, MAPK cascades and the mitochondinal pathway [12-14].

The over-expression of Bax in mitochondria induces the opening of pores in the inner mitochondrial membrane, MPT [9]. This induction of MPT caused cytochrome *c* to release into the cytoplasm, where it formed a complex with Apaf-1 and procaspase-9; thus, caspase-9 was activated, causing downstream death signal transduction [15]. In this study, the inhibition of mitochondrial permeability transition, or MPT, with cyclosporin A partially inhibited the cell death augmented by z-VAD-fmk. The expression level of Bax was increased when L929 cells were cultured with z-VAD-fmk and TNFα; however, the expressions of Bcl-2 and cytochrome c were not altered. In addition, caspase-9 inhibitor z-LEHD-fmk did not influence TNFα-induced cell death indicating that postmitochondrial caspase-9 was not activated in this process. These results suggested that Bax over-expression promoted MPT in mitochondria, but did not promote cytochrome c release into the cytoplasm and so neither the activation of caspase-9. It is well known that caspase-9 activats the downstream caspase-3, and that caspase-3 then cleaves PARP, a nuclear enzyme, to an 85-KDa product [16]. In this study, 20 μM DPQ, the inhibitor of PARP, reduced that cell death induced by z-VAD-fmk plus 20 ng/ml TNFα, and treatment with z-VAD-fmk and 20 ng/ml TNFα at 18 h, caused PARP protein cleavage to the 85 KDa fragment. All of these results suggest that an unknown signaling pathway(s) might exist in the post-mitochondrial processes to the downstream PARP which degrades in a caspase-independent manner, and that some protease(s) different from caspase-3 was responsible for PARP degradation in this case.

The MAPK family mainly consists of ERK, JNK and p38 MAPK. Activation of the distinct MAPK subtype cascades depends upon the types of cells and the stimuli, and the functional role of each MAPK subtype which may vary according to the cell type [13]. The ERK pathway is predominantly activated by mitogens through a Ras-dependent mechanism, and it is required for cell proliferation and differentiation; however, JNK and p38 are activated by pro-inflammatory cytokines and various environmental stresses [18, 19]. The results of this study showed that both JNK and phosphorylated JNK expression were markedly increased and JNK inhibitor SP600125 had partially inhibited cell death at 12 h, indicating that JNK activation may mediate z-VAD-fmk-augmented L929 cell death. However, at 18 h the inhibitive function of JNK inhibitor SP600125 had become weaker. This may result because z-VAD-fmk augments more necrosis than apoptosis, and necrotic cells releasing cytotoxic matters into the culture medium and initiating cell death through another as yet undetermined signal pathway. Whether this function of JNK in the cell death

is associated with the pre-mitochondrial signal pathway remains to be elucidated.

In summary, the mechanism by which z-VAD-fmk augments TNFα-induced L929 apoptotic and necrotic cell death is in part due to JNK activation and the pre-mitochondrial signal pathway, accompanied by the downstream protein PARP degradation. In addition to apoptosis, there is another pattern of cell death, autophagy, characterized by membrane-bound vacuoles that target organelles and proteins to the lysosome for degradation [19]. It has been reported that z-VAD-fmk promoted autophagy through activation of JNK in L929 cells [20]. However, in our study, z-VAD-fmk alone barely activated JNK, and z-VAD-fmk partially inhibited caspase-3 and -8 activities. Therefore, z-VAD-fmk might both augment TNFα-induced cell death by inhibiting caspase-3 and -8 activities and have a second cytotoxic effect against L929 cells through another unidentified pathway.

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