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Short communication

ACTIVATION OF THE INTRINSIC AND EXTRINSIC PATHWAYS IN HIGH PRESSURE-INDUCED APOPTOSIS OF MURINE ERYTHROLEUKEMIA CELLS

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Abstract: We previously demonstrated that caspase-3, an executioner of apoptosis, is activated in the pressure-induced apoptosis of murine erythroleukemia (MEL) cells (at 100 MPa). Here, we examined the pathway of caspase-3 activation using peptide substrates and caspase inhibitors. Using the substrates of caspases-8 and -9, it was found that both are activated in cells under high pressure. The production of nuclei with sub-G1 DNA content in 100 MPa-treated MEL cells was suppressed by inhibitors of caspases-8 and -9, and pan-caspase. In 100 MPa-treated cells, pan-caspase inhibitor partially prevented the cytochrome *c* release from the mitochondria and the breakdown of mitochondrial membrane potential. These results suggest that the intrinsic and extrinsic pathways are activated in apoptotic signaling during the high pressure-induced death of MEL cells.

Key words: Apoptosis, Caspases, Cytochrome *c*, Flow cytometry, Membrane potential, High pressure

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Abbreviations used: Ac-IETD-MCA – acetyl-IIe-Glu-Thr-Asp-4-methylcoumaryl-7-amide; Ac-LEHD-CHO – acetyl-Leu-Glu-His-Asp-aldehyde; Ac-LEHD-MCA – acetyl-Leu-Glu-His-Asp-4-methylcoumaryl-7-amide; CAD – caspase-activated deoxyribonuclease; DTT – dithiothreitol; HEPES – 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; MEL – murine erythroleukemia; NP-40 – nonidet P-40; PBS – phosphate-buffered saline; PI – propidium iodide; PMSF – phenylmethanesulfonyl fluoride; RNase A – ribonuclease A; ROS – reactive oxygen species; UV – ultraviolet; z-IETD-fmk – benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone; z-VAD-fmk – benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone

INTRODUCTION

To maintain tissue homeostasis in multicellular organisms, unwanted cells undergo cell death and are eliminated from the tissue. Recent research has revealed the pathways of apoptotic and non-apoptotic cell death [1-3]. Apoptosis is programmed and caspase-dependent cell death, and it is a physiological process that removes virus-infected or surplus cells [4]. All forms of apoptosis are characterized by structural properties such as cell shrinkage, condensation of nuclei, and loss of microvilli [1-4]. The non-apoptotic pathway is caspase-independent, and cell deaths such as necrosis and autophagy are involved in this pathway [1]. The properties of necrosis are cellular swelling and organelle degradation [1-3]. In its final stages, the cell membrane is disrupted so inflammation occurs due to the release of the cellular contents. It was recently shown that autophagy also participates in programmed cell death in a caspase-independent manner [1]. The morphology of autophagic cells is distinct from those of apoptotic and necrotic cells.

In apoptotic signaling, there are two caspase-dependent pathways, i.e. extrinsic and intrinsic [5]. The extrinsic pathway is activated in Fas-induced apoptosis. In this pathway, the Fas ligand, a member of the tumor-necrosis factor family, binds to the cell-surface death receptor Fas. This ligand-receptor interaction recruits the adaptor protein FADD (Fas-associating protein with death domain), which in turn recruits procaspase-8 [2]. Oligomerization of procaspase-8 leads to its autocatalytic activation. The activated caspase-8 directly activates effector caspases such as caspase-3 [6, 7]. Furthermore, caspase-8 cleaves the N-terminal domain of Bid, an apoptosis-promoting member of the Bcl-2 family [7]. Truncated Bid translocates to the mitochondria and induces the breakdown of mitochondrial membrane potential and the release of cytochrome *c* [7]. Thus, caspase-8 is the apical caspase in the extrinsic pathway.

The intrinsic pathway is activated by various cellular stresses, including serum deprivation. The release of cytochrome *c* from the mitochondria into the cytosol is another pathway to activate caspase-3 via caspase-9 [8, 9]. The release of cytochrome *c* from the mitochondria is mediated by Bcl-2 family members such as Bax and Bcl-2 [10]. Pro-apoptotic members like Bax and Bid facilitate the cytochrome *c* release, whereas the anti-apoptotic members such as Bcl-2 and Bcl-XL prevent its release [10]. Thus, the mitochondria play a central role in the intrinsic pathway.

Apoptosis is induced upon the exposure of cells to high pressures. For instance, mammalian cells such as MEL cells [11] and human lymphoblasts [12] undergo apoptosis when exposed to a pressure of 100 MPa. Interestingly, living organisms have been found in deep-sea environments where the pressure reaches 110 MPa. Thus, it is interesting to examine how apoptosis in mammalian cells is induced by high pressure. Previously, we demonstrated that caspase-3 is activated in high pressure-induced apoptosis [11]. However, the pathway of caspase-3 activation remains unclear. In this paper, we report that the intrinsic and extrinsic pathways are activated in high pressure-induced apoptosis of MEL cells.

MATERIALS AND METHODS

Chemicals

Acetyl-Leu-Glu-His-Asp-4-methylcoumaryl-7-amide (Ac-LEHD-MCA) and acetyl-Leu-Glu-His-Asp-aldehyde (Ac-LEHD-CHO) were obtained from the Peptide Institute, Inc. Acetyl-IIe-Glu-Thr-Asp-4-methylcoumaryl-7-amide (Ac-IETD-MCA) was obtained from the BIOMOL Research Lab, and benzyloxycarbonyl-Val-Ala-Asp(OMe)-fluoromethyl ketone (z-VAD-fmk) and benzyloxycarbonyl-Ile-Glu(OMe)-Thr-Asp(OMe)-fluoromethyl ketone (z-IETD-fmk) from R&D Systems Inc. Dithiothreitol (DTT), 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), leupeptin hemisulfate monohydrate, streptomycin, and Triton X-100 were purchased from Wako Chemicals, and penicillin G, propidium iodide (PI), ribonuclease A (RNase A) and Rhodamin 123 from Sigma. Nonidet P-40 (NP-40) and phenylmethanesulfonyl fluoride (PMSF) were from Nacalai tesque, and RPMI-1640 medium was from Nissui Pharmaceutical Co. All the other chemicals were of reagent grade.

Cell culture and pressure treatment

MEL cells (cell line 745A) were maintained in RPMI-1640 medium containing 10% fetal calf serum, streptomycin (0.1 mg/ml), and penicillin G (100 U/ml) at 37ºC in a CO_2 (5 %) incubator. The cells (0.5-1.0 x 10^6 cells/ml) in the medium were subjected to a pressure of 100 MPa for 30 min at 37ºC [13].

Detection of apoptosis by flow cytometry

MEL cells were preincubated in the presence of caspase inhibitors for 60 min at 37ºC, exposed to a pressure of 100 MPa, and then incubated in the presence of caspase inhibitors for 90 min at atmospheric pressure (0.1 MPa) and 37ºC. The concentration of all the caspase inhibitors used was 50 μM. For z-VAD-fmk only, a concentration of 100 μM was also used. After culture, the suspensions were centrifuged for 5 min at 250 x g and 4ºC. The cells were washed twice with phosphate-buffered saline (PBS: 136 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.4) and then fixed with 70% ethanol overnight at -20ºC. The samples were washed with PBS and treated with RNase A (100 μg/ml) in PBS for 20 min at 37ºC. After treatment, the cells were washed once in PBS and stained with PI (50 μg/ml) for 10 min at room temperature. Flow cytometric analysis was performed using an EPICS XL System II (Coulter).

Measurements of caspase activity

For the measurement of the caspase-8 and -9 activities, 100 MPa-treated cells were cultured at atmospheric pressure for 90 min at 37ºC. After the culture, the cells were washed twice with chilled PBS, and then treated with aqueous solution containing 1% Triton X-100 and 1% NP-40. The lysate was incubated for 30 min at 4ºC, and centrifuged for 5 min at 17,000 x g and 4ºC. The supernatant and a substrate (Ac-IETD-MCA for caspase-8 and Ac-LEHD-MCA

for caspase-9) in 100 mM HEPES-KOH, 5 mM DTT, 10% NP-40 and 10% sucrose, pH 7.4 were mixed, and the mixture (0.5 ml) was incubated for 60 min at 37ºC. After incubation, 2 ml of sodium acetate (1 M, pH 4.2) was added into the reaction mixture. The released 7-amino-4-methylcoumarin (AMC) was measured at 380 nm excitation and 460 nm emission.

Measurements of the mitochondrial membrane potential and cytochrome *c*

MEL cells were preincubated in the presence of z-VAD-fmk (100 μM) for 30 min at 37ºC, exposed to a pressure of 100 MPa, and then cultured in the presence of z-VAD-fmk (100 μM) for 90 min at atmospheric pressure and 37ºC. After the culture, the cells were washed three times with PBS. For the measurement of mitochondrial membrane potential, the cells were suspended in PBS containing Rhodamine 123 (10 μM) and incubated for 15 min at 37ºC. The membrane potential was measured using a flow cytometer (EPICS XL System II, Coulter).

To isolate the mitochondria, the cells were suspended in mitochondrial isolation buffer (250 mM sucrose, 20 mM HEPES-KOH, 10 mM KCl, 1 mM EGTA, 1 mM EDTA, 1.5 mM $MgCl_2$, 1 mM DTT, 0.1 mM PMSF, 10 μg/ml leupeptin) and stood for 30 min at 0ºC. The samples were homogenized by douncing forty times in a Dounce homogenizer, and centrifuged at 800 x g for 10 min at 4ºC. The supernatants were centrifuged at 20,000 x g for 15 min at 4ºC. The pellets were used to measure cytochrome *c* within the mitochondria. The cytochrome *c* was measured using an ELISA kit (Quantikine M, R&D Systems, Inc.)

RESULTS

The suppression of high pressure-induced apoptosis by caspase inhibitors

To examine the pathway of caspase-3 activation, MEL cells exposed to a pressure of 100 MPa were cultured in the presence of caspase inhibitors, and analyzed via flow cytometry (Fig. 1A). The apoptotic cells appeared to have dominantly sub-G1 DNA content (Fig. 1A-b). The high pressure-induced apoptosis was significantly suppressed by the inhibitors of the pan-caspases (Fig. 1A-c and 1B), caspase-8, and caspase-9 (Fig. 1C). Of these inhibitors, the pan-caspase inhibitor z-VAD-fmk was most effective (Fig. 1C). The inhibition effect of z-VAD-fmk at 100 μM was almost the same as that at 50 μM (data not shown). Additionally, when the activities of caspases-8 and -9 were examined using fluorescence substrates, the caspase-8 activity in extracts prepared from 100 MPa-treated MEL cells increased 5.6-fold compared with that from cells not subjected to high pressure, whereas the caspase-9 activity increased 3.8-fold. These results suggest a possibility of the contribution of caspases-8 and -9 to the activation of caspase-3.

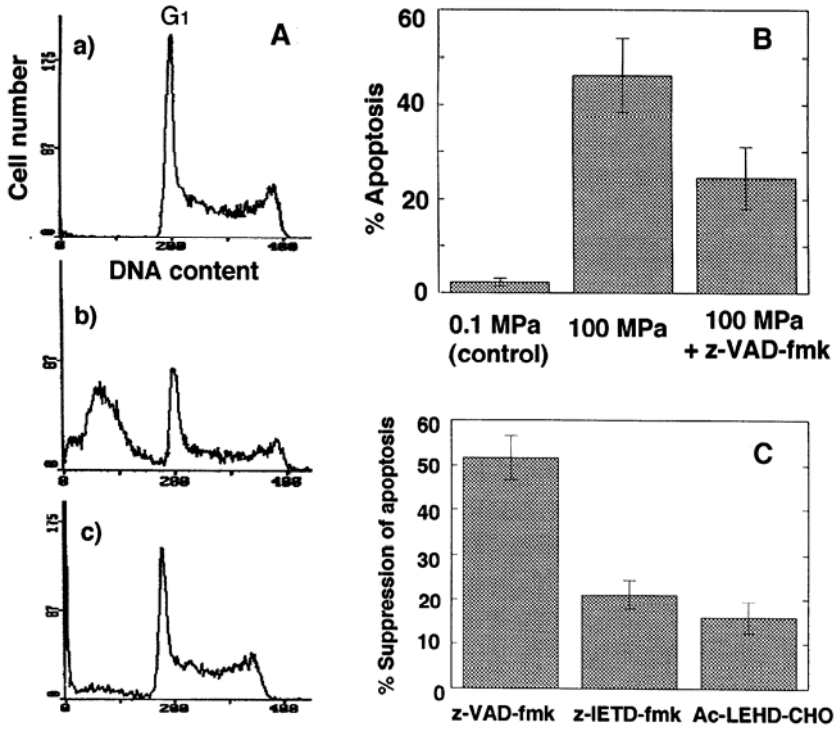

Fig. 1. The inhibition of apoptosis by caspase inhibitors in 100 MPa-treated MEL cells. A – Flow cytometric analysis of high pressure-induced apoptosis. The 100 MPa-treated cells were cultured in the absence (b) or presence (c) of 50 μM z-VAD-fmk for 90 min under atmospheric pressure (0.1 MPa). (a) – Cells not subjected to high pressure (control). B – Apoptosis (%) quantified via flow cytometric analysis. The values are the means ± SD for three experiments. C – Suppression (%) of high pressure-induced apoptosis by the inhibitors of pan-caspases, caspase-8, or caspase-9. The concentration of all the caspase inhibitors was 50 μM. The values are the means ± SD for three experiments.

Mitochondrial membrane potential in high pressure-treated MEL cells

Positively charged lipophilic molecules such as Rhodamine 123 are partitioned between the cell and the surrounding medium depending on the mitochondrial membrane potential. Therefore, these charged dyes are plentifully incorporated into polarized mitochondrial membranes, but poorly into depolarized ones. Thus, the normal cells with polarized mitochondrial membranes are expected to show strong fluorescence intensity, whereas apoptotic cells that have a breakdown of mitochondrial membrane potential show weak fluorescence intensity. Therefore, changes in the mitochondrial membrane potential in MEL cells were examined using Rhodamine 123 (Fig. 2A). In the 100 MPa-treated MEL cells, the breakdown of mitochondrial membrane potential was observed in parts of the cell population. Such a reduction in membrane potential was considerably recovered by z-VAD-fmk.

Release of cytochrome *c* from the mitochondria in high pressure-treated MEL cells

The release of cytochrome *c* from the mitochondria in high pressure-treated cells was examined using the ELISA method (Fig. 2B). In 100 MPa-treated MEL cells, about 70% of cytochrome *c* was released from the mitochondria. However, this release of cytochrome *c* was suppressed by about 40% by z-VAD-fmk.

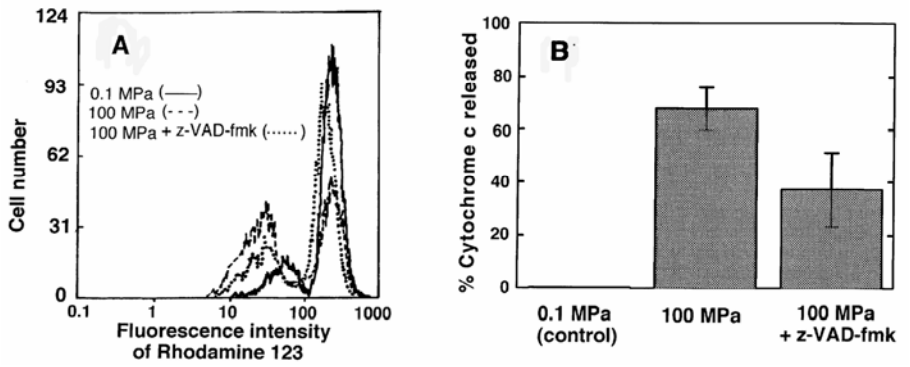

Fig. 2. Mitochondrial membrane damage to 100 MPa-treated MEL cells. MEL cells exposed to a pressure of 100 MPa were cultured in the absence or presence of z-VAD-fmk (100 μM) for 90 min under atmospheric pressure (0.1 MPa). A – The mitochondrial membrane potential of high pressure-treated cells. For the measurements of membrane potential, these cells were stained with Rhodamine 123. B – Release of cytochrome *c* from the mitochondria in 100 MPa-treated MEL cells. The values are the means ± SD for three experiments.

DISCUSSION

We previously showed that there is caspase-3 activation in the high pressure-induced apoptosis of MEL cells [11]. In this study, the pathway of caspase-3 activation was analyzed using peptide substrates and caspase inhibitors. Several lines of evidence show the contribution of caspases-8 and -9 to the signaling pathway of 100 MPa-induced apoptosis.

The breakdown of mitochondrial membrane potential and the release of cytochrome *c* from the mitochondria are interesting events in apoptosis. The response of the cells to high pressure is dependent on the cell cycle. MEL cells in the G1- or G2-phase are stable to a pressure of 80 MPa, whereas cells in the S-phase are sensitive to that pressure [13]. That explains why the breakdown in the membrane potential is observed only in a part of the MEL cell population. In high pressure-treated cells, the breakdown in the membrane potential is prevented by z-VAD-fmk. Here, it is useful to compare our results with those for apoptosis induced by other methods. In apoptosis induced by ionizing radiation in Jurkat cells, a loss of mitochondrial membrane potential is observed [6]. However, this loss is unaffected by z-VAD-fmk, indicating the contribution of a caspase-independent pathway to this event [6]. Thus, the apoptotic signaling to

the mitochondria induced by high pressure is different from that induced by ionizing radiation.

One pathway of caspase-3 activation is associated with the release of cytochrome *c* from the mitochondria [4, 7]. The released cytochrome *c* binds to Apaf-1 (apoptotic protease activating factor-1). This oligomeric cytochrome *c*-Apaf-1 complex recruits and activates caspase-9. Then, caspase-9 activates the executioner caspase-3 [4, 7]. In 100 MPa-treated MEL cells, the release of cytochrome *c* occurs in parts of the cell population. Provided that the mitochondrial outer membranes are disrupted by a pressure of 100 MPa, cytochrome *c* would be released from the mitochondria of all the cells. However, no such release of cytochrome *c* is observed. This suggests that the cytochrome *c* release from the mitochondria in 100 MPa-treated MEL cells is not due to the disruption of the outer mitochondrial membranes by high pressure, but is a response to apoptotic signals. Such a release of cytochrome *c* is partially prevented by z-VAD-fmk. This suggests that Bid, a pro-apoptotic factor, remains inactive due to the inhibition of caspase-8 by z-VAD-fmk [14]. Furthermore, the cytochrome *c* release being insensitive to z-VAD-fmk suggests the existence of a caspase-independent pathway such as the translocation of Bax from the cytosol to the mitochondrial membranes [4, 7, 9]. In 100 MPa-treated MEL cells, we demonstrated that caspase-9 is activated, and that the apoptosis is suppressed by the caspase-9 inhibitor. These results suggest that the intrinsic pathway is activated in high pressure-induced apoptosis of MEL cells. Another pathway of caspase-3 activation is associated with caspase-8 [6, 15]. In 100 MPa-treated MEL cells, we demonstrated caspase-8 activation and reduction of the nuclei with sub-G1 DNA content by the caspase-8 inhibitor. These results suggest that the extrinsic pathway is also activated in high pressure-treated MEL cells.

Active caspase-3, an executioner of apoptosis, cleaves the inhibitor of caspase-activated deoxyribonuclase (ICAD) which forms a complex with caspase-activated deoxyribonuclease (CAD) to inhibit its DNase activity [16]. The released CAD enters the nucleus and degrades chromosomal DNA. In this study, the fragmentation of DNA was monitored by flow cytometry. Cells with degraded DNA have sub-G1 DNA contents. The production of nuclei with sub-G1 DNA contents is inhibited, but not completely, by z-VAD-fmk or caspase-3 inhibitor Ac-DEVD-CHO (200 μM) [11]. Thus, it seems likely that caspase-independent DNA degradation, as seen with necrosis, also occurs in 100 MPa-treated MEL cells. Similar results for necrotic cell death are reported for 100 MPa-treated lymphoblasts [12].

In terms of analysis of the signalling pathway, UV-induced apoptosis is ahead of pressure-induced apoptosis. It is well known that UV irradiation induces DNA damage such as thymine dimers and nucleotide deletion [17, 18]. Such DNA damage induces the activation of ATR (ATM- and Rad3-related) protein kinase and in turn Chk1, a protein kinase needed for the DNA damage G2 checkpoint control [19]. If DNA damage is severe, the apoptotic pathway via the

mitochondria is activated. On the other hand, it is unclear what the apoptotic stimulus for high pressure is. By contrast to the UV situation, DNA is stable against high pressure. For instance, 80 MPa-treated *Xenopus* nuclei are able to replicate DNA in *Xenopus* extracts [20]. It is known that oligomeric proteins are dissociated under high pressures [21]. So, it is of interest to examine the influence of high pressure on multiprotein complexes participating in DNA replication. Further, reactive oxygen species (ROS) also induce apoptosis and the production of ROS is enhanced by pressure treatment [22]. Thus, data to understand how apoptosis is induced by high pressure needs to be accumulated. Further work is to investigate the pathway of caspase-8 activation and the caspase-independent pathway of cytochrome *c* release in 100 MPa- induced apoptosis.

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