Original Paper

Involvement of Caspases 3, 8, and 9 Signaling Pathways in Hyperthermia Induced Apoptosis in HL-60 Cells

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Abstract : The mechanism of hyperthermia-induced apoptosis in HL-60 cells was investigated. HL-60 cells were heated at 43°C for 1 h and then incubated at 37°C for 4 h. During and after the hyperthermia treatments, apoptosis induced by hyperthermia was assessed by observing morphologic changes and DNA fragmentation. A significant and rapid induction of apoptosis in HL-60 cells treated at 43°C for 1 h was associated with morphological changes, and DNA fragmentation was demonstrated. These results suggest that hyperthermia treatment at 43°C has the potential to induce apoptosis in HL-60 cells. The induction of apoptosis by a hyperthermia treatment was associated with the activation of caspase-3 and was completely suppressed by a caspase-3 inhibitor. These observations suggest that the activation of caspase-3 by hyperthermia treatment is essential for the commitment of cells to undergo apoptosis. Furthermore, inhibitors of caspase-8 and -9 blocked caspase-3 activation and hyperthermia induced apoptosis. These results may imply that caspase-8 and -9 mediate caspase-3 activation during hyperthermia-induced apoptosis in HL-60 cells.

Key Words : apoptosis, hyperthermia, caspase-3, caspase-8, caspase-9

Introduction

Apoptosis can be initiated by a wide variety of intracellular and extracellular stimuli and constitutes a mechanism for the removal of unnecessary, aged or damaged cells. Cells undergoing apoptosis exhibit morphological changes which are characteristic of the apoptotic process. In this process, dying cells typically shrink, and widespread membrane blebbing occurs, followed by chromatin condensation and DNA fragmentation. The cell further dissociates into membrane-enclosed vesicles called apoptotic bodies which are rapidly and completely ingested by neighboring cells and phagocytes^{1,2)}.

The cellular mechanism leading to apoptosis is associated with the activation of a family of cysteine-dependent aspartate-directed proteases, called caspases. All of the caspases are synthesized as inactive proenzymes and then release active subunits when they are catalytically activated³⁾. The active caspases hydrolyze a number of key structural and housekeeping proteins during the progression of apoptosis, such as poly (ADPribose) polymerase (PARP), DNA-dependent protein kinase, protein kinase Cd, and U1 small ribonucleoproteins⁴⁻⁶⁾.

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Caspase-3 has been identified as a key executioner involved in apoptosis induced by many stimuli⁷⁾, and is also required for the occurrence of nuclear changes associated with apoptosis to develop, such as chromatin condensation⁸⁾. Although caspase-3 can be directly activated by caspase-8, the farthest upstream caspase involved in TNF- or Fas-initiated proapoptotic signaling, caspase-3 is also indirectly activated by caspase-8 in a process involving cytochrome c release and caspase-9 activation⁹⁾.

Hyperthermia has been considered as a potent modality for cancer treatment¹⁰⁾. Hyperthermia increases the effectiveness of currently available cancer treatments such as chemotherapy and radiation¹¹⁾. The combination of hyperthermia with radiation permits a decrease in the therapeutic radiation dose used¹²⁾. Recently it has been reported that hyperthermia can trigger apoptosis in malignant cells^{13–15)}. However, the mechanism leading to apoptosis triggered by hyperthermia treatment is not well understood. The initiating events of hyperthermia-induced apoptosis such as caspase activation are poorly defined. It would be of interest to know the role of caspase pathways in hyperthermia-induced apoptosis. The aim of this study was to learn more about the mechanism through which hyperthermia triggers apoptosis, with emphasis on the caspase pathway.

Materials and Methods

Chemicals

Trypan blue, propidium Iodide and agarose were purchased from Sigma (St Louis, MO, USA). RNase A and Proteinase K were purchased from WAKO (Tokyo, Japan). The fluorogenic caspase substrates acetyl-Asp-Glu-Val-Asp -aminotri fluoromethyl coumarin (Ac-DEVD-AFC), and acetyl-Ile-Glu-Thr-Asp- aminotri fluoromethyl coumarin (Ac-IETD-AFC) were purchased from MBL (Tokyo, Japan). The caspase inhibitor peptides, z-Asp-Glu-Val-Asp-fluoromethyl ketone (DEVD-FMK), z-Ile-Glu-Thr-Asp-fluoromethyl ketone (IETD-FMK) and z-Leu-Glu-His-Asp-fluoromethyl ketone (LEHD-FMK) were purchased from MBL (Tokyo, Japan), dissolved to make a stock solution in DMSO, and stored at -20°C . All the other reagents were analytical grade commercial products.

Cell culture

Human promyelocytic leukemia HL-60 cells were obtained from the RIKEN Gene Bank (Tokyo, Japan). Cells were maintained in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS, GIBCO BRL, Tokyo, Japan), 100 U/ml Penicillin G, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 2 mM-glutamine (Sigma), and grown in a humidified 5% CO_2 atmosphere at 37°C .

Hyperthermia treatments

Hyperthermia treatments were performed by immersion of plastic culture tubes containing 3 ml of a cell suspension (1×10^6 cells/ml) in a water bath (BF-22, Yamato, Tokyo, Japan) at 37, 42, 43 or 44°C . The temperature of the cell suspension was monitored with a digital thermometer (Anritsu, Tokyo, Japan) coupled with a 0.8-mm thermocouple. After the treatments, HL-60 cells were incubated at 37°C for the indicated times. When required, the inhibitors for caspases were added to the cell suspension.

Evaluation of apoptosis and cell damage

Apoptosis is characterized by morphological changes such as membrane blebbing, cell shrinkage, and the presence of apoptotic bodies. To detect the occurrence of apoptotic and necrotic cell death, HL-60 cells were stained with a trypan blue solution during or after the treatments, and examined using a phase contrast microscope (Olympus, Tokyo, Japan) at a 400X magnification. Cells which excluded trypan blue and showed membrane blebbing and cell shrinkage were considered apoptotic, while cells which were trypan blue-stained and which also exhibited a “balloon-like” morphology were considered necrotic. The fraction of apoptotic or necrotic cell was determined by counting the number of cells showing these morphological changes on a hemocytometer glass plate. Cell viability was checked immediately before each series of treatments, and cell suspensions with cell viability levels above 99% were used. This number of intact viable cells before each exposure was considered to be an essential standard to insure the validity of the data obtained after each treatment.

Electrophoretic analysis of DNA fragmentation

After an appropriate length incubation following each treatment, the cells were harvested, washed in phosphate-buffered saline (PBS) and then lysed in 100 μ l of 0.1M phosphate-citrate buffer solution. Following lysis, the samples were centrifuged at 16,000 $\times$ g for 5 min. The supernatants were then treated with 200 μ g/ml DNase-free RNase (2 μ l) at 37°C for 1 h, followed by a treatment with 1 μ g/ml proteinase K (1 μ l) at 50°C for 1 h. The samples were electrophoresed at 50 V for 3 h in 1.5% (w/v) agarose gels complemented with 1 μ g/ml ethidium bromide. Separated DNA fragments (DNA ladders) were visualized using a UV transilluminator. The size of DNA fragments was determined by comparisons with DNA molecular weight markers: 100 bp DNA (Invitrogen, Carlsbad, CA, USA).

Measurement of caspase-3 and -8 activities

Caspase-3 and -8 activities were assayed with a fluorogenic technique using specific peptide substrates: Ac-DEVD-AFC for caspase-3 and Ac-IETD-AFC for caspase-8. Treated cells were washed with 50 mM PBS and then resuspended in buffer containing 50 mM Tris/HCL (pH 7.4), 1 mM EDTA, and 10 mM EGTA. The cell lysates were centrifuged at 800 $\times$ g for 5 min and the supernatant was incubated with

Fig. 1. Morphological observation of apoptosis induction with phase contrast microscopy (a) cells treated at 37, and (b) cells treated at 43°C.

50 μ M of peptide substrate at 37°C for 2 h. The formation of 7-amino-4-trifluoromethylcoumarin (AFC) was measured using a fluorescence spectrophotometer (Hitachi F-3000, Tokyo, Japan) with excitation at 400 nm and emission at 505 nm. The enzyme activity was measured immediately before each series of treatments. The activity measured before a hyperthermia exposure was considered to be the control for the activity determinations made after each treatment. Caspase activities were expressed as the ratio of released AFC measured in treated cells to the level measured in untreated control cells.

Results

Morphological changes

Apoptosis induction was examined by observing morphological changes under phase contrast microscopy after a 1 hour hyperthermia treatment. The morphological changes seen in cells treated at 37°C and 43°C are shown in Fig. 1. No significant morphological changes were observed in control cells treated at 37°C, the normal incubation temperature (Fig. 1a). Treatment at 43°C clearly produced membrane blebbing and cell shrinkage (Fig. 1b). For a more detailed examination of morphologic changes in apoptotic cells, electron microscopy was used. However, phase contrast microscopy can be used for the observation of major apoptotic changes such as cell shrinkage, membrane blebbing, and

apoptotic body formation. Since morphologic changes were easily and clearly observed in hyperthermia-treated cells as shown in Fig.1, the morphological features of apoptosis were assessed only with phase-contrast microscopy in this study.

Apoptosis induction

The apoptotic fractions of HL-60 cell populations during hyperthermia treatments are shown in Fig. 2a.

This apoptotic fraction of the cell population was plotted versus the duration of the heat exposure for temperatures of 42, 43 and 44°C. The results at 37°C without hyperthermia treatment are also plotted versus duration of exposure. The apoptotic fraction for cells subjected to all treatments was less than 2% when the treatments were initiated. The apoptotic fractions in cells treated at 42, 43 and 44°C increased as the exposure duration increased. No significant apoptotic induction was observed in control cells at 37°C. The apoptotic fraction in the cells treated at 43°C

Fig. 2. Apoptotic fraction of HL-60 cells (a) during and (b) after hyperthermia treatments. Cells were treated at 37 (○), 42 (□), 43 (●) and 44°C (■) for 1 h. Each point and vertical bar represents the mean ± S.D. of three experiments.

was significantly higher than at 37, 42 and 44°C during the exposures. After a 1 h exposure duration at 43°C, the apoptotic fraction increased to 45%.

The apoptotic fractions of HL-60 cells after hyperthermia treatment at 42, 43 and 44°C were plotted versus incubation time in Fig. 2b. The results at 37°C without hyperthermia treatment are also plotted versus the incubation time. The necrotic fractions of cells after 42, 43 and 44°C hyperthermia were $1.69 \pm 0.99\%$, $1.72 \pm 0.67\%$ and $20.1 \pm 1.54\%$ ($n=3$), respectively. The apoptotic fractions in the cells treated at 42 and 43°C decreased after treatments with increasing incubation time. Interestingly, the apoptotic fraction in the cells treated at 44°C was at its maximum after 1 h of treatment and then decreased with increasing incubation time.

DNA fragmentation

Fig. 3 shows a characteristic DNA ladder observed in HL-60 cells treated at 43°C for 1 h (lanes 4-8). Immediately after hyperthermia treatments, the DNA ladder was not obvious (lane 4). It became clearly visible after a 1 h incubation following the hyperthermia treatment (lane 5). No obvious DNA ladder was observed when cells were treated at 37°C (lane 3).

Fig. 3. Agarose gel electrophoresis of DNA. Lane 1 represents marker DNA. Lane 2 represents DNA from cells before the treatments. Lane 3 represents DNA from cells maintained at 37°C for 1 h and then incubated at 37°C for 4 h. Lanes 4-8 represent DNA from cells treated with 43°C hyperthermia for 1 h and then incubated at 37°C for 0, 1, 2, 3 and 4 h respectively.

Fig. 4. Caspase-3 and -8 activities of HL-60 cells during hyperthermia treatments. Cells were treated at 43°C for 1 h. Each point and vertical bar represents the mean $\pm$ S.D. of three experiments.

Fig. 5. Caspase-3 and -8 activities of HL-60 cells after hyperthermia treatments. Cells were treated at 43°C for 1 h and then incubated at 37°C for 4 h. Each point and vertical bar represents the mean $\pm$ S.D. of three experiments.

Fig. 6. Effect of caspase inhibitors on hyperthermia-induced apoptosis. Cells were treated at 43°C for 1 h. Each point and vertical bar represents the mean $\pm$ S.D. of three experiments.

Caspase-3 and 8 Activation

To see if caspases are activated by hyperthermia treatment in HL-60 cells, the enzymatic activities of caspase-3 and -8 were measured using fluorescent peptide substrates. The activities of caspase-3 and -8 in HL-60 cells during hyperthermia treatment at 43°C were plotted versus exposure duration in Fig. 4a and b respectively.

The activities were determined by measuring 7-amino-4-trifluoromethylcoumarin (AFC) fluorescence intensity. As shown in Fig. 4a and b, the activities of both enzymes increased as with increasing exposure duration. The degree of caspase-8 activation was lower than that seen for caspase-3. The activities of caspase-3 and -8 in HL-60 cells after hyperthermia treatment at 43°C were plotted versus the incubation time in Fig. 5a and b respectively.

The cellular activities of both enzymes decreased gradually with increasing incubation time after hyperthermia treatment at 43°C.

Effect of caspase inhibitors

The effect of caspase inhibitors on both, hyperthermia induced apoptosis and caspase-3 activation was tested, and the results are shown in Fig. 6 and 7.

Both, the apoptotic fraction and caspase-3 activity, were completely suppressed in the presence of either 50 μ M DEVD-FMK or 50 μ M IETD-FMK, but not completely suppressed in the presence of 50 μ M LEHD-FMK.

Discussion

Cell shrinkage and membrane blebbing, characteristic of apoptotic cells, were clearly observed in cells treated at 43°C for 1 h, while

no significant morphologic changes were observed in control cells treated at 37°C (Fig. 1). The apoptotic fraction in cells treated at 43°C for 1 h was significantly higher than in cells exposed to 37, 42 and 44°C (Fig. 2). The highest apoptotic fraction was observed when cells were treated with hyperthermia at 43°C for 1 h. Membrane blebbing, one of the most visible changes in the apoptotic process, was observed, and cells with blebs were scored after the treatments. At longer times after the blebbing process had begun, the cells became round and some blebs detached from the cells. These detached blebs are considered to be so-called apoptotic bodies,

and these apoptotic bodies eventually disintegrated into smaller fragments. This observation may account for the decreasing number of cells showing membrane blebbing at times longer than 1 h after hyperthermia treatment. The increase in temperature from 43°C to 44°C leads to a decrease in the incidence of apoptosis and to an increase in necrosis. These results show that HL-60 cells are likely to undergo necrosis instead of apoptosis during treatment with temperatures above 44°C. It has been reported that necrosis is a consequence of membrane disruption after hyperthermia treatment. The change of membrane fluidity after a 44°C hyperthermia treatment may thus contribute to heat-induced necrotic cell death^{16–17}. On the basis of these results, a 43°C hyperthermia temperature was used for subsequent experiments.

The fragmentation of DNA in linker regions between nucleosomes yielded fragments which were multiples of 180–200 bp, a size which is a typical hallmark of apoptosis¹⁸. Agarose gel electrophoresis was used to examine the production of apoptotic DNA fragments, and DNA ladder formation after hyperthermia treatment at 43°C was observed (Fig. 3). These results demonstrate that hyperthermia treatment at 43°C for 1 h causes a significant induction of apoptosis in HL-60 cells, associated with morphological changes and DNA fragmentation. A typical DNA ladder was not obvious immediately after exposure, but was apparent after a 1 h post-hyperthermia incubation. This observation suggests that DNA fragmentation was caused by an enzymatic process rather than by direct thermal damage to HL-60 cells. In this study, membrane blebbing was observed during hyperthermia treatments. In contrast, DNA fragmentation was caused after hyperthermia treatment. Though many investigators have detected each event for identifying apoptosis, few reports have discussed the time course of these apoptotic events. Okayama *et al* reported that DNase γ activation is directly related to DNA fragmentation during apoptosis. DNase γ activation occurred in condensed chromatin in round-shaped cells, and not in cells showing blebbing, indicating that DNase γ activation occurred in the late part of the blebbing stage^{19–20}.

Caspase-3 is an important enzyme required for the execution of the final phase of apoptosis and it

Fig. 7. Effect of caspase inhibitors on hyperthermia-induced caspase-3 activation. Cells were treated at 43°C. Each point and vertical bar represents the mean $\pm$ S.D. of three experiments.

is active in the cells undergoing apoptotic death^{21–25}). Significant activation of caspase-3 was demonstrated during hyperthermia treatment at 43°C for 1 h (Fig. 4a). Induction of apoptosis by hyperthermia treatment was associated with activation of caspase-3, and DEVD-FMK has been reported to act as an inhibitor of caspase-3²⁶). Since both, apoptosis induction and caspase-3 activation, were completely suppressed by DEVD-FMK, it is likely that the activation of caspase-3 is an integral part of the pathway mediating apoptosis induced by hyperthermia treatment (Fig. 6 and 7). It has been shown previously that HL-60 cells rapidly undergo apoptosis during treatment with temperatures above 43°C. Gianni et al reported that the rapid induction of apoptosis in acute promyelocytic leukemia NB4 cells is directly due to caspase-3 proteolysis. They suggested that the activation of caspase-3 may be a major determinant of the initiation and of the rate of progression of apoptosis. The results reported here for rapid apoptosis induction associated with caspase-3 activation also support this hypothesis. The aim of future work will be to determine the exact mechanisms through which hyperthermia treatment rapidly activates apoptotic cell death^{27–28}).

Distinctive caspase-8 activation was also observed during hyperthermia treatment. These data suggest that hyperthermia stimulates activation of caspase-8. The activation of caspase-8 accompanied the activation of caspase-3 during the apoptotic process (Fig. 2 and 4). Caspase-8 appeared to be activated to a lower extent than caspase-3 when the same concentration of either substrate was used (Fig. 5). This may imply that only a small amount of caspase-8 may exist in these cells, or that only part of caspase-8 is activated by hyperthermia treatment. Recently, two pathways have been proposed for activation of caspase-3 by caspase-8. One involves the direct activation of caspase-3 by caspase-8, and the other operates through a mitochondria-mediated mechanism where caspase-8 potentiates the release of cytochrome c from mitochondria, with the subsequent activation of caspase-3 by cytochrome c with the aid of caspase-9⁹). The significant inhibition of apoptotic induction and caspase-3 activation by IETD-FMK, a caspase-8 inhibitor²⁹), suggests that caspase-8 may act as a proximal signaling protease that is responsible for an upstream activation of caspase-3 by hyperthermia treatment. However, how caspase-8 is activated by hyperthermia treatment has yet to be determined. The remarkable inhibition of apoptotic induction and of caspase-3 activation by LEHD-FMK, a caspase-9 inhibitor³⁰) further suggests that caspase-9 might also play a role in amplifying caspase-3 activation.

In conclusion, a significant and rapid induction of apoptosis was seen in HL-60 cells treated with hyperthermia, and this apoptosis induction was associated with morphological changes and DNA fragmentation. The induction of apoptosis by hyperthermia treatment was associated with the activation of caspase-3, and apoptosis induction appeared to be completely blocked by a caspase-3 inhibitor. These observations suggest that the activation of caspase-3 by hyperthermia treatment is essential for cellular commitment to undergo apoptosis. Furthermore, a caspase-8 inhibitor almost completely blocked hyperthermia-induced apoptosis and caspase-3 activation. Apoptotic induction and caspase-3 activation were also remarkably inhibited in the presence of a caspase-9 inhibitor. These results may imply that caspase-8 and -9 mediate caspase-3 activation during hyperthermia-induced apoptosis in HL-60 cells. Since the induction of apoptosis is considered to be an important mechanism with potential antitumor effects, a further understanding of the mechanism of hyperthermia-induced apoptosis could provide useful information for the hyperthermia treatment of tumors.

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References

- 1) Kerr J.R.F., Wyllie A.H., Currie A.R. : Apoptosis : a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br J Cancer*, 26 : 239-257, 1972.
- 2) Dive C., Gregory C.D., Phipps D.J., Evans D.L., Milner A.E., Wyllie A.H. : Analysis and discrimination of necrosis and apoptosis (programmed cell death) by multiparameter flow cytometry. *Biochim Biophys Acta*, 1133 : 275-285, 1992.
- 3) Salvesen G.S., Dixit V.M. : Caspases : intracellular signaling by proteolysis. *Cell*, 91 : 443-446, 1997.
- 4) Liu X., Zou H., Slaughter C., Wang X. : A heterodimeric protein that functions downstream of caspase-3 to trigger DNA fragmentation during apoptosis. *Cell*, 89 : 175-184, 1997.
- 5) Kischkel F.C., Hellbardt S., Behrmann I., Germer M., Pawlita M., Krammer P.H., Peter M.E. : Cytotoxicity-dependent APO-1 (FAS/CD95) -associated protein form a death-inducing signaling complex (DISC) with the receptor. *EMBO J*, 14 : 5579-5588, 1995.
- 6) Asbkenazi A., Dixit V.M. : Death receptors : signaling and modulation. *Science*, 281 : 1305-1308, 1998.
- 7) Xue D., Shaham S., Horvitz H.R. : The *Caenorhabditis elegans* cell-death protein CED-3 is a cysteine protease with substrate specificities similar to those of the human CPP32 protease. *Genes Dev*, 10 : 1073-1083, 1996.
- 8) Brancolini C., Lazarevic D., Rodriguez J., Schneider C. : Dismantling cell-cell contacts during apoptosis is coupled to a caspase-dependent proteolytic cleavage of beta-catenin. *J Cell Biol*, 139 : 759-771, 1997.
- 9) Li H., Zhu H., Xu C.J., Yuan T. : Cleavage of BID by caspase8 mediates the mitochondria damage in the Fas pathway of apoptosis. *Cell*, 94 : 491-501, 1998.
- 10) Dewey W.C., Thrall D., Gillette E.L. : Hyperthermia and radiation-a selective thermal effect on chronically hypoxic tumor cells in vivo. *Int J Radiat Oncol Biol Phys*, 123 : 463-474, 1997.
- 11) Bull J.M.C. : An update on the anticancer effects of a combination of chemotherapy and hyperthermia. *Cancer Res*, 44 : 4853-4856, 1984.
- 12) Takahashi A., Ohnishi T. : What is the critical hyperthermia target in cancer cells? *Jpn J Hyperthermic Oncol*, 22 : 229-237, 2006.
- 13) Cui Z.-G., Wada S., Kondo T., Fujiwara Y., Zhao Q.-L., Ogawa R., Nagaki Y., Shoji M., Tabuchi Y., Yuki H., Feril L.B.Jr : Enhancement of hyperthermia-induced apoptosis by modification of intracellular oxidative stress. *Jpn J Hyperthermic Oncol*, 21 : 71-80, 2005.
- 14) Kameda K., Kondo T. : The role of intracellular Ca^{2+} in apoptosis induced by Hyperthermia and its enhancement by verapamil in U937 cells. *Int J Radiat Oncol Biol Phys*, 49 : 1369-1379, 2001.
- 15) Arai Y., Kondo T., Tanabe K., Zhao Q.L., Li F.J., Ogawa R., Li M., Kasuya M. : Enhancement of hyperthermia-induced apoptosis by local anesthetics on human histiocytic lymphoma U937 cells. *J Biol Chem*, 277 : 18986-18993, 2002.
- 16) Harmon B.V., Corder A.M., Collins R.J., Gobe G.C., Allen J., Allan D.J., Kerr J.F. : Cell death induced in a murine mastocytoma by 42-47 degrees C heating in vitro : evidence that the form of death changes from apoptosis to necrosis above a critical heat load. *Int J Radiat Biol*, 58 : 845-858, 1990.
- 17) Vania R.K., Ian R.B. : The effect of hyperthermia on the induction of cell death in brain, testis, and thymus of the adult and developing rat. *Cell Stress Chaperones*, 7 : 73-90, 2002.
- 18) Wyllie A.H. : Glucocorticoid-induced thymocyte apoptosis is associated with endogenous endonuclease activation.

- Nature, 284 : 555-556, 1980.
- 19) Okamoto K., Mizuno M., Nakahara N., Natsume A., Yoshida J., Mori T., Hori S., Kobayashi H. : Process of apoptosis induced by TNF-alpha in murine fibroblast Ltk-cells: continuous observation with video enhanced contrast microscopy. *Apoptosis*, 7 : 77-86, 2002.
 - 20) Collins J.A., Schandi C.A., Young K.K., Vesely J., Willingham M.C. : Major DNA fragmentation is a late event in apoptosis. *J Histochem Cytochem*, 45 : 923-934, 1997.
 - 21) Hengartner M.O. : The biochemistry of apoptosis. *Nature*, 407 : 770-776, 2000.
 - 22) Lin S., Fujii M., Hou D.X. : Rhein induce apoptosis in HL-60 cells *via* reactive oxygen species-independent mitochondrial death pathway. *Arch Biochem Biophys*, 418 : 99-107, 2003.
 - 23) Huang S., Lynch M.C., Kochevar I.E. : Caspase-8 mediates caspase-3 activation and cytochrome c release during singlet oxygen-induced apoptosis of HL-60 cells. *Exp Cell Res*, 250 : 203-212, 1999.
 - 24) Yaizu H., Adachi Y., Taketani S., Tokunaga R., Fukuhara S., Ikehara S. : A crucial role of caspase 3 and caspase 8 in paclitaxel-induced apoptosis. *Mol Cell Biol Res Commun*, 2 : 36-41, 1999.
 - 25) Granville D.J., Carthy C.M., Jiang H., Shore G.C., McManus B.M., Hunt D.W. : Rapid cytochrome c release, activation of caspases 3, 6, 7 and 8 followed by Bap31 cleavage in HeLa cells treated with photodynamic therapy. *FEBS Lett*, 437 : 5-10, 1998.
 - 26) Schempp C.M., Simon-Haarhaus B., Termeer C.C., Simon J.C. : Hypericin photo-induced apoptosis involves the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and activation of caspase-8. *FEBS Lett*, 493 : 26-30, 2001.
 - 27) Katschinski D.M., Boos K., Schindler S.G., Fandrey J. : Pivotal role of reactive oxygen species as intracellular mediators of hyperthermia-induced apoptosis. *J Biol Chem*, 275 : 21094-21098, 2000.
 - 28) Gianni M., de The H. : In acute promyelocytic leukemia NB4 cells, the synthetic retinoid CD437 induces contemporaneously apoptosis, a caspase-3-mediated degradation of PML/RARalpha protein and the PML retargeting on PML-nuclear bodies. *Leukemia*, 13 : 739-749, 1999.
 - 29) Garcia-Calvo M., Peterson E.P., Leiting B., Ruel R., Nicholson D.W. : Thornberry NA. Inhibition of human caspases by peptide-based and macromolecular inhibitors. *J Biol Chem*, 273 : 32608-32613, 1998.
 - 30) Potokar M., Milisav I., Kreft M., Stenovec M., Zorec R. : Apoptosis triggered redistribution of caspase-9 from cytoplasm to mitochondria. *FEBS Lett*, 544 : 153-159, 2003.
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