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Necessity of interleukin-1β converting enzyme cascade in taxotere-initiated death signaling

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Abstract

Taxotere is a new type chemotherapeutic agent which targets tubulin. In the present study, we investigated the molecular machinery of taxotere-initiated death signaling. Taxotere induced cell death in mouse fibroblast L929 cells. Cell morphological analysis revealed that this effect showed characteristics of apoptotic and necrotic cell death. To further examine taxotere-induced cell death, we investigated the direct involvement of caspase. When cells were pretreated with the synthesized tetrapeptide inhibitor of caspase, YVAD-CHO (Ac–Tyr–Val–Ala–Asp-aldehyde: inhibitor of interleukin-1β converting enzyme (ICE) subfamily) or DEVD-CHO (Ac–Asp–Glu–Val–Asp-aldehyde: inhibitor of CPP32 subfamily), taxotere-induced cell death was prevented. In addition, time course experiments demonstrated that activation of the ICE subfamily preceded activation of the CPP32 subfamily in taxotere-initiated death signaling, suggesting the direct involvement of the ICE cascade in taxotere-initiated death signaling. On the basis of these results, we suggest that taxotere causes the initiation of ICE cascade in its death signaling pathway and that the down-stream site of taxotere-initiated death signaling is the same as that of other chemotherapeutic agents. © 1998 Elsevier Science B.V.

Keywords: Chemotherapeutic agent; Taxotere; Cell death; Interleukin-1β converting enzyme cascade

1. Introduction

Programmed cell death is an essential process for cell homeostasis and is encountered in various physiological phenomena and disease states (Wyllie et al., 1980; Nagata and Golstein, 1995; Suzuki et al., 1996a,b). Two morphologically distinct processes have been identified, namely 'apoptotic cell death' and 'necrotic cell death' (Wyllie et al., 1980). Apoptotic cell death is characterized by condensation and fragmentation of nuclei, loss of plasma membrane microvilli, condensation of cytoplasm and fragmentation of chromosomal DNAs into 180 bp oligomers, while necrotic cell death is predominantly characterized by cell membrane destruction, such as abnormal cell membrane permeability (Wyllie et al., 1980; Shimizu et al., 1996; Suzuki, 1997). Cell death is induced by various factors and conditions, and a number of drugs, including many chemotherapeutic agents, also initiate death signaling in target cells.

The CED-3 death gene, identified from *Caenorhabditis elegans*, shows high similarity to interleukin-1β converting enzyme (ICE) (Yuan et al., 1993). The important role of ICE/CED-3 as the death mediator in intracellular death signaling has been demonstrated (Miura et al., 1993). Recently, 10 genes showing a close similarity to ICE/CED-3 (ICE/CED-3 homologue) have been identified and termed caspase (Alnemri et al., 1996). There are three subfamilies of caspase, termed ICE-, CPP32- and ICH-1-subfamily. The involvement of the ICE cascade in death receptor Fas-initiated death signaling has been reported (Enari et al., 1996). Both ICE and CPP32 subfamilies are activated in the process of Fas-initiated death signaling, with activation of the ICE subfamily preceding that of the CPP32 subfamily (Enari et al., 1996). Because the ICE or CPP32 subfamily catalyze the proteolysis of pro-interleukin-1β (IL-1β) or poly (ADP-ribose) polymerase (PARP), the aldehyde-conjugated synthesized tetrapeptide which codes the cleavage site of pro IL-1β (Ac–Tyr–Val–Ala–Asp-aldehyde: YVAD-CHO) or PARP (Ac–Asp–Glu–Val–Asp-aldehyde: DEVD-CHO) can be used as a specific inhibitor of the ICE or CPP32 subfamily

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(Fernandes-Alnemri et al., 1994; Enari et al., 1995; Nicholson et al., 1995; Hasegawa et al., 1996).

We and others have reported that caspase plays a dominant role in cell death induced by chemotherapeutic agents, such as camptothecin and its derivative CPT-11, VP-16 and adriamycin (Mashima et al., 1995; Suzuki and Kato, 1996; Suzuki et al., 1996c, 1997a). These reagents induce cell death by inhibition of type I or II topoisomerase or by insertion into DNA (Glisson and Ross, 1987; Mashima et al., 1995; Suzuki and Kato, 1996; Suzuki et al., 1996c, 1997a). In contrast, the complex plant alkaloid taxol has a unique taxane ring structure which appears to affect microtubule binding to the polymeric microtubule form of tubulin and has been developed as a chemotherapeutic agent (Woods et al., 1995). Taxol induces cell death by triggering machinery different from that of other chemotherapeutic agents. We therefore investigated the molecular machinery of another tubulin-targeting chemotherapeutic agent taxotere.

2. Cells and procedures

2.1. Cell line

Mouse fibroblast L929 cells were maintained in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal calf serum (Gibco BRL) in a humidified atmosphere of 5% CO_2 and 95% air. L929 cells readily divide, so this cell line is useful for the investigation of chemotherapeutic agent-induced cell death.

2.2. Peptide and chemicals

YVAD-MCA, YVAD-CHO, DEVD-MCA and DEVD-CHO were purchased from Peptide Lab. (Osaka, Japan). Taxotere was synthesized at the New Product Research Laboratories IV, Daiichi Pharmaceutical Co., Japan, and dissolved in dimethylsulfoxide (DMSO). This solution was diluted with 0.01% cremophor EL (Sigma, St. Louis, MO) in phosphate-buffered saline (PBS) and used for experiments.

2.3. Cell morphological analysis

To analyze cell morphology, cells were stained with Wright–Giemsa and Hoechst 33342-propidium iodide (PI). L929 cells were cultured on chamber slides (NUNC, Naperville, IL), incubated with taxotere and then stained. The morphology of Wright–Giemsa-stained cells was examined under a light microscope. Hoechst 33342 and PI were purchased from Molecular Probes (Eugene, OR) and were used as previously described (Hasegawa et al., 1996). Cells stained with 10 μM reagent for 10 min were examined under a nonconfocal fluorescence microscope.

2.4. Assay of cell viability

Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT) assay as previously described (Mossman, 1983; Suzuki and Kato, 1996). After treatment, 10 μl of PBS-diluted MTT (5 mg/ml) was added to each well and the plate was incubated for 4 h. Acid-isopropanol (0.04 N HCl in isopropanol 100 μl/well) was added. The plate was maintained at room temperature for 5 min and then read on a microtiter plate reader (Titerteck, Fukuoka, Japan) at A570/A690.

Cell viability was also assessed by the Hoechst 33342 staining procedure. After incubation, cells were collected and stained with Hoechst 33342 at 10 μM for 10 min and then observed under a nonconfocal fluorescence microscope. Cell viability was indicated by the proportion of cells carrying intact nuclei relative to the total number of cells (about 5000 cells). Three wells per group were used for experiments and experiments were repeated 5 times.

2.5. Preparation of cell extracts and assay of enzyme

Preparation of cell extracts and assay of enzymatic activity were performed as previously described (Hasegawa et al., 1996) with some modification (Suzuki et al., 1997a,b). Cells were collected, washed with PBS and suspended in PBS–EDTA (pH 7.4). After addition of 10 μM digitonin (Sigma), cells were incubated at 37°C for 10

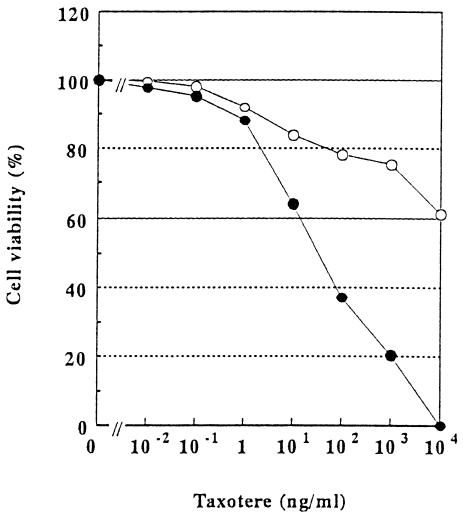

Fig. 1. Effect of taxotere in mouse fibroblast L929 cells. Cells were treated with various concentrations of taxotere for 24 h. After treatment, the cell viability of each group was measured by MTT assay (open circle) or Hoechst 33342 staining analysis (closed circle). Bars are S.E. of 5 samples.

min. Lysates were collected by centrifugation (15 000 rpm/5 min) and the protein concentration was determined with a DC protein assay kit (Bio Rad, Richmond, CA).

Protein concentrations were adjusted to 30 μg/ml and aliquots (2 ml) were incubated with 10 μl of YVAD-MCA (50 μM) or DEVD-MCA (50 μM) at 37°C for 15 min for

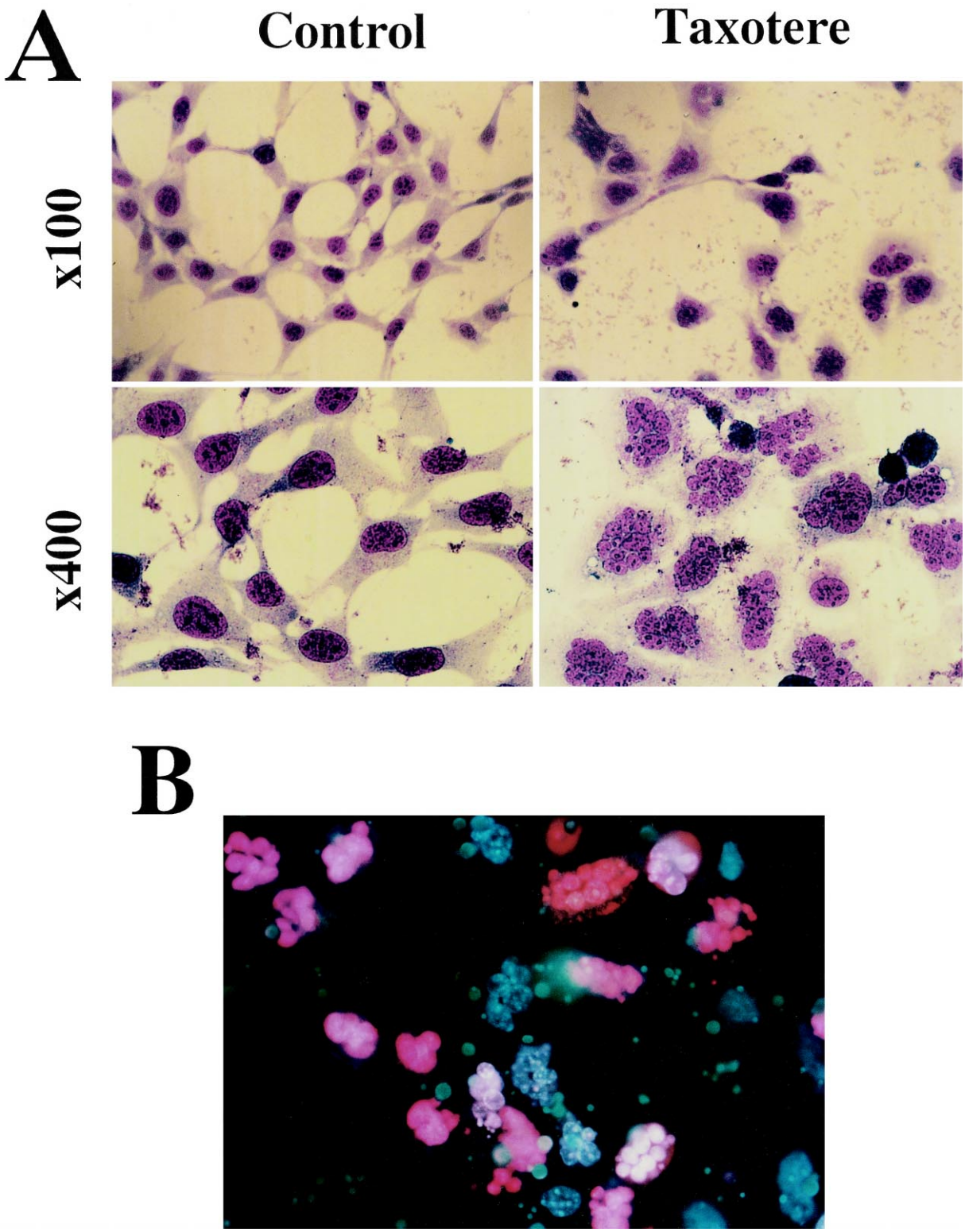

Fig. 2. Morphological analysis of taxotere-treated cells. (A) Wright–Giemsa staining analysis. Cells were treated with (Taxotere) or without (Control) 1 μg/ml taxotere for 24 h and then stained with Wright–Giemsa. (B) Hoechst 33342/PI staining analysis. Cells treated with 1 μg/ml taxotere for 24 h were stained with Hoechst 33342 (blue)/PI (pink).

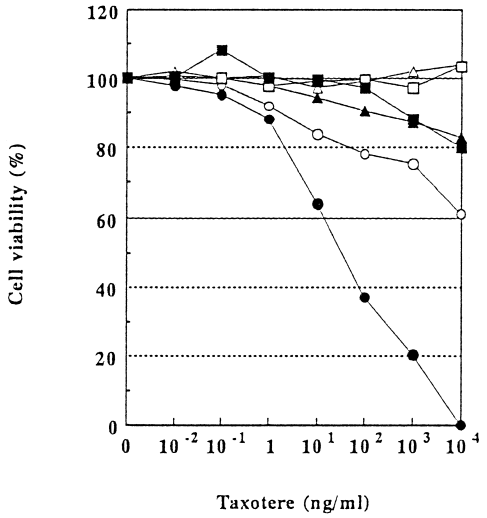

Fig. 3. Effect of caspase inhibitors YVAD-CHO and DEVD-CHO on taxotere-induced cell death. Cells pretreated with or without (circle) 600 μM YVAD-CHO (square) or 100 μM DEVD-CHO (triangle) for 2 h were treated with various concentrations of taxotere for 24 h. As control, L929 cells were pretreated with DMSO for 2 h and then treated with taxotere. After treatment, the cell viability of each group was measured by MTT assay (open) or Hoechst 33342 staining analysis (close). Bars are S.E. of 5 samples.

enzyme assay and the release of amino-4-methylcoumarin was monitored with a spectrofluorometer. One unit was defined as the amount of enzyme required to release 0.22 nmol amino-4-methylcoumarin per min at 37°C.

3. Results

3.1. Effect of taxotere in mouse fibroblast L929 cells

To examine an effect of taxotere in L929 cells, cells were incubated with various concentration of taxotere for 24 h. When cell viability was measured with MTT assay and Hoechst 33342 staining procedures, dose-dependent cytolytic activity was encountered (Fig. 1).

3.2. Death characterization of taxotere-induced cytolysis

We demonstrated that taxotere shows the dose-dependent cytolytic activity in mouse fibroblast L929 cells. Therefore, Wright–Giemsa and Hoechst 33342/PI staining analyses were performed to examine whether taxotere-induced cytolytic activity is due to apoptotic cell death or necrotic cell death. When the cells were treated with taxotere, the cell body-atrophy and nuclear fragmentation were seen (Fig. 2A). This result suggests that taxotere induces apoptotic cell death. In contrast, most taxotere-treated cells were stained with PI (Fig. 2B) and the condensation of chromosomal DNA was not encountered (Fig. 2B). Thus, these results showed that taxotere induced both apoptotic and necrotic fashions. We therefore describe taxotere-induced cytolysis as 'taxotere-induced cell death' in the present report.

3.3. Involvement of caspase family in taxotere-induced cell death

We and others have demonstrated the direct involvement of the caspase family in cell death induced by chemotherapeutic agents (Mashima et al., 1995; Suzuki and Kato, 1996; Suzuki et al., 1996c, 1997a). Therefore, cells were pretreated with YVAD-CHO (ICE subfamily inhibitor) or DEVD-CHO (CPP32 subfamily inhibitor) before taxotere treatment to examine the possible involvement of caspase family in taxotere-induced cell death. When cell viabilities were measured with MTT assay and Hoechst 33342 staining analyses, pretreatment of cells with YVAD-CHO (600 μM for 2 h) or DEVD-CHO (100 μM for 2 h) prevented taxotere-induced cell death (Fig. 3), suggesting the direct involvement of caspase family in taxotere-induced cell death.

3.4. ICE cascade in taxotere-induced cell death

Our present data demonstrated the direct involvement of caspase family in taxotere-induced cell death. Recently, the sequential activation of the ICE subfamily and CPP32 subfamily in the death signaling pathway, namely ICE cascade, has been reported as death-triggering system (Enari et al., 1996; Suzuki et al., 1997a). To further examine the involvement of the caspase family, especially the ICE subfamily and the CPP32 subfamily, in taxotere-induced cell death, time-course changes in each activity were measured. When cells were treated with taxotere, activation of the ICE subfamily reaching a peak 3 h preceded activation

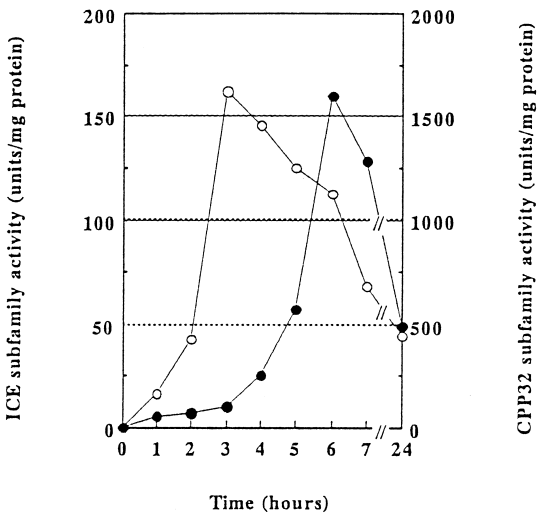

Fig. 4. Time-course of ICE- and CPP32-subfamily activity in taxotere-induced cell death. Cells were treated with 1 μg/ml taxotere for indicated periods and the proteolytic activity of the ICE-(open) or the CPP32-(close) subfamily was measured. Bars are S.E. of 5 samples.

of the CPP32 subfamily reaching a peak 6 h (Fig. 4). These results indicated that ICE cascade mediates the death signaling pathway initiated by taxotere.

4. Discussion

In the present study, we investigated the molecular machinery of death signaling initiated by the chemotherapeutic agent taxotere, which interacts with components of the cytoskeleton, such as tubulin (Woods et al., 1995). When mouse fibroblast L929 cells were incubated with various concentrations of taxotere for 24 h, MTT assay and Hoechst 33342 staining analysis revealed that taxotere induced dose-dependent cytolysis in the cells (Fig. 1). To characterize this cytolytic activity, Wright–Giemsa staining analyses were performed. When the cells were treated with taxotere, atrophy of the cell body and nuclear fragmentation were seen (Fig. 2A), suggesting that taxotere induces apoptotic cell death. To further characterize this taxotere-induced cytolysis, Hoechst 33342/PI staining was done. Most taxotere-treated cells were stained with PI (Fig. 2B), while condensation of chromosomal DNA was not encountered (Fig. 2B). In general, apoptotic cell death is characterized by nuclear fragmentation and condensation of chromosomal DNAs and necrotic cell death by cell membrane damage, such as abnormal cell membrane permeability (Wyllie et al., 1980; Shimizu et al., 1996; Suzuki, 1997). Our present results demonstrate that taxotere induced nuclear fragmentation and cell membrane damage, but not chromosomal DNA condensation, suggesting that taxotere induces both apoptotic and necrotic cell death. We therefore describe taxotere-induced cytolysis as 'taxotere-induced cell death' in the present report.

Caspase, especially the ICE- and CPP32-subfamilies, acts as the common mediator of apoptotic and necrotic death signaling (Shimizu et al., 1996; Suzuki, 1997) and plays a dominant role in the death signaling initiated by various chemotherapeutic agents, such as CPT and its derivative CPT-11, VP-16 and adriamycin (Mashima et al., 1995; Suzuki and Kato, 1996, Suzuki et al., 1996a,b,c). To investigate the direct involvement of caspases in taxotere-initiated death signaling, the effects of two synthesized tetrapeptide inhibitors of caspase, YVAD-CHO (inhibitor of ICE subfamily) and DEVD-CHO (inhibitor of CPP32 subfamily), were examined. Pretreatment of cells with either YVAD-CHO (600 μM for 2 h) or DEVD-CHO (100 μM for 2 h) prevented taxotere-induced cell death (Fig. 3). These results suggest that both the ICE- and CPP32-subfamilies play an important role in taxotere-initiated death signaling.

This prevention of cell death by both caspase subfamily inhibitors led us to the possible involvement of the ICE cascade, namely the sequential activation of ICE- and CPP32-subfamilies (Enari et al., 1996), in taxotere-initiated death signaling. We therefore examined the time course of changes in ICE- and CPP32-subfamily activity. Results showed that activation of the ICE subfamily preceded activation of the CPP32 subfamily, with ICE subfamily activity reaching a peak 3 h after the start of incubation whereas CPP32 subfamily activity reached a peak level at 6 h (Fig. 4). These observations suggest that the ICE cascade is present and plays an important role as the death mediator in taxotere-initiated death signaling.

In the present study, we demonstrated that the chemotherapeutic agent taxotere induces ICE cascade-mediated cell death in mouse fibroblast L929 cells. The dead cells showed nuclear fragmentation and necrotic cell membrane damage, but no chromosomal DNA condensation. We and other researchers have previously demonstrated the direct involvement of caspase in cell death induced by various chemotherapeutic agents. Each agent triggers death signaling by distinct pathways: CPT and its derivative CPT-11 inhibit type-I topoisomerase (Mashima et al., 1995; Suzuki and Kato, 1996; Suzuki et al., 1996c, 1997a), VP-16 inhibits type-II topoisomerase (Glisson and Ross, 1987; Kohn et al., 1987; Zweling, 1989; Walker et al., 1991; Mashima et al., 1995), and adriamycin has a multiple triggering system to induce cell death (Mashima et al., 1995). In contrast, these death signaling pathways, including that of taxotere, involve the same machinery, namely ICE cascade, at a down-stream site. In addition, the Fas ligand/Fas and TNF-α/TNF-R1 system, which are involved in cell death under various physiological and disease conditions (Nagata and Golstein, 1995; Suzuki et al., 1996a,b), are also mediated by caspase activation at a down-stream site (Enari et al., 1995; Hasegawa et al., 1996; Tewari and Dixit, 1995). We therefore suggest that various death signaling pathways are commonly mediated by caspase at a down-stream site. In the present study, we demonstrated the direct involvement of the ICE cascade in taxotere-induced cell death using mouse fibroblast L929 cells. The same phenomenon, namely the essential role of the ICE cascade in taxotere-induced cell death, was demonstrated in other human and rat tumor cell lines (Suzuki, unpublished data). Therefore, we suggest that the ICE cascade is activated by taxotere to cause cell death.

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