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# Caspase3-like Death Protease Is Activated in CTLL2 Cells by Interleukin-2 Deprivation

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**Abstract** Cytokine deprivation-induced apoptosis can abrogate by the appropriate survival factors. Because the mechanism of Interleukin (IL)-2 deprived apoptotic cell death remains unclear, we here show the apoptosis in CTLL2 cells correlates with an increase of the activity of caspase3-like protease(s). Inhibition of caspase3-like protease(s) with caspase protease inhibitors (Z-VAD, Z-EVD, and Z-LPD) blocks typical apoptotic morphological abnormalities in CTLL2 cells. Interestingly, Bcl-$X_L$ protein was decreased by IL-2 deprivation in the cells. These results suggest that caspase3-like protease(s), not caspase1, plays an important role in apoptosis execution of CTLL2 cell death.

**Key words**: Caspase3-like protease, Apoptosis, Interleukin-2, CTLL2 cells

## Introduction

Apoptosis is a fundamental process of development, tissue degeneration, and removal of unwanted cells within a multicellular organism [1]. Caspases are clearly activated in apoptotic cell death and appear to be required for certain aspects of apoptosis. During apoptosis, many cellular proteins undergo caspase-dependent degradation [2]. Of them, caspases3-like proteases are responsible for the cleavage of their substrates at the onset of apoptosis. Many cytotoxic stimuli can cause apoptosis with a similar mechanism. It is now known that many inducers of apoptosis converge on the caspase activation, which then appear to launch the terminal and execution stages of apoptosis. Although the relevance of cleavage of structural proteins, like poly (ADP-ribose) polymerase (PARP), DNA-protein kinase (PK), gelsolin, fodrin, actin, and lamin, is easily conceivable [2-7], the functional importance of these and other cleavages, such as those of signaling molecules is not yet investigated. It is, however, widely assumed that the caspase-specific cleavage of these proteins is responsible for the various hallmarks of apoptosis such as nuclear fragmentation, cytoplasmic membrane blebbing, and DNA fragmentation [8-10].

In this study, we examined the involvement of caspase3-like death protease(s) in CTLL2 cells induced by IL-2 deprivation [11,12]. Originally, IL-2 was found to be a potent lymphoid cell growth factor that exerts its biological activity primarily on T cells. By IL-2 withdrawal, the expression of Bcl-$X_L$ protein decreased, but it was recovered by the inhibitors of caspase proteases such as Z-EVD, Z-VAD, and Z-LPD. We also examined whether or not caspase3-like protease(s) is involved in CTLL2 cell apoptosis via a signal pathway of Bcl-2related protein cleavage, and the possibility that radical oxygen species is responsible for the IL-2depleted CTLL2 cell apoptosis.

## Materials and methods

### Cell line

Murine IL-2-dependent T cells, CTLL2 were cultured at 37℃ in a humidified atmosphere of 5% $CO_2$ and 95% air in RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 μg/ml of penicillin and streptomycin, and 10 units/ml of mouse IL-2.

### Reagents

Mouse IL-2 (Genzyme, Cambridge, MA) was used for maintenance of the cells as a concentration of 10 units/ml. Benzyloxycarbonyl-Glu-Val-Asp-CH2OC(O)-2,6,-dichloro benzene (Z-EVD), benzyloxycarbonyl-Val-Ala-Asp-$CH_2$OC (O)-2,6,-dichlorobenzene (Z-VAD), and benzyloxycarbonyl-Leu-Pro-Asp-$CH_2$OC(O)-2,6,-dichlorobenzene (Z-LPD) [as specific caspase inhibitors] were used to inhibit apoptosis in CTLL2 cells. The fluorogenic substrates of proteases were obtained from Peptide Institute (Osaka, Japan) and used

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for the substrates of proteases. 2,2,6,6-Tetramethylpiperidine-N-oxyl (TEMPO), L-ascorbic acid, and N-acetylcysteine (NAC) were purchased from Sigma Co. (St. Louis, MO). Other reagents were of analytical grade.

### DAPI staining

CTLL2 cells were incubated with medium alone or medium containing IL-2 for 24 h followed by washing twice with phosphate buffered saline (PBS). After fixing with methanol/acetic acid (3:1), the cells were applied to slide glass and stained with 4',6-diamidino-2-phenylindole(DAPI; 1 μg/ml) for 30 min in dark room. The morphological changes were visualized by using a fluorescence microscope equipped with UV-2A filter [8].

### Measurement of protease activity

CTLL2 cells were incubated with medium alone or medium containing IL-2 for 12 h. Then the cells were harvested and lysed with the lysis buffer (10 mM HEPES, pH 7.4), 2 mM EDTA, 0.1% CHAPS, and 5 mM dithiothreitol). The cell lysate was then incubated with 20 mM of the fluorogenic substrate of proteases (YVAD-amino-methylcoumarin [AMC] and DEVD-AMC) in ICE buffer (20 mM HEPES [pH 7.5], 10% glycerol, 2 mM dithiothreitol) for 15 min at 37°C. The AMC released from the fluorogenic substrates was measured with the excitation at 380 nm and the emission at 460 nm using a spectrophotometer [13].

### Apoptosis inhibition by inhibitors

CTLL2 cells were incubated with or without IL-2 (10 units/ml). In some experiments, Z-VAD, Z-EVD, and Z-LPD were added to the culture medium at an appropriate time [8,14]. TEMPO, N-acetylcysteine (NAC), and L-ascorbic acid (LAA) were also added to the culture medium. Viable cells were counted using trypan blue exclusion assay.

### Western blot analysis

Western blot was carried out as described previously [15]. Briefly, cells were treated with or without IL-2 were solubilized with lysis buffer containing 2% NP-40 and 0.2% SDS under reduced conditions. Then the cell lysates were applied to a 10-20% gradient polyacrylamide gel. The electrophoresed proteins were transblotted onto a nitrocellulose membrane. After blocking the membrane were incubated with anti-Bcl-$X_L$ monoclonal antibody (Transduction Lab., Lexington, KY) and then incubated with a peroxidase-conjugated antibody followed by developing using the ECL detection system (Amersham, Buckinghamshire, UK).

## Results and discussion

Caspases from human origin are homologs of *ced-3*, a gene product of *Caenorhabditis elegans*. The caspases include caspase1/ICE, caspase2/Nedd2/Ich1L, caspase3/Yama/apopain, caspase4/Ich2/TX/ICErelII, caspase5/TY/ICErelIII, caspase6, caspase7, caspase8/MACH/FLICE/Mch5, caspase9, caspase10, caspase11, caspase12, caspase13, and caspase14 are involved in apoptotic cell death [8, 16-19]. The proteases inactivated by treatment of some potent inhibitors of caspases, which is known to suppress apoptosis. During apoptosis, some substrates of these caspases including PARP, actin, gelsolin, fodrin, and lamin were cleaved by caspases and the cleaved products can accelerate further activation of apoptotic transducers, although the activation mechanism remains unclear. Various cytokines were known to inhibit the apoptosis by transmitting a survival signal(s). IL-2-dependent cytotoxic T-cell line, CTLL2, underwent apoptosis by the IL-2 withdrawal [20], but they could survive and grow by the addition of IL-2. Other cytokines or growth factors, such as IL-3, IL-5, IL-6, IL-7, IL-9 had similar effects on these factor-dependent cell lines [21,22].

### IL-2 withdrawal induces apoptosis in CTLL2 cells

In order to investigate the involvement of caspases during IL-2depleted apoptosis, we used a murine CTLL2 cell line. CTLL2 cells can grow when IL-2 was added to the culture medium (Fig. 1A, closed circles). The viable cells proportionally increased by addition of IL-2 (Fig. 1A closed circles, and 1B). When CTLL2 cells were cultured without IL-2, they underwent apoptosis with typical nuclear and DNA fragmentation [1, 23] (Fig. 1A, 1C, and data not shown). However, CTLL2 cells evaded apoptosis when IL-2 was added to the culture medium within 2 hours (data not shown). In addition, staurosporine, an inhibitor of general tyrosine kinases, inhibited significantly the cell growth in a time-dependent manner (Fig. 1A, open triangles) while cyclosporin A, an inhibitor of protein kinases, did not (Fig. 1A, closed triangles). Therefore, these results suggest that IL-2 depletion induces CTLL2 cells to die by apoptosis.

### Expression of Bcl-$X_L$ protein during apoptosis

It is well known that fluorogenic-labelled tetrapeptides (YVAD-AMC and DEVD-AMC) can be cleaved by caspase1 (ICE) and caspase3 (CPP32), respectively, during apoptosis. We so examined that the activity of caspase-family proteases using these tetrapeptides which were the specific substrates of caspase-family proteases [17]. When the cell lysates from CTLL2 cells, which were cultured alone for 12 h, were incubated with the fluorogenic substrates of caspase-family proteases, the AMC was liberated from IL-2supplemented CTLL2 cells did not contain such protease activity (Fig. 2). Therefore, the addition of IL-2 suppressed the activation of caspase3 protease in CTLL2 cells.

### Suppression of caspase3-like protease activation by IL-2

To identify a molecule(s) that was associated with the apoptosis-suppression by IL-2, we investigated the activity

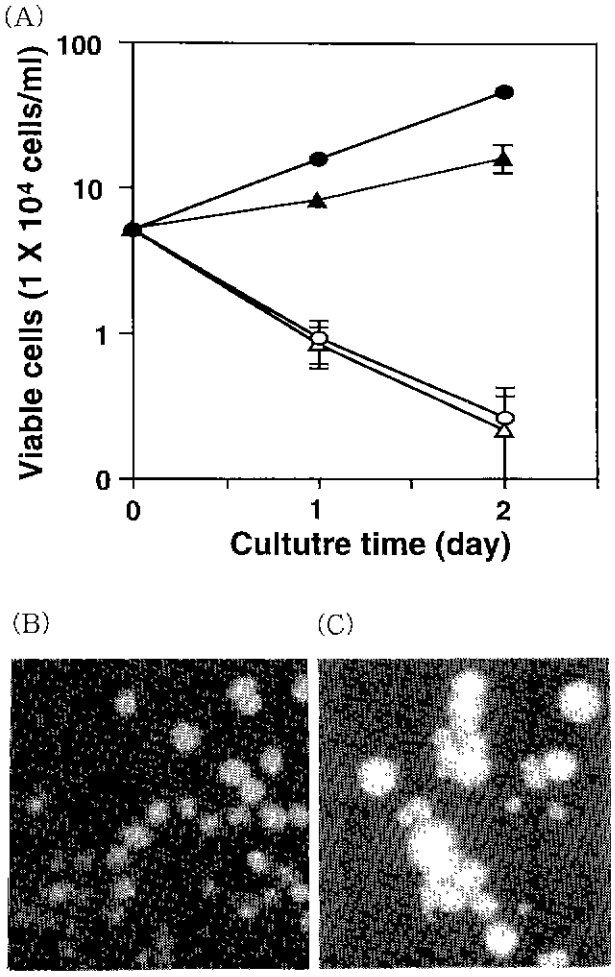


**Fig. 1.** Cell growth of CTLL2 cells by IL-2. IL-2-dependent CTLL2 cells (1 x $10^5$ cells/ml) were seeded in 24 wells, and cultured with (closed circles) or without (open triangles) IL-2 (10 units/ml). Staurosporine (10 ng/ml) or cyclosporine A (1 μM) was also added to the IL-2-supplemented culture medium. The viable cell numbers were counted using the trypan blue dye exclusion assay.

of caspase-family protease(s). Caspase-family proteases were known to participate in the induction of apoptosis because overexpression of these proteases can induce apoptosis [24-26]. When CTLL2 cells were cultured alone for 12 h in the presence of Z-LPD, that is a novel inhibitor of caspase-family proteases [7,27], the decrease of viable cell numbers in CTLL2 cells was inhibited in a dose-dependent manner (Fig. 3, and data not shown). The other inhibitors, such as Z-VAD, Z-EVD, and Z-Asp also inhibited the decrease of viable cells at a concentration of 100 mg/ml (Fig. 3, and not shown). The DNA fragmentation occurred in CTLL2 cells was also inhibited by the addition of the inhibitors (unpublished observations). These results indicate that the specific inhibitors for caspase3 inhibited the induction of apoptosis caused by caspase3 in CTLL2 cells.

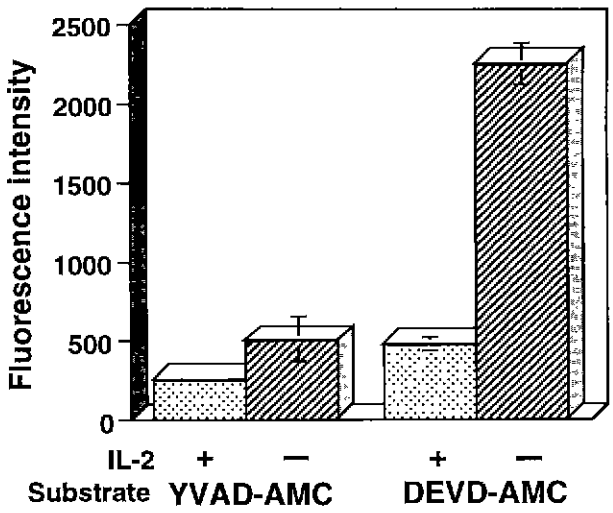


**Fig. 2.** Activation of caspase3-like protease in CTLL2 cell lysates by IL-2 withdrawal. IL-2-dependent CTLL2 cells were cultured with IL-2 upto 1 x $10^7$ cells/ml. To remove remaining IL-2, the cells were centrifuged in 15-ml tubes. The cells were cultured with or without murine IL-2 for 12 h at a concentration of 1 x $10^5$ cells/ml. After incubation, the cells were transferred and washed two times with PBS. Then ICE buffer was added to each sample before protein assay. AMC assay was carried out as previously described in "Materials and methods" using two specific proteinase substrates.

## Inhibition of Bcl-$X_L$ protein change in CTLL2 cells

We next tested the expression of *bcl-2* related proteins by

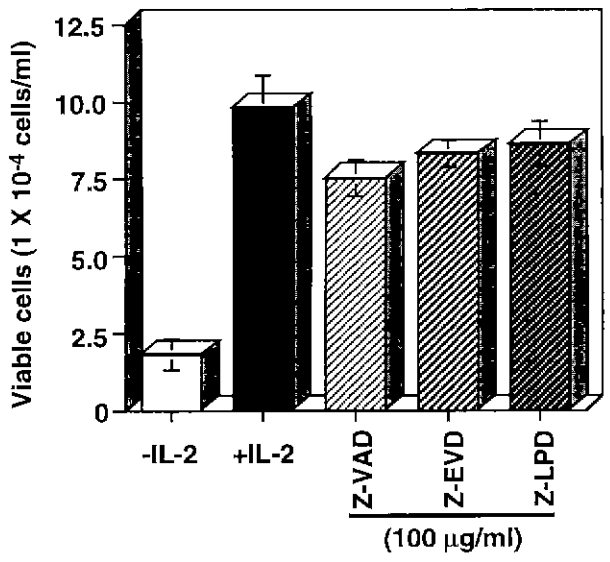


**Fig. 3.** Effect of caspase3-like protease inhibitors on IL-2-depleted apoptosis. CTLL2 cells were cultured as described in Fig. 1. Three kinds of caspase-family protease inhibitors, Z-EVD, Z-VAD, and Z-LPD, were added to 24 wells at 100 μg/ℓ of each inhibitor. After 12 h later, the viability was counted by trypan blue exclusion assay.

western blot analysis when CTLL2 cells were exposed to the depletion of IL-2. Bcl-2-related proteins are key molecules in certain cell lines during apoptosis. The *bcl-2* gene was originally cloned from the breakpoint of a t(14:18) chromosomal translocation, which is present in many human B cell lymphomas [28]. The *bcl-2* has been shown to prevent apoptosis induced by growth-factor deprivation in certain hematopoietic cell lines. Recently, Bcl-2 protein is found to regulate the ICE (caspase1)-mediated apoptosis. Although Bcl-2 protein does not directly interact with ICE (caspase1)-family proteases, Bcl-2 could inhibit the apoptosis of the cells transfected with these proteases. Furthermore, Bcl-2 could suppress the activation of ICE (caspase1)-family proteases.

We could not confirm the change of Bcl-2 expression when IL-2 was depleted from CTLL2 cells for 24 h (data not shown). CTLL2 cells did not undergo apoptosis when they were cultured without IL-2 for 6 h, and the expression level of Bcl-$X_L$ protein was similar (data not shown). However, interestingly, when the cells were exposed to IL-2 deprivation for 24 h, the Bcl-$X_L$ protein was degraded into some minor fragments (Fig. 4). We found two fragmented bands (about 18-20 kDa) that were degraded and reactive to the anti-mouse Bcl-$X_L$ specific monoclonal antibody in the CTLL2 cells lysates. This pattern of additional bands did not appear in the cell lysates of CTLL2 cells cultured in the IL-2 supplemented medium. The expression levels of other Bcl-2-related protein, Bag-1, Bax, Bad, and Bak were not changed during the CTLL2 cell apoptosis [29]. These results suggest that IL-2-depleted cell apoptosis cause Bcl-$X_L$ to additional fragments by the exposure of 24 h, indicating that the appearance of the 18-20 kDa fragments was closely related to the level of apoptosis in CTLL2 cells (see Fig. 4).

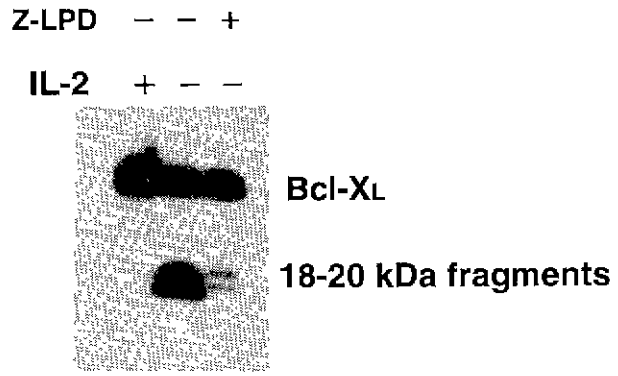


**Fig. 4.** Decreased expression of Bcl-$X_L$ protein during IL-2-depleted apoptosis. CTLL2 cells were cultured in RPMI medium containing IL-2 (lane 1), or in IL-2-depleted medium (lane 2). CTLL2 cells were cultured with 100 μg/ml of Z-LPD in the IL-2-depleted medium (lane 3). After cultivation for 24 h, 40 μg/lane of the cell lysates were electrophoresed and blotted onto a nitrocellulose membrane, as described in "Materials and Methods". The membrane was incubated with an anti-mouse Bcl-X-specific monoclonal antibody for 1 h. After washing three times with washing buffer (see Materials and Methods), the membrane was incubated with a peroxidase-conjugated secondary antibody and developed with ECL mixture.

## Involvement of ROS on IL-2-depleted apoptosis

There are many reports that reactive oxygen species (ROS) produced by various death inducers in many cell lines can trigger apoptosis [30]. Recently, it was reported that oxygen radicals were also important components of apoptosis in *Saccharomycetes* system. Madeo *et al.* have found that apoptosis can be induced in the yeast *Saccharomycetes cerevisiae* by depletion of glutathione or by low external doses of hydrogen peroxide, suggesting that the generation of oxygen radicals is a key event in the ancestral apoptotic pathway [31]. Therefore, we examined whether or not ROS is involved in apoptosis by examining the cell viability and morphology when the CTLL2 cells were treated without IL-2. As shown in Fig. 5, we counted the viable cell numbers when CTLL2 cells were incubated to free radical spin trap TEMPO or antioxidants. IL-2 was added to the culture medium and denoted as a positive control. Unexpectedly, TEMPO (1 μM) rescued the cell death (up to 85%) by inhibiting a signal pathway regarding apoptosis. But antioxidants used, such as L-ascorbic acid, NAC, and catalase did not affect the cell viability (see Fig. 5., and data not shown) [11,32]. Many reports showed that free radical spin traps inhibited the apoptosis by suppressing the production of radical oxygen species [33]. IL-2-depleted cells also may produce ROS, but addition of IL-2 can evade apoptosis by reducing the ROS production. At present, we do not know

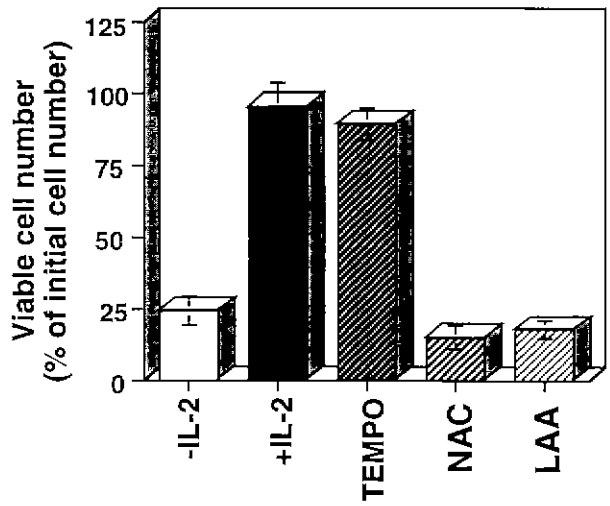


**Fig. 5.** Effect of free radical-spin traps on IL-2-depleted apoptosis. CTLL2 cells were cultured for 12 h in the abensence or in the presence of IL-2 (10 units/ml). TEMPO (1 μM), N-acetylcysteine (NAC; 100 μM), and L-ascorbic acid (LAA; 100 μM) were added to the culture medium as described in "Materials and Methods". The viable cells were counted by trypan blue exclusion assay. Results denoted SD of three determinations.

the exact mechanism why antioxidants such as L-ascorbic acid, NAC, and catalase can not suppress the apoptosis in CTLL2 cells. Further studies regarding on the relationship between the production of ROS and mechanism of apoptosis inhibition especially in that the elucidation of apoptotic induction mechanism caused by ROS production is responsible for understanding of signal transduction of IL-2-depleted apoptosis.

In conclusion, we demonstrated that caspase3-like protease (s) plays an important role in induction of apoptosis by activating the enzyme(s) induced by IL-2 deprivation. Bcl-$X_L$ protein expression was decreased during apoptosis of CTLL2 cells in the absence of IL-2, indicating that Bcl-$X_L$ is a candidate of caspase3-like protease substrate for triggering of apoptotic execution program in CTLL2 cells.

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