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Caspase-3–like Death Protease is Inhibited by Interleukin-7

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Abstract Highly metastatic mouse T-lymphoma CS21 cells can grow *in vitro* when cocultured with CA12 lymph node stromal cells, but they undergo apoptotic cell death when separated from CA12 stromal cells. It has been found that cysteine and interleukin-7 (IL-7) as antiapoptotic soluble factors that produced by CA12 stromal cells. In this study, we report that an ICE family protease is activated in CS21 cells when separated from CA12 stromal cells and cultured alone. Enzyme purification using an avidin affinity column revealed that the involved cysteine protease possessed caspase3-like death protease activity. In addition, when IL-7 was added to CS21 cell culture, the protease activity could not be detected during partial purification of the enzyme. Taken together, these results strongly suggest that the caspase3-like protease activation is suppressed by IL-7 as an antiapoptotic factor that leads to abrogation of apoptosis execution.

Key words: Apoptosis; Interleukin-7; caspase3-like death protease; enzyme purification; T-lymphoma

Introduction

It is now evident that cell death by apoptosis plays pivotal roles in cell homeostasis especially in immune system and in cancer progression [22,31,33]. Although several genes involved in apoptosis have been identified, the molecular mechanisms by which these gene-regulated sequential apoptotic events occur are poorly understood [36,37].

We have previously prepared and characterized mouse malignant T-lymphoma CS21 cells that are highly metastatic to auxillary lymph nodes when innoculated s.c. into the right flank of BALB/c mice [35]. CS21 cells grew in vitro when cells were cocultured with CA12 lymph node stromal cells but eventually underwent apoptosis after separation from the stromal cells [15]. CA12 stromal cells exerted bi-directional regulations on the growth of CS21 lymphoma cells. They promoted the growth of CS21 cells by direct contact and/or by a soluble factor(s). We recently were successful in raising mAbs that inhibited CS21 cell adhesion to CA12 stromal cells. The resultant mAbs MCS-5 and -19, which recognize a Mr 168,000 and a Mr 23,000 protein, respectively, suppressed apoptotic cell death of CS21 cells even after the cells were separated from CA12 cells [9]. Therefore, we proposed that cell adhesion molecules, such as CD45 [12] and Thy-1 (CD 90) [10], play an important role in CS21 cell survival. Besides these adhesion molecules, the involvement of soluble factor(s) on apoptosis of these cells has a significant meaning in that the deprivation of these soluble factors cause apoptosis to cells by transmitting death execution signal(s). In the course of examining the soluble factors responsible for CS21 cell proliferation, we recently identified cysteine [19] and IL-7 [20] as antiapoptotic soluble factors produced by CA12 stromal cells.

Although several papers on human Interleukin-1β-converting enzyme (ICE) and ICE-family (especially CPP32 and CPP32-like, now called caspase3 and caspase3-like) proteases, playing a critical role in apoptotic hierarchical cascade, were reported [1,5,6,32,34], murine caspase3 or caspase3-like death proteases during apoptosis of tumor cells have not yet been fully understood. We, therefore, compared the protease by purifying the enzyme according to the method described for human CPP32 purification [30]. We, here, report that a caspase3-like death protease is activated during apoptosis of CS21 lymphoma cells when separated from coculture with CA12 stromal cells, and the activity is inhibited by adding IL-7.

Materials and Methods

Cell lines and cell culture

CS21 lymphoma cells and CA12 lymph node stromal cells

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were established, as described previously [9,15]. These cells were cultured at 37℃ in a humidified atmosphere of 5% CO_2-95% air in RPMI 1640 medium supplemented with 5% heat-inactivated fetal calf serum (Biocell, Carson, CA) and kanamycin at 100 μg/ml.

Reagents

The fluorogenic substrates, acetyl-L-tyrosyl-L-valyl-L-alanyl-L-aspartyl-7-amino-4-methylcoumarin (YVAD-AMC) and acetyl-L-aspartyl-L-glutamyl-L-valyl-L-aspartyl-7-amino-4-methylcoumarin (DEVD-AMC), were obtained from Bachem Chemical Co., and used for the substrates of proteases. Avidin-agarose column (Pierce, Rockfold, IL) and biotinyl-DEVD-CHO (Peptide Institute) were used for affinity column chromatography. Other reagents were all commercially available.

Analysis of DNA fragmentation

CS21 cells (1 x 10^6 cells/ml) were suspended in 20 ml of 50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 0.5 mg/ml proteinase K (Sigma). After incubation at 50℃ for 1 h, a 10 ml aliquot of 0.5 mg/ml RNase A solution was added to a mixture of 10 ml of the 70℃-preheated solution containing 10 mM EDTA (pH 8.0), 1% (w/v) low-melting point agarose (Sigma), 0.25% bromophenol blue, and 40% sucrose. The DNA was analyzed by electrophoresis in 2% agarose gels followed by ethidium bromide staining and photographing on a UV illuminator.

ELISA for IL-1 β

Fifty ml of samples (CS21 cells at 0 h and 24 h, respectively) were added into precoated the wells and then gently agitate to mix and incubate at 37℃. After 1 h incubation, the solutions were aspirated from the wells and washed 4 times with the washing buffer. Fifty ml of the antiserum to anti-rabbit mouse IL-1 β were added and incubated for 1 h, followed by conjugate HRP-conjugated goat anti-rabbit IgG as a second antibody (PerSeptive Diagnostics, MA). Washing four times with the buffer was subjected before adding substrate for 15 min. The absorbance at 450 nm was read with a spectrophotometer (Beckmann).

Estimation of protease activity

The cell lysate was incubated with 20 μM of the fluorogenic substrate of proteases (YVAD-AMC or DEVD-AMC) in ICE buffer [20 mM HEPES (pH 7.5), 10% glycerol, 2 mM dithiothreitol] for various time at 37℃. The AMC released from the fluorogenic substrates were measured with the excitation at 380 nm and the emission at 460 nm using a fluorescence spectrophotometer (Model F-2000, Hitachi, Tokyo).

Purification of the caspase3-like death protease

The cytosol fraction from CS21 cells that possessed apoptosis-inducing activity was prepared and applied with an avidin-agarose column to remove avidin-binding proteins. The pass-through fraction was reacted with the biotinyl−DEVD-CHO (Peptide Institute) for overnight at 4℃. The conjugate was then applied to a new avidin-agarose column and washed, as described previously [30]. The enzyme was eluted by perfusing the column with D-biotin (Sigma). The eluted samples were concentrated, dialyzed, electrophoresed, and stained with silver reagent. The activity of the enzyme was estimated by incubating with DEVD-AMC as described above.

Silver staining

After washing an electrophoresized gel twice with PBS, the gel was stained with silver staining kit (Daiichi Chemical, Tokyo) as described in an user's manual.

Results

Suppression of the caspase-family protease activation by IL-7

Caspase-family proteases were known to take part in apoptosis induction because overexpression of these proteases could induce apoptosis [24,34]. We first examined whether or not caspase-family proteases were involved in the apoptosis which occurred in the isolated culture of CS21 cells. As shown in Fig. 1, the apoptotic DNA ladder that occurred in the isolated culture of CS21 cells were inhibited by the addition of benzyloxycarbonyl-L-glutamyl-L-valyl-L-aspartyl

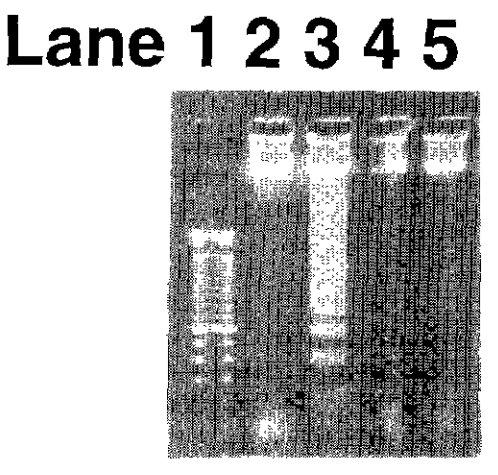

Fig. 1. Suppression of apoptosis in CS21 cells by an inhibitor of caspase-family proteases. CS21 cells were isolated and cultured for 0 h (Lane 2), 24 h (Lane 3) with medium alone, medium containing 10 mM (Lane 4) or 100 μM (Lane 5) Z-EVD. The harvested cells were incubated with proteinase K and RNAse, respectively, for 2 h at 50℃, and analyzed with a UV transilluminator, as describe in "Materials and Methods." The molecular size marker was the 100-base pair DNA ladder (Lane 1).

CH_2OC (O)-2, 6-dichlorobenzene (Z-EVD; 10-100 μM). Moreover, we also confirmed by FACS analysis that the apoptotic bodies (18.2%) of 24 h-cultured CS21 cells decreased to 6.8% by the inhibitor (100 μM), while the apoptotic percentage of fresh isolate CS21 cells were only 3.5% (data not shown). Therefore, we conjectured that an ICE-family protease might be involved in the induction of CS21 cell apoptosis. As ICE can cleave pro-IL-1β to IL-1β, we examined whether CS21 cell lysate containing the death enzyme activity can cleave it. As shown in Table 1, even though the CS21 cell were treated without IL-7 for 24 h, the amount of IL-1β produced was not changed. Moreover, we tried to detect the IL-1β by western blotting, but we could not detect the band of mature IL-1β (data not shown). These results suggest that the involved protease is not ICE (caspase1) but ICE-related protease.

Caspase3-like death protease is activated during CS21 cell apoptosis

We next examined the activity of caspase-family protease using fluorogenic tetrapeptides (YVAD-AMC and DEVD-AMC) as the substrates of the proteases [20,23,30]. When the lysates were prepared from CS21 cells which were cultured alone for 24 h and then incubated for various time periods with the fluorogenic substrates of caspase-family proteases, the AMC was liberated from DEVD-AMC (the specific substrate of CPP32/Yama/Apopain) with a time-dependent manner but not from YVAD-AMC (the specific substrate of caspase1) (Fig. 2), indicating that a caspase3-like death protease is activated during CS21 cell apoptosis. Interestingly, the cell lysates from IL-7-treated CS21 cells lost the activity to cleave DEVD-AMC (data not shown).

The activity of caspase3-like death protease is inhibited by IL-7

The DEVD-AMC-cleaving protease expressed in the CS21 cell lysate might be a mouse homolog of caspase3 because the activity of the protease was inhibited by the addition of DEVD-CHO, but not by the addition of YVAD-CHO [6, 20, 24, 30, 34]. These results sugest that IL-7 suppresses CS21 cell apoptosis by inhibiting the activation of the caspase3-like protease expressing in CS21 cells. Recently, mouse CPP32 has been cloned [14], but its homolog has not yet been identified. To demonstrate whether the caspase3-like protease is participating in executing CS21 cell apoptosis,

Table 1. Failure to cleavage of pro-IL-1β to IL-1β during apoptosis of CS21 lymphoma cells.

	IL-1β (pg/ml)
Medium alone	752.9±16.8*
CS21 cells, 0 h	765.8±12.2
CS21 cells, 24 h	717.8±40.8

*Results are the mean±S.D.

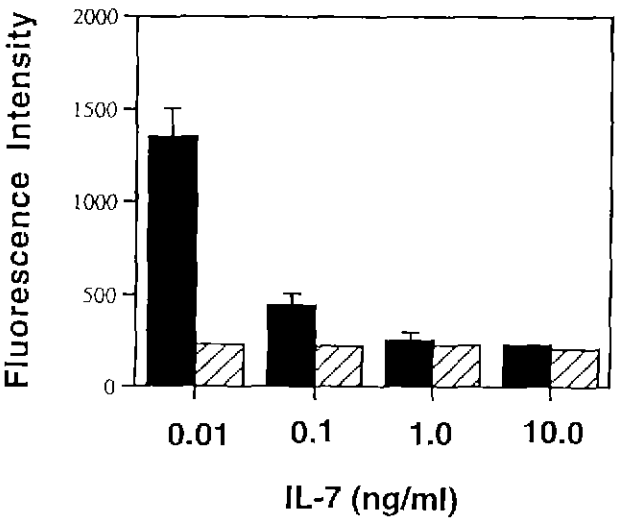

Fig. 2. Detection of caspase3-like death activity in CS21 cell lysate. CS21 cells were isolated and cultured for various time periods. The cell lysates were incubated with DEVD-AMC (black columns) or YVAD-AMC (dotted columns) for 15 min at 37℃. The AMC liberated from the substrates were measured. The vertical bars represent standard deviation of triplicates.

we tried to purify the enzyme using a biotinylated derivative of the Ac-DEVD-CHO [30]. At first, CS21 cells isolated from coculture plates were incubated for 24 h and then the lysis buffer was added to solubilize the cells. After 20 min incubation on ice, the reaction mixture was centrifuged and collected as a cytosolic fraction. To carry out enzyme purification efficiently, we used avidin-agarose column as an affinity column. Because many proteins were contained in CS21 cytosolic fractions, we examined whether the pass-through of avidin-agarose column after incubation with CS21 cell lysates possess the caspase3 protease activity. The wash-out fractions possessed the caspase3 activity (data not shown). By this procedure, avidin-binding proteins in the cell lysates could be discarded. The resultant pass-through fractions mixed with biotinylated DEVD-applied to a new avidin column. The column was washed with 10-volume of ICE buffer, and then 20 mM D-biotin was used for elution of biotinyl DEVD-CHO-enzyme complexes. The complexes were dialyzed three times with 2 liters of ICE buffer containing 0.1% BSA in every 12 h. The dialyzed enzyme solution was examined for DEVD-AMC cleavage assay. As shown in Fig. 3A, fractions 4, 5, 6 did not contain ICE activity, but did the caspase3 protease activity. After concentrating fraction 4, 5, and 6 in a Centricon C-10, the enzyme was electophoresized and stained with silver agents (Fig. 3B). We confirmed that this caspase3-like death proenzyme was detected (Fig. 3B, lane 5), and the enzyme is composed of a larger subunit p17 and a 1 or 2 smaller subunit(s) p12-p13. Interestingly, the death protease activity of IL-7-treated CS21 cells was not detected (Fig. 3B, lane

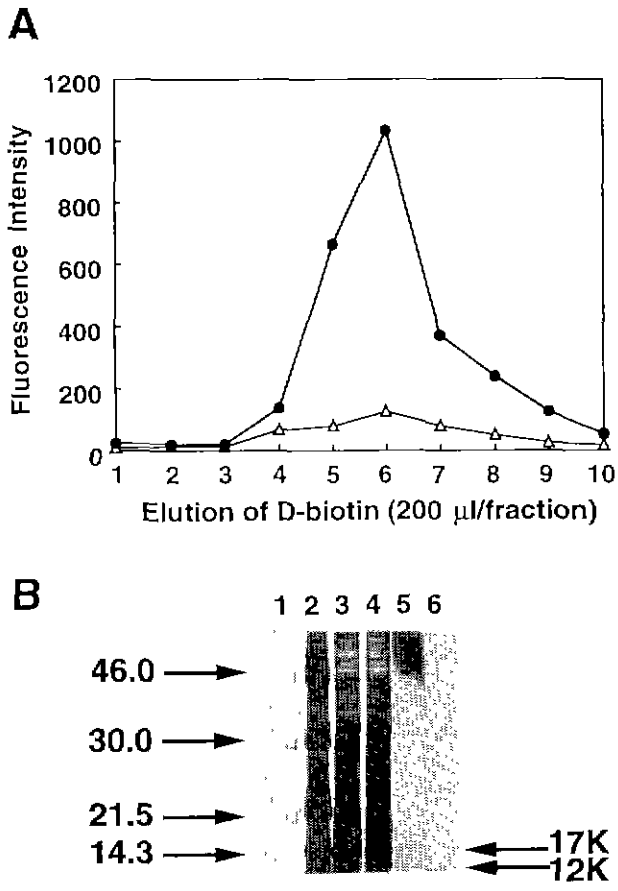

Fig. 3. Partial purification of the caspase3-like death protease in CS21 cells. (A), After passing through an avidin affinity column in order to remove other proteins, the fraction was incubated with 200 *nM (final concentration after racemization)* of biotinyl—DEVD-CHO, then applied to an avidin-agarose column and washed with 10-volume of ICE standard buffer. The enzyme was eluted by perfusing the column with 20 mM D-biotin. Each fraction was dialyzed with ICE standard buffer containing 0.1% BSA *for 36 h by changing the buffer three times. The caspase3-like* death protease activity was measured with DEVD-AMC (closed circles) or YVAD-AMC (open triangles). The data represent the mean values of triplicate determinations. (B), Silver staining of the purified caspase3-like death protease. Samples were resolved *on a 10-20% SDS-polyacrylamide gel, and protein bands were* visualized by silver staining. Molecular marker (lane 1), CA12 cytosolic fraction (lane 2), pass-through of biotinyl—DEVD-CHO (lane 3), eluted with D-biotin with (lane 5), or without (lane 4) dialysis, and IL-7 added (lane 6) were applied.

6), suggesting that IL-7 inhibits the CPP32-like protease activation. Taken together, these results indicate that the caspase3-like protease is associated with CS21 cell apoptosis and IL-7, as an antiapoptotic factor produced by CA12 stromal cells, relieves CS21 cells from apoptotic cell death.

Discussion

IL-7 is a 25-kDa cytokine secreted by bone marrow stromal cells that could stimulate the proliferation and differentiation of pre-B cells[28]. IL-7 was subsequently shown to affect the cell growth of T-cell lineage in the presence of concanavalin A or phorbol myristate acetate [28,29]. IL-7 also had a growth-promoting effect on precursor B- and T-acute lymphoblastic leukemia cells [11]. Because the CS21 cells were mouse malignant T-lymphoma cells, it might be reasoned that IL-7 could promote CS21 cell growth. Various cytokines were known not only to promote cell growth but also to inhibit apoptosis by transmitting a survival signal (or signals). For example, CTLL-2, an IL-2—dependent cytotoxic T-cell line, underwent apoptosis when IL-2 was withdrawn but could survive and grow when IL-2 was added (unpublished results). As the apoptosis-inhibitory effect of IL-7 in lymphoma cells has not yet been fully examined, we investigated the effect using IL-7—dependent CS21 cells and found that IL-7 is a cytokine that possesses the ability to inhibit apoptosis [20]. CA12 lymph node stromal cells produced IL-7 [20], and the secreted IL-7 contributed to the growth and the suppression of CS21 cell apoptosis. In this paper, we further demonstrated that the caspase3-like death protease is inhibited by IL-7.

Apoptotic cysteine proteases that have homology to CED-3, an apoptosis-related gene product in *Caenorhabditis elegans*, have been identified and characterized. These caspase-family proteases, such as ICE, Nedd-2/Ich1, CPP32/Yama/Apopain, TX/ICH-2/ICErel-II, ICErel-III/TY, Mch2, Mch3/ICE-LAP3/CMH-1, FLICE/Mch5/MACH, CE-LAP6/Mch6 and Mch4 (see Ref. 25 as the nomenclature of ICE/CED-3 protease family), play an important role in apoptosis induction because overexpression of these proteases can induce apoptosis [3,6-8,18,20,24-27,30]. CS21 cell apoptosis was inhibited by the addition of Z-EVD, an inhibitor of ICE-family proteases (Fig. 1, Lane 4 and 5). ICE-family proteases might be involved in the apoptosis induction in CS21 cells because the protease activity that cleave the DEVD-AMC was detected in CS-21 cells during induction of CS-21 cell apoptosis. We suggest that the protease might be a mouse homolog of CPP32 because the activity contained in the CS21 cell lysate was inhibited by the addition of DEVD-CHO and iodoacetamide but not by the addition of YVAD-CHO, E-64, and aprotinin [6,20,30,34].

Recently, however, the mouse caspase3 has been cloned [14]. We, therefore, tried to compare the protease by purifying the enzyme according to the method described for human caspase3 purification [24]. Because the protease was not abundant in CS21 cells, we could not obtain a sufficient amount to be able to determine the N-terminal sequence. However, the purified enzyme could cleave DEVD-AMC but not YVAD-AMC, as observed in the cell lysate of CS21 cells (Fig. 3A). Furthermore, an identical inhibition profile was found between the purified protease (Fig. 3 and data not shown) and human caspase3 [30]. These results indicate that the apoptosis-inducing protease in CS21 cells is a mouse

homolog of caspase3. Because the activity of a caspase3-like death protease was not detected in the CS21 cell lysate cultured with IL-7, IL-7 inhibited CS21 cell apoptosis by suppressing the activation of the caspase3-like death.

Sequence alignment of these cysteine proteases indicates that the caspase3 protease is the most closely related protease to CED-3, a nematodal apoptosis-inducing gene product. All known ICE and ICE-like proteases including caspase3 and caspase3-like proteases [4,13,16,21] are conserved and involved in binding the carboxylate side chain of aspartic acid during apoptosis execution. Analysis of the genomic DNA sequence of mouse CPP32 β indicates that it contains a genomic structure distinguishable from that of human or mouse ICE (caspase1). Although there is no many report on involvement of caspase3 in mouse or human disease [17], the development of caspase-family protease-deficient mice can make a clear analysis of the role that the proteases have crucial key function in the normal development, maintenance of tissue homeostasis and moreover progression of cancer.

In conclusion, we demonstrated that a murine caspase3-like death protease is associated with apoptosis of T-lymphoma cells. IL-7 clearly inhibited CS21 cell apoptosis by suppressing the activation of a caspase3-like protease. The study with *in vivo* testing of anti−IL-7 monoclonal antibody will give important information about the biological significance of IL-7 on T-lymphoma metastasis. We are anticipating that IL-7 and our previously identified molecules (such as CD45, Thy-1, and cysteine) may be good tools for metastatic prevention in CS21 cells in that lymphatic metastasis is more difficult for circumvention than blood vessel-mediated metastasis.

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