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Mechanism underlying cytotoxicity of thialysine, lysine analog, toward human acute leukemia Jurkat T cells

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Abstract

We first report the mechanism for the inhibitory effect of the lysine analog, thialysine on human acute leukemia Jurkat T cells. When Jurkat T cells were treated with thialysine (0.32–2.5 mM), apoptotic cell death along with several biochemical events such as mitochondrial cytochrome *c* release, caspase-9 activation, caspase-3 activation, degradation of poly (ADP-ribose) polymerase, and DNA fragmentation was induced in a dose- and time-dependent manner. However, these thialysine-induced apoptotic events were significantly abrogated by an ectopic expression of Bcl-xL, which is known to block mitochondrial cytochrome *c* release. Decylubiquinone, a mitochondrial permeability transition pore inhibitor, also suppressed thialysine-induced apoptotic events. Comparison of the thialysine-induced alterations in the cell cycle distribution between Jurkat T cells transfected with Bcl-xL gene (J/Bcl-xL) and Jurkat T cells transfected with vector (J/Neo) revealed that the apoptotic cells were mainly derived from the cells accumulated in S and G2/M phases following thialysine treatment. The interruption of cell cycle progression in the presence of thialysine was accompanied by a significant decline in the protein level of cdk4, cdk6, cdc2, cyclin A, cyclin B1, and cyclin E. These results demonstrate that the cytotoxic activity of thialysine toward Jurkat T cells is attributable to not only apoptotic cell death mediated by a mitochondria-dependent death signaling pathway, but also interruption of cell cycle progression by a massive down-regulation in the level of cdks and cyclins.

Keywords: Lysine analog; Thialysine; Apoptosis; Mitochondrial cytochrome *c*; Caspase cascade; Bcl-xL; Cell-cycle arrest; cdks; Cyclins

1. Introduction

Thialysine, *S*-2-aminoethylcysteine, is a lysine analog with the 4-methylene group substituted by a sulfur atom. Since the structural similarity of thialysine to lysine is remarkable, it can effectively compete with lysine for lysyl tRNA activation and for incorporation into cellular proteins. Previously it has been shown that thialysine is toxic against microorganisms including bacteria [1,2] and yeasts [3,4], and reduces the growth rate and plating efficiency of Chinese Hamster Ovary (CHO) cells [5]. These toxic effects of thialysine appear to be reversible by the addition of lysine. Several studies have proposed the possible application of amino acid analogs to pharmacological treatment of malignant conditions including cancers [6–12]. For instance, the L-arginine analog, L-canavanine has been reported to possess growth retardation activity toward tumor cells in culture and experimental tumors *in vivo* [6,7,11]. Synergic antitumor effects from a combination of L-canavanine with 5-fluorouracil or γ-irradiation have been demonstrated, indicating that L-canavanine may modulate the chemo- or radio-sensitivity of tumors [8,9]. In addition, it has been shown that the amino acid analog, L-2,4 diaminobutyric acid accumulates in hepatoma cells, which causes hyperosmosis with subsequent cell lysis [12]. Although these suggest that the inhibitory mode of amino

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Abbreviations: PARP, poly (ADP-ribose) polymerase; FasL, Fas ligand; FBS, fetal bovine serum; 2-ME, β-mercaptoethanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PMSF, phenylmethylsulfonyl fluoride; MOPS, 3-(*N*-morpholino)propanesulfonic acid; MES, 2-(*N*-morpholino)ethanesulfonic acid; kDa, kilodalton; cdk, cyclin-dependent kinase; Rb, retinoblastoma protein.

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acid analogs toward tumors may vary depending on the analog types and cell types, a primary mechanism underlying the antitumor activity of amino acid analogs, leading to cell damage, is thought to be their incorporation into cellular proteins in substitution for the intact forms and subsequent induction of structurally aberrant proteins with impaired function or degradation [13–15]. Chemotherapy employing antineoplastic drugs often relies on the difference of the mitotic rate between tumor and normal cells in order to confine its toxic effect to the tumor. In this regard, amino acid analogs have been simply considered to possess a potency as chemotherapeutic agents because their incorporation into cellular proteins, which result in an inhibitory effect on cell growth, can be more significant in tumor cells than in normal cells. However, the inhibitory activity of amino acid analogs on the growth of tumor cells, and the underlying inhibitory mechanisms requisite for evaluating their potency as a chemotherapeutic agent, remain largely unknown. Since the induction of apoptosis in tumor cells can lead to their own destruction, apoptosis has been implicated as an efficient mechanism by which malignant tumor cells are removed when treated with antineoplastic drugs. As a potential mechanism in the drug-induced apoptosis, upregulation of Fas ligand (FasL) and/or Fas expression with subsequent induction of apoptotic cell death through activation of Fas signaling has been implicated [16–19]. Mitochondrial release of cytochrome *c*-mediated activation of caspase–cascade has also been implicated in chemotherapeutic agent-induced apoptosis [20,21]. However, the involvement of apoptotic cell death in the inhibitory activity of amino acid analogs against tumor cells is still poorly elucidated.

In a previous study, we found that the cytotoxicity of L-canavanine toward human acute leukemia Jurkat T cells is attributable to induced apoptosis [22]. L-Canavanine-induced apoptosis appeared to accompany mitochondrial cytochrome *c*-independent activation of caspase-3, which could be interrupted by an ectopic expression of Bcl-2 or Bcl-xL, suggesting that L-canavanine may cause apoptotic cell death of Jurkat T cells by triggering a conserved caspase cascade, leading to caspase-3 activation without involving the mitochondrial cytochrome *c* release. Since mitochondria are known to play an important role in the commitment of apoptosis provoked by many physiological and non-physiological signals [23–25], our previous results raised a question that the mitochondria-independent pathway converging to caspase-3 activation plays a central role in amino acid analog-induced apoptosis regardless of the analog types. In the present study, to understand the mechanism by which amino acid analogs induce apoptotic cell death, we investigated a lysine analog, thialysine-induced apoptotic signaling pathway in Jurkat T cells, focusing on the mitochondrial cytochrome *c*-mediated activation of caspase cascade. In addition, we compared a thialysine-mediated alteration in the cell cycle distribution of Jurkat T cells transfected with Bcl-xL gene (J/Bcl-xL) and Jurkat T cells transfected with vector (J/Neo) to investigate whether thialysine arrests cell cycle progression. The results demonstrate that thialysine induces apoptotic cell death in Jurkat T cells *via* a mitochondria-dependent death signaling pathway including mitochondrial cytochrome *c* release and activation of caspase-9 and -3, which is negatively regulated by Bcl-xL. The results also demonstrate that thialysine can arrest cell cycle progression by inducing down-regulation of the protein levels of several positive cell cycle regulators, indicating that cytotoxic activity of thialysine toward malignant Jukart T cells is due to induced apoptosis as well as the cell-cycle arrest.

2. Materials and methods

2.1. Reagents, antibodies, and cells

Thialysine and decylubiquinone were purchased from Sigma Chemical. The ECL Western blotting kit was from Amersham. Anti-cytochrome *c* antibody was purchased from Pharmingen. Anti-caspase-9, anti-caspase-3, anti-PARP, and anti-Bcl-xL were from Santa Cruz Biotechnology. Monoclonal anti-FasL antibody was purchased from Transduction Laboratories. A broad-spectrum caspase inhibitor (z-VAD-fmk) was purchased from Calbiochem. Human acute leukemia Jurkat T cell line E6.1 was supplied by Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA). Jurkat T cell clones JT/Neo, JT/Bcl-2, J/Neo and J/Bcl-xL were supplied by Dr. Dennis Taub (Gerontology Research Center, NIA/NIH). Jurkat T cells were maintained in RPMI 1640 (Life Technologies) containing 10% fetal bovine serum (FBS, UBI), 20 mM HEPES (pH 7.0), 5×10^{-5} M β-mercaptoethanol (2-ME), and 100 μg/mL gentamycin.

2.2. Cytotoxicity assay

The cytotoxic effect of thialysine on Jurkat T cells was analyzed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay reflecting cell viability or by counting viable cell numbers after staining of cells with trypan blue. For MTT assay, Jurkat T cells (5×10^4) were added to a serial dilution of thialysine in 96-well plates. After incubation for 16 hr, 50 μL of MTT solution (1.1 mg/mL) was added to each well and incubated for an additional 4 hr. After centrifugation, the supernatant was removed from each well and then 150 μL of DMSO was added to dissolve the colored formazan crystal produced from MTT. The OD values of the solutions were measured at 540 nm by a plate reader. The equivalent culture was stained with trypan blue and unstained cells were counted in order to estimate the viable cell number by dye exclusion.

2.3. DNA fragmentation analysis

Apoptotic DNA fragmentation induced in Jurkat T cells following thialysine treatment was determined as previously described [26]. Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (0.5% Triton X-100, 5 mM EDTA, 10 mM Tris–HCl, pH 7.4) for 20 min on ice. After centrifugation for 15 min at 14,000 rpm, the supernatant was collected and treated for 2 hr at 50° with proteinase K and subsequently with RNase for 4 hr at 37°. After extraction with an equal volume of buffer-saturated phenol, the DNA fragments were precipitated with 2.5 vol. of ethanol in the presence of 0.5 M NaCl and visualized following electrophoresis on a 1.2% agarose gel.

2.4. Flow cytometric analysis

Cell cycle progression of Jurkat T cells following thialysine treatment was analyzed by Flow cytometry as described elsewhere [27]. Approximately 1×10^6 cells were suspended in 100 μL of PBS, and 200 μL of 95% ethanol were added while vortexing. The cells were incubated at 4° for 1 hr, washed with PBS, and resuspended with 12.5 μg of RNase in 250 μL of 1.12% sodium citrate buffer (pH 8.45). Incubation was continued at 37° for 30 min before staining the cellular DNA with 250 μL of propidium iodide (50 μg/mL) for 30 min at room temperature. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content, based on increased red fluorescence.

2.5. Preparation of cell lysate and Western blot analysis

Cellular lysates were prepared by suspending 5×10^6 Jurkat T cells in 200 μL of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS), and 2.5 μg/mL proteinase inhibitor E-64, pH 7.2). The cells were disrupted by sonication and extracted at 4° for 30 min. An equivalent amount of protein lysate (20–30 μg) was denatured with SDS sample buffer, and subjected to electrophoresis on 4–12% SDS gradient polyacrylamide gel with MOPS buffer. For detection of caspase-3 activation and mitochondrial cytochrome *c* release, protein lysates were electrophoresed on 10% SDS polyacrylamide gel with 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer. The proteins were electrotransferred to Immobilon-P membranes (Millipore Corporation). Detection of each protein was carried out with an ECL Western blotting kit (Amersham) according to the manufacturer's instructions.

2.6. Detection of mitochondrial cytochrome c in cytosolic protein extracts

To assess the mitochondrial cytochrome *c* release in Jurkat T cells following thialysine treatment, cytosolic protein extracts were obtained. Briefly, approximately 5×10^6 cells treated with thialysine were washed with cold PBS three times and then suspended in 0.5 mL of a lysis buffer (250 mM sucrose, 10 mM KCl, 1.5 mM $MgCl_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride (PMSF), 2.5 μg/mL E-64, and 20 mM HEPES, pH 7.2) The cells were allowed to swell on ice for 30 min and were homogenized with a Dounce homogenizer with 50 strokes. The homogenates were centrifuged at 1000 *g* for 10 min at 4°, and the supernatants were centrifuged at 15,000 *g* for 15 min at 4°. The supernatants were harvested as cytosolic extracts free of mitochondria, and analyzed for alterations in the level of mitochondrial cytochrome *c* release by Western blotting.

3. Results and discussion

3.1. Apoptotic effect of thialysine on Jurkat T cells

To understand the mechanisms underlying the cytotoxicity of thialysine, its effect on Jurkat T cell line E6.1 was investigated. When Jurkat T cells were treated with thialysine at various concentrations ranging from 0.32 to 2.5 mM for 20 hr, cell viability determined by MTT assay appeared to decline significantly in a dose-dependent manner (Fig. 1A). After treatment with 0.32 mM thialysine, the viability remained at the level of 40%. Cell viability declined, however, to a minimal level (20%) in the range of 1.25–2.5 mM thialysine. Under these conditions, apoptotic DNA fragmentation was easily detectable with a maximum level at concentrations ranging from 0.63 to 2.5 mM thialysine (Fig. 1B). To assess apoptotic change in the nuclear morphology of Jurkat T cells following treatment with thialysine, the cells treated with 1.25 mM thialysine for 20 hr were stained with DAPI. Typical apoptotic bodies were detected in Jurkat T cells after thialysine treatment (Fig. 1C). The time course of induced apoptotic DNA fragmentation was also investigated following the treatment of 1.25 mM thialysine. As shown in Fig. 1D, a significant level of apoptotic DNA fragmentation began to be detectable at 12 hr and reached a maximum level 20 hr after thialysine treatment. These results demonstrate that thialysine can induce apoptotic DNA fragmentation of Jurkat T cells in a concentration- and time-dependent manner, and suggest that the cytotoxic effect of thialysine is attributable to induced apoptosis.

3.2. Involvement of mitochondrial cytochrome c-mediated activation of caspase cascade in thialysine-induced apoptosis

The activation of caspase-3 through proteolytic degradation of a 32-kDa pro-enzyme into a 19-kDa activated form is often required for apoptosis induced by many different stimuli [28]. Recently we have found that

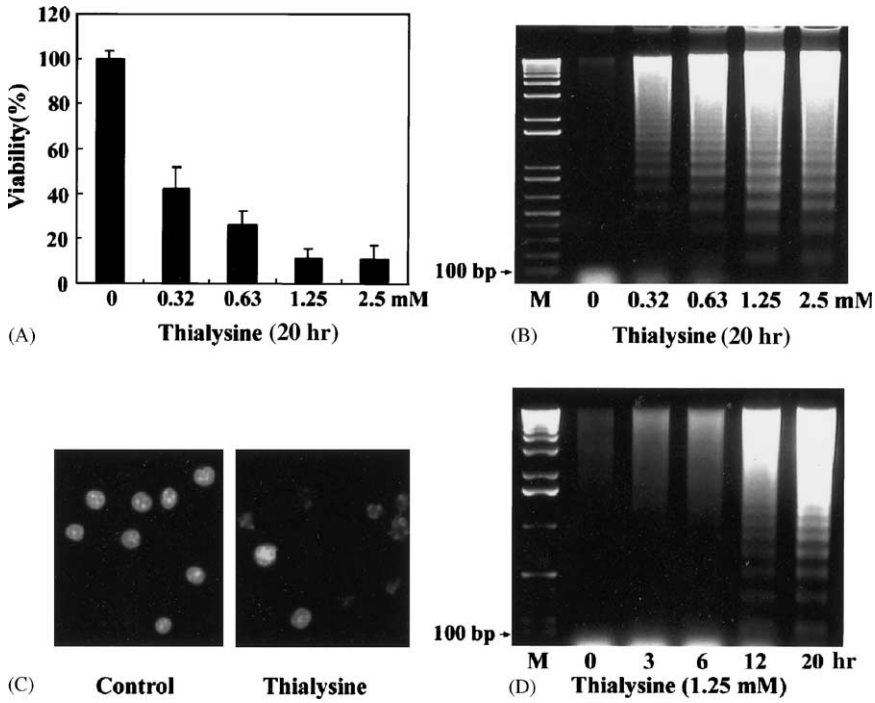

Fig. 1. Effect of thialysine on cell viability (A), apoptotic DNA fragmentation (B) and nuclear morphology (C), and kinetic analysis of thialysine-induced apoptotic DNA fragmentation (D) in Jurkat T cells. Continuously growing Jurkat T cells (5×10^4) were incubated with indicated concentrations of thialysine in a 96-well plate for 20 hr and the final 4 hr were incubated with MTT. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Equivalent cultures were prepared and the cells were collected to analysis apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis. To assess nuclear apoptotic change in Jurkat T cells exposed to 1.25 mM thialysine for 20 hr, the cells were fixed with cold ethanol and then stained with DAPI (1 μg/mL). For time course of thialysine-induced apoptotic DNA fragmentation, Jurkat T cells (5×10^6) were incubated with 1.25 mM thialysine for indicated times and processed to analyze apoptotic DNA fragmentation.

L-canavanine, an L-arginine analog, induces apoptotic cell death in Jurkat T cells *via* caspase-3 activation in the absence of mitochondrial cytochrome *c* release, indicating the involvement of mitochondria-independent caspase-3 activation in L-canavanine-induced apoptosis. To determine whether this phenomenon can be extended to thialysine-induced apoptosis in Jurkat T cells, we investigated the change in the pro-caspase-3 (32 kDa) as well as active capase-3 (19 kDa) level by Western blot analysis of Jurkat T cells, following thialysine treatment for 20 hr. As shown in Fig. 2A, the expression of pro-caspase-3 was easily detectable in continuously growing Jurkat T cells. Although there was no detectable alteration in the level of pro-caspase-3 by 0.32 mM thialysine, it declined in a concentration-dependent manner in the presence of thialysine ranging from 0.63 to 2.5 mM. At a concentration of 0.32 mM thialysine, the active caspase-3 was detected as a very faint band. At concentrations of 0.63–2.5 mM thialysine, however, the level of active caspase-3 appeared to increase in accordance with the reduction of pro-caspase-3 level induced by thialysine. As a downstream target of active caspase-3 during induction of apoptosis, poly (ADP-ribose) polymerase (PARP) has been reported to be cleaved into two fragments [29]. This cleavage of PARP by active caspase-3 is proposed as a marker of apoptosis in many

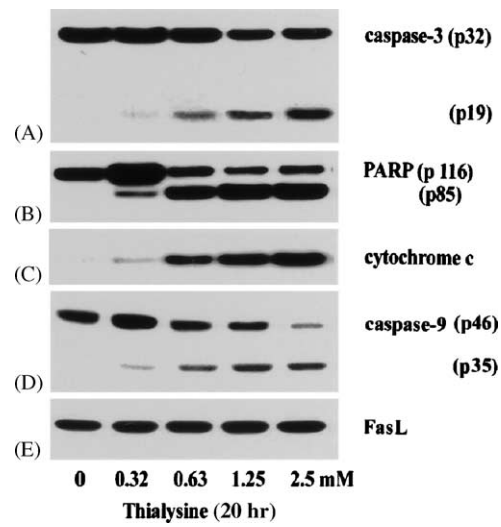

Fig. 2. Western blot analysis of caspase-3 activation (A), cleavage of PARP (B), mitochondrial cytochrome c release (C), caspase-9 activation (D), and expression level of FasL (E) in Jurkat T cells after treatment with thialysine. The cells (~5×10^6 cells) were incubated at a concentration of 4×10^5 mL^{-1} with indicated concentrations of thialysine for 20 hr and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Section 2 using the ECL Western blotting detection system.

experimental models. When the degradation of PARP was investigated by Western blot analysis, the cleavage of PARP was detected along with activation of caspase-3 in the presence of 0.32–2.5 mM thialysine (Fig. 2B). Sequentially, we investigated whether thialysine-induced apoptosis accompanies the mitochondrial cytochrome *c* release. Although there was barely detectable cytochome *c* in the cytosolic fraction of continuously growing Jurkat T cells, the level of cytosolic cytochrome *c* released from mitochondria increased significantly in the presence of thialysine ranging from 0.32 to 2.5 mM (Fig. 2C). This suggests that thialysine-induced apoptosis involves a mitochondria-dependent activation of caspase-3. Further these results suggest that although two different amino acid analogs, thialysine and L-canavanine [22], can induce both caspase-3 activation and PARP degradation, a thialysine-mediated apoptotic pathway leading to caspase-3 activation is distinct from the pathway mediated by L-canavanine.

Several studies have shown that a chemical-induced apoptotic signaling pathway involves the action of cytochrome *c* released from mitochondria [30–32]. The released cytochrome *c* together with apoptotic protease-activating factor-1 (Apaf-1) is known to activate caspase-9 in the presence of dATP, and the latter then activates caspase-3 [33,34]. In order to elucidate the signaling intermediates that are correlated with the mitochondrial cytochrome *c* release in the thialysine-induced apoptotic pathway, we examined whether caspase-9 activation occurs along with the thialysine-mediated mitochondrial cytochrome *c* release. Since the activation of caspase-9 proceeds through proteolytic degradation of the inactive pro-enzyme (46 kDa) into the activated form (35 kDa), the alteration in the level of the pro-enzyme and active enzyme after thialysine treatment was investigated by Western blot analysis. As shown in Fig. 2D, in the presence of 0.63–2.5 mM thialysine, a significant decrease in the level of pro-caspase-9 was detected as well as caspase-9 activation in accordance with the mitochondrial cytochrome *c* release. On the other hand, there was no change in the level of FasL following exposure to 0.63–2.5 mM thialysine, suggesting that Fas-death signaling may not be associated with thialysine-induced apoptosis. Kinetic analysis of these apoptotic events following the treatment of 1.25 mM thialysine also showed that the activation of capsase-9, and caspase-3, and the cleavage of PARP began to be detectable at 12 hr and increased by 20 hr in accordance with the pattern of induced apoptotic DNA fragmentation after thialysine treatment (Fig. 3). It is noteworthy that thialysine-induced apoptosis in Jurkat T cells appears to occur more rapidly than L-canavanine-induced apoptotis, because Jurkat T cells do not undergo apoptosis until 24 hr after treatment of L-canavanine (data not shown). These results indicate that the thialysine treatment in Jurkat T cells cause cytochrome *c* release from mitochondria and the subsequent activation of a cytochrome *c*-dependent caspase cascade, leading to PARP cleavage and apoptotic DNA fragmentation.

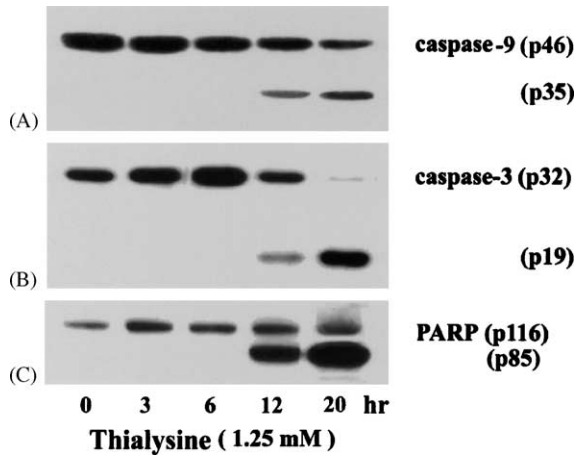

Fig. 3. Time course of activation of caspase-9 (A), and caspase-3 (B), and cleavage of PARP (C) in Jurkat T cells following treatment with 1.25 mM thialysine. Continuously growing Jurkat T cells (5×10^6) were incubated with 1.25 mM thialysine for indicated times, and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Section 2.

3.3. Effect of Bcl-xL on thialysine-induced apoptotic cell death

Our results demonstrate that thialysine-induced apoptosis accompanies mitochondrial cytochrome *c* release, and activation of caspase-9 and -3. It remains unclear, however, whether these thialysine-mediated cellular biochemical events are prerequisites for apoptotic cell death. Recently it has been reported that antiapoptotic regulatory protein Bcl-2 and its homolog Bcl-xL can protect cells from apoptosis induced by diverse signals such as Fas ligation, ionizing radiation, hypoxia, or chemotherapeutic agents [35–39]. The antiapoptotic role of Bcl-2 and Bcl-xL is initially known to center around their prevention of caspase-3 activation through blocking cytochrome *c* release from mitochondria [40,41].

We decided to take advantage of this antiapoptotic role of Bcl-xL to examine whether the mitochondrial cytochrome *c* release and the subsequent activation of caspase cascade are crucial steps for thialysine-induced apoptosis. In this regard, we investigated the effect of ectopic overexpression of Bcl-xL on the thialysine-induced mitochondrial release of cytochrome *c*, and cytochrome *c*-mediated apoptotic events including caspase-9 activation, caspase-3 activation, PARP cleavage, and DNA fragmentation by employing Jurkat T cells transfected with Bcl-xL gene (J/Bcl-xL) and Jurkat T cells transfected with vector (J/Neo). As shown in Fig. 4A, the cytotoxicity of thialysine to Jurkat T cells at concentrations ranging from 0.32 to 2.5 mM was significantly reduced by ectopic expression of Bcl-xL. Similarly, thialysine-induced apoptotic DNA

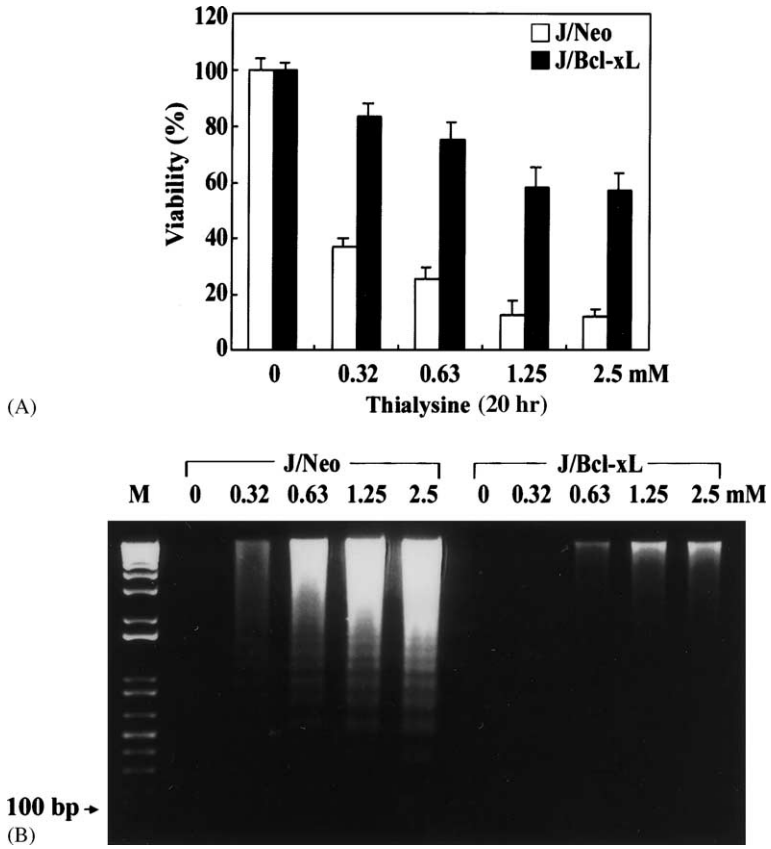

Fig. 4. Inhibitory effect of Bcl-xL on cytotoxicity (A), and apoptotic DNA fragmentation (B) induced by thialysine. Both Jurkat T cells overexpressing Bcl-xL (J/Bcl-xL) and control cells (J/Neo) were incubated at a density of 5×10^4 per well with various concentrations of thialysine in 96-well plates. After incubation for 16 hr, MTT was added for an additional 4 hr. The cells were processed to assess the cell viability. Equivalent cultures were prepared and the cells were collected to analyze apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis.

fragmentation also declined, indicating that the protection effect of Bcl-xL on the cytotoxicity of thialysine is mainly due to its inhibition of apoptotic DNA fragmentation (Fig. 4B). The ectopic expression of Bcl-xL in Jurkat T cells was confirmed by Western blot analysis (Fig. 5A). When the effect of ectopic expression of Bcl-xL on the thialysine-mediated mitochondrial cytochrome *c* release into cytosol was investigated by Western blot analysis, cytochrome *c* release inducible in the presence of 0.63–2.5 mM thialysine appeared to be markedly reduced by Bcl-xL (Fig. 5B). Under these conditions, thialysine-mediated capase-9 activation, caspase-3 activation, and the degradation of PARP declined to an undetectable or barely detectable level (Fig. 5C–E). These results indicate that the mitochondrial cytochrome *c* release with subsequent activation of the caspase cascade are negatively regulated by Bcl-xL, and are thus required for thialysine-induced apoptotic DNA fragmentation. The cytotoxicity of thialysine to Jurkat T cells at concentrations ranging from 0.32 to 2.5 mM was effectively blocked by a broad-spectrum caspase inhibitor (z-VAD-fmk), confirming a role of the caspase cascade in the death (data not shown). To better understand that thialysine-mediated activation of caspase-9 and -3 occurs downstream of the induced mitochondrial cytochrome *c* release, we employed decylubiquinone, a mitochondrial permeability transition pore inhibitor, which is known to suppress chemical-induced mitochondrial cytochrome *c* release [42]. After Jurkat T cells were pretreated with decylubiquinone at concentrations of 25, 50 or 100 μM for 2 hr, the cells were exposed to 0.63 mM thialysine for 20 hr. As shown in Fig. 6A, the cytotoxic effect of thialysine was markedly reduced in the presence of decylubiquinone. The addition, however, of decylubiquinone up to 100 μM did not affect the viability of Jurkat T cells. Since the inhibitory effect of decylubiquinone on the cytotoxicity of thialysine reached a maximum level at a concentration of 50 μM, Jurkat T cells were treated with various concentrations of thialysine together with 50 μM decylubiquinone and analyzed to detect any changes in the level of thialysine-mediated activation of caspase-9, and -3, and the degradation of PARP. Treatment with decylubiquinone appeared to attenuate several cytochrome *c*-dependent apoptotic events such as caspase-9 activation, caspase-3 activation, and PARP degradation (Fig. 6B–D). These results demonstrate that thialysine causes mitochondrial cytochrome *c* release leading to sequential activation of caspase-9, and -3 in Jurkat T cells. Previously we have shown that the L-arginine analog,

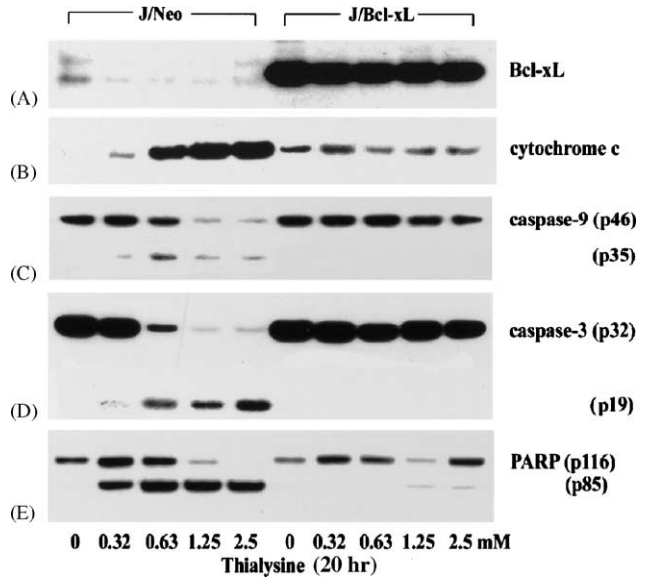

Fig. 5. Western blot analysis of ectopic overexpression of Bcl-xL (A), and its inhibitory effect on mitochondrial cytochrome *c* release (B), caspase-9 activation (C), caspase-3 activation (D), and cleavage of PARP (E) induced by thialysine. Both Jurkat T cells overexpressing Bcl-xL (J/Bcl-xL) and control cells (J/Neo) were incubated for 20 hr at a density of 4×10^5 per well with indicated concentrations of thialysine and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Section 2.

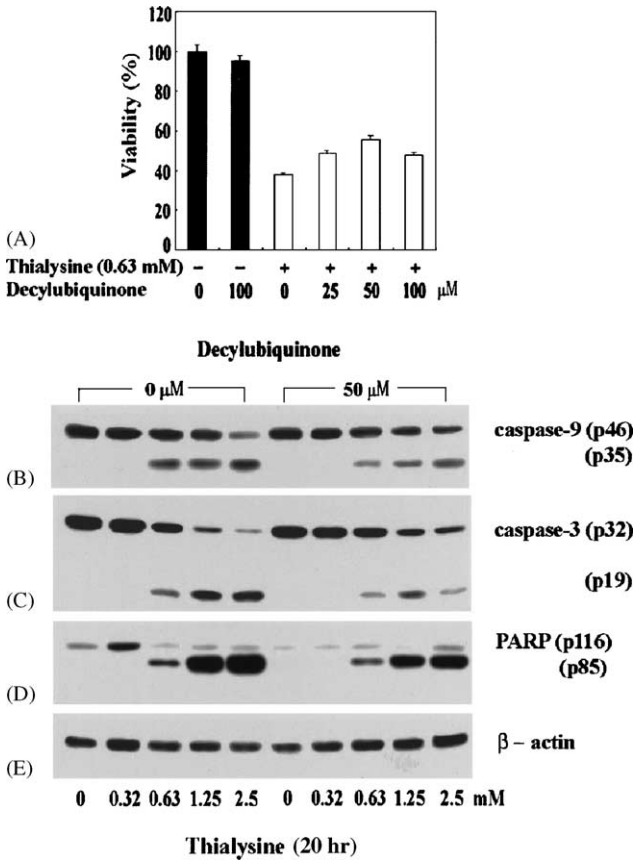

Fig. 6. Effect of decylubiquinone on cytotoxicity (A), caspase-9 activation (B), caspase-3 activation (C), and PARP cleavage (D) induced by thialysine. Jurkat T cells were incubated at a density of 5×10^4 per well with various concentrations of thialysine in the presence of 50 μM of decylubiquinone in 96-well plates. After incubation for 16 hr, MTT was added for an additional 4 hr. The cells were processed to assess the cell viability. Equivalent cultures were prepared and the cells were collected to make cell lysates. Equal amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Section 2.

L-canavanine induces apoptotic cell death of Jurkat T cells *via* mitochondrial cytochrome *c*-independent caspase-3 activation [22]. These previous results together with the present results suggest that caspase-3 activation is commonly involved in amino acid analog-mediated apoptosis. It is likely, however, that there are at least two different apoptotic pathways leading to caspase-3 activation in amino acid analog-induced apoptosis: a mitochondrial cytochrome *c*-independent pathway for L-canavanine and a mitochondrial cytochrome *c*-dependent pathway for thialysine.

3.4. Thialysine-induced alteration of cell cycle distribution

Although the ectopic expression of Bcl-xL protects cells from thialysine-induced apoptotic DNA fragmentation by blocking the mitochondrial cytochrome *c* release with resultant activation of the caspase cascade, cell viability measured by MTT assay still appears to decline to the level of 60% in the range of 1.25–2.5 mM (Fig. 4A). Since these results raised the possibility that cytotoxic effect of thialysine on Jurkat T cells is not exerted only by inducing apoptotic DNA fragmentation but also by causing growth arrest, we decided to investigate whether the treatment of thialysine leads to a change in the cell cycle progression of Jurkat T cells. Both J/Bcl-xL and J/Neo cells were analyzed by Flow cytometry after treatment of various concentrations of thialysine for 20 hr. When J/Bcl-xL cells were treated with thialysine at concentrations of 0.32–2.5 mM for 20 hr, there was a barely detectable increase in the level of sub-G1 peak representing apoptotic cells, confirming the protective effect of Bcl-xL on thialysine-induced apoptotic DNA fragmentation (Fig. 7A). As compared to the continuously growing J/Bcl-xL cells, the cells treated with 0.32 mM thialysine showed an increase in the level of both S and G2/M cells in proportion to the reduction in the level of G1 cells. However, only G2/M cells appeared to increase at concentrations of 0.63–2.5 mM thialysine. Under the same conditions, J/Neo cells showed that there was a significant enhancement in the level of sub-G1 cells in a dose-dependent manner, in accordance with the results from apoptotic DNA fragmentation analysis (Fig. 7B). Comparison of the thialysine-induced alterations in the cell cycle distribution between J/Bcl-xL and J/Neo cells suggested that the apoptotic cells might be mainly derived from the cells accumulated in the

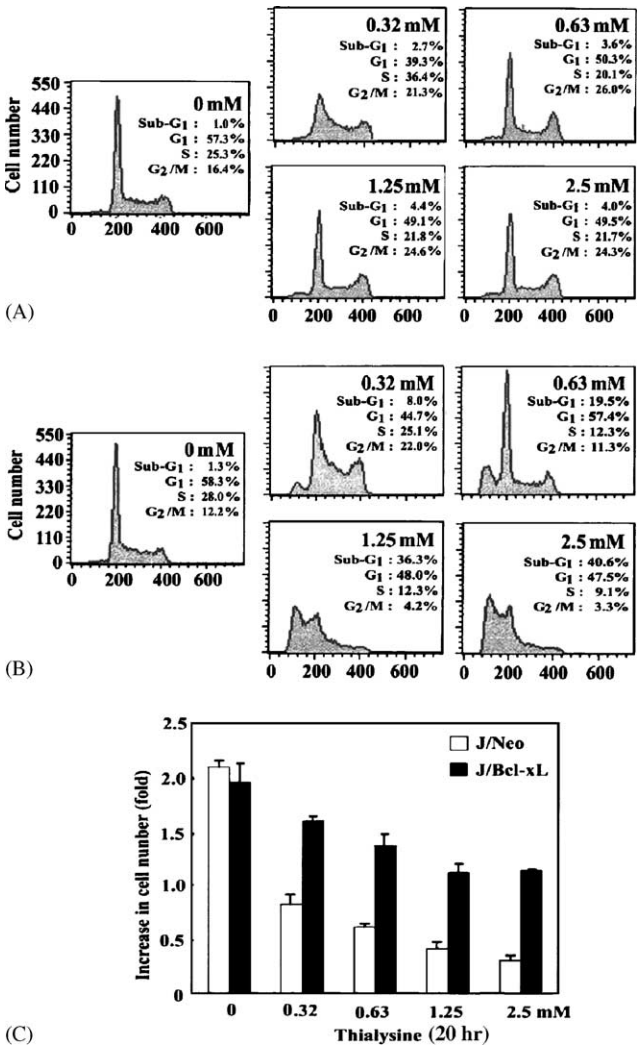

Fig. 7. Flow cytometric analysis of the cell cycle distribution in Jurkat T cells transfected with Bcl-xL gene (J/Bcl-xL) (A) and Jurkat T cells transfected with vector (J/Neo) (B) after treatment with various concentrations of thialysine for 20 hr, and viable cell number of equivalent cultures (C). After Jurkat T cells were incubated in the presence of thialysine under individual conditions, the cells were harvested. The analysis of cell cycle distribution was performed on an equal number of cells (2×10^4) by flow cytometry after the staining of DNA by propidium iodide. The viable cell number was also counted after staining of the cells by trypan blue.

S and G2/M phase following thialysine treatment. Under these conditions, the number of viable cells for J/Bcl-xL or J/Neo were also measured after staining with trypan blue. As shown in Fig. 7C, the initial cell number (0.5×10^6 mL^{-1}) of J/Bcl-xL increased to 1.02×10^6 mL^{-1} in the absence of thialysine, indicating that the cells were able to complete one round of the cell cycle within 20 hr and thus the cell number increased by 2.0-fold. The initial cell number of J/Bcl-xL in the presence of 0.32, 0.63, 1.25, 2.5 nM thialysine was enhanced by 1.6, 1.4, 1.1, and 1.1-fold, respectively. Since the apoptotic DNA fragmentation accompanying the mitochondrial cytochrome *c*-dependent activation of the caspase cascade was barely detectable in J/Bcl-xL cells following thialysine treatment, these results confirm that thialysine causes an interruption in the cell cycle progression of J/Bcl-xL cells. On the other hand, although the initial cell number of J/Neo was increased by 2.1-fold without thialysine, it was significantly reduced in the presence of thialysine in a dose-dependent manner, and only 84–31% of the initial cell numbers remained alive at concentrations of 0.32–2.5 mM thialysine. These results support our prediction that the majority of J/Neo cells, which failed to complete the S and G2/M phase in the presence of thialysine, may undergo apoptotic cell death. In order to understand the mechanism underlying the thialysine-mediated interruption of the cell cycle, the effect of thialysine on the protein levels of retinoblastoma (Rb), cyclin-dependent protein kinases (cdks), and cyclins was investigated by Western blot analysis. As shown in Fig. 8, the proteins specific for Rb, cdk4, cdk6, cdk2, cdc2, cyclin A, cyclin B1, and cyclin E were easily detectable in Jurkat T cells, and these protein levels except cdk2, whose protein level remains unchanged in the presence of thialysine, significantly and concomitantly declined in the presence of thialysine in a dose-dependent manner. Since the progression of each phase of the mammalian cell cycle is governed to a large extent by the sequential activation and inactivation of a series of cdks

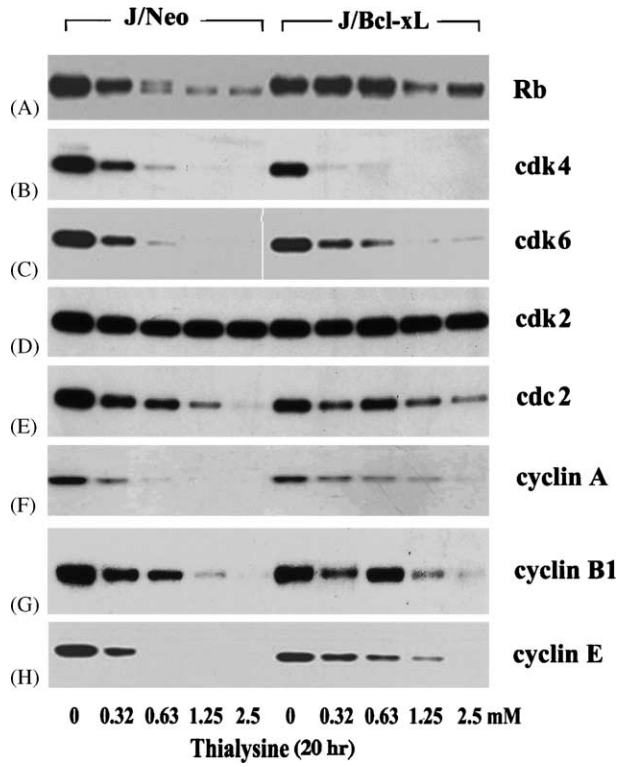

Fig. 8. Western blot analysis of the protein levels of Rb (A), cdk4 (B), cdk6 (C), cdk2 (D), cdc2 (E), cyclin A (F), cyclin B1 (G), and cyclin E (H) in Jurkat T cells overexpressing J/Bcl-xL gene (J/Bcl-xL) or control cells (J/Neo). Both Jurkat T cells overexpressing Bcl-xL (J/Bcl-xL) and control cells (J/Neo) were incubated for 20 hr at a density of 4×10^5 per well with indicated concentrations of thialysine and prepared for the cell lysates. Equivalent amounts of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Section 2.

[43,44], these results suggest that the down-regulation of cdks as well as cyclins by thialysine leads to the perturbation and subsequent inappropriate regulation of the cell cycle progression. The loss of catalytic activity of cdks due to the down-regulation of both cdks and cyclins in the presence of thialysine was further evidenced by our finding that the level of hyperphosphorylated Rb declined in the presence of thialysine. Recently, it has been reported that L-canavanine is able to arrest human lung adenocarcinoma A549 cells in the G1 phase by inducing upregulation of the negative cell cycle regulators p53 and $p21^{WAF1}$ accompanying the failure of phosphorylation of Rb protein [45]. However, our present results apparently demonstrate that thialysine increases the number of cells in the S and G2/M phases rather than the cells in the G1 phase, when thialysine-induced apoptosis is blocked by an ectopic overexpression of Bcl-xL. At the same time, there was no detectable increase in the level of negative cell cycle regulatory proteins such as p53, $p21^{WAF1}$, and $p27^{Kip1}$ in Jurkat T cells following thialysine-treatment (data not shown). Previous results and our results suggest that the mechanism underlying the inhibitory effect of amino acid analogs on cell cycle progression may vary depending on the types of cells and amino acid analogs.

Taken together, these results demonstrate that the inhibitory activity of thialysine toward Jurkat T cells is attributable to not only apoptotic cell death mediated by a mitochondria-dependent death signaling pathway but also interruption of cell cycle progression by a massive down-regulation in the level of cdks and cyclins. In addition, the results indicate that a thialysine-induced commitment of apoptosis is negatively regulated by Bcl-xL through its protective roles against the mitochondrial cytochrome *c* release with a resultant activation of the caspase cascade. These findings will be useful for evaluating the potency of amino acid analogs including thialysine as the chemotherapeutic agent for cancer.

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