Introduction

L-Canavanine is a naturally occurring L-arginine analog found in many leguminous plants, which appears to play an important role in plants as a mechanism of chemical defense against a variety of organisms [18]. Previous studies have shown that L-canavanine is toxic against microorganisms including viruses [17], bacteria, and yeasts [22], and causes growth retardation of plants as well as animal cells [6,13,21,26,30]. Since structural similarity of L-canavanine to L-arginine is remarkable enough for L-canavanine to compete with L-arginine for arginyl tRNA activation, a primary mechanism underlying these toxic effects is thought to be incorporation of L-canavanine into cellular proteins in substitution for L-arginine and subsequent induction of the formation of structurally aberrant proteins with impaired function or degradation [19,20]. This concept has been supported by the data showing that the toxic effects of L-canavanine can be reversed by the addition of L-arginine.

Chemotherapy employing antineoplastic drugs often relies on the difference in the mitotic rate between tumor and normal cells in order to confine its toxic effect to the tumor. In this context, L-canavanine has been considered to possess the potency as a chemotherapeutic agent because its incorporation into cellular proteins, which result in an inhibitory effect on cell growth, can be more significant in tumor cells than in normal cells. Previously, several studies have proposed the possible application of L-canavanine to pharmacological treatment of malignant conditions including tumors. For instance, L-canavanine possesses growth retardation activity toward tumor cells in culture and ex-
peripheral tumors in vivo [6,26,30]. Synergic antitumor effects upon combination of L-canavanine with 5-fluorouracil or γ-irradiation have also been reported, indicating that L-canavanine may modulate the chemosensitivity or radiosensitivity of tumors [5,25]. L-Canavanine has been shown to arrest human lung adenocarcinoma A549 cells in the G1 phase by inducing upregulation of the negative cell cycle regulators p53 and p21WAF1, which is accompanied by the failure of phosphorylation of retinoblastoma (Rb) protein [3]. Since L-canavanine can be hydrolyzed by arginase to urea and canaline that inactivates vitamin B6-containing enzymes, it has been suggested that the conversion of L-canavanine to its toxic metabolite canaline may also be associated with inhibition of tumor cell growth in the presence of L-canavanine [24].

In a previous study, we demonstrated an involvement of apoptotic cell death in the inhibitory activity of L-canavanine against human acute leukemia Jurkat T cells [8]. The L-canavanine-induced apoptosis appeared to be accompanied by activation of procaspase-3, which could be interrupted by the overexpression of Bcl-2 or Bcl-xL, suggesting that L-canavanine caused apoptotic cell death of Jurkat T cells by triggering a conserved caspase cascade, leading to caspase-3 activation and PARP degradation, which could be regulated by Bcl-2 or Bcl-xL. However, the precise mechanism underlying the apoptogenic effect of L-canavanine on tumor cells, which is requisite for evaluating its potency as a chemotherapeutic agent, still remains largely unknown.

In order to understand further L-canavanine-induced apoptotic mechanism of tumor cells, in the present study, we have investigated L-canavanine-induced apoptotic signaling pathway including activation of multiple caspases, and its modulation by protein tyrosine kinase p56k using Jurkat T cells, the p56k-deficient Jurkat clone JCaM1.6, and p56k-stable transfectant JCaM1.6/1ck. The results demonstrate that L-canavanine induces apoptotic cell death of Jurkat cells through provoking mitochondrial membrane potential (Δψm) reduction, caspase-9 activation, and subsequent activation of caspase-3, and -7, leading to degradation of poly (ADP-ribose) polymerase (PARP), which can be significantly suppressed by p56k.

Materials and Methods

Reagents, antibodies, and cells
L-Canavanine and 3,3′-dihexyloxycarbocyanine iodide (DiOC6) were purchased from Sigma Chemical (St. Louis, MO, USA). The ECL Western blotting kit was purchased from Amersham (Arlington Heights, IL, USA), and Immobilon-P membrane was obtained from Millipore Corporation (Bedford, MA, USA). Anti-caspase-9, anti-caspase-8, and anti-caspase-7 were purchased from Cell Signaling (Beverly, MA, USA). Anti-PARP, anti-Bid, and anti-β-actin were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Monoclonal anti-caspase-3, anti-Fas and anti-FasL antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). The broad-range caspase inhibitor z-VAD-fmk, the caspase-9 inhibitor z-LEHD-fmk, and the caspase-3 inhibitor z-DEVD-fmk were obtained from BD Sciences (Chicago, IL, USA), and the caspase-12 inhibitor z-ATAD-fmk and the caspase-4 inhibitor z-LEVAD-fmk were obtained from Biovision (Mountain View, CA, USA). Human Jurkat T cell clone E6.1, Jurkat T cells clone JCaM1.6, Jurkat T cell clone A3, and FADD-deficient Jurkat T cell clone I2.1, and caspase-8-deficient Jurkat T cell clone I9.2 were purchased from ATCC (Manassas, VA, USA). Jurkat T cells were maintained in RPMI 1640 containing 10% FBS, 20 mM HEPES (pH 7.2), 5×10−5 M β-mercaptoethanol, and 100 μg/ml gentamycin. For the culture of both JT/Bcl-2 and JT/Neo cells, G418 was added to the RPMI1640 medium at a concentration of 300 μg/ml.

Cytotoxicity assay

The cytotoxic effect of L-canavanine on Jurkat T cells was analyzed by MTT assay. For MTT assay, Jurkat T cells (5×10⁵) were added to the serial dilution of L-canavanine in 96-well plates. At 32 hr after incubation, 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. OD values of the solutions were measured at 540 nm by a plate reader.

DNA fragmentation analysis

Apoptotic DNA fragmentation induced in Jurkat T cells following nocodazole treatment was determined by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis as previously described [9].

Flow cytometric analysis

Cell cycle distribution of Jurkat T cells following exposure
to L-canavanine was analyzed by flow cytometry as described elsewhere [10]. After fixation with 67% ethanol at 4°C for 1 hr, the cells (1×10^6) were washed with PBS, and treated with RNase (50 μg/ml) at 37°C for 30 min before staining of the cellular DNA with propidium iodide (50 μg/ml) for 20 min. The stained cells were analyzed on a FACScan flow cytometer for relative DNA content, based on increased red fluorescence.

Measurement of mitochondrial membrane potential disruption
Changes in the mitochondrial membrane potential (Δψm) following treatment with L-canavanine was measured after staining with 3,3′-dihexylxocarbocyanine iodide (DiOCl) [31]. After treatment with L-canavanine, the cells were harvested and incubated with PBS containing 50 nM DiOCl for 1 min at 37°C. Analysis was done with flow cytometry.

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 MgCl2, 25 mM MOPS, 1 mM phenylmethylsulfonyl fluoride, 2.5 μg/ml proteinase inhibitor E-64, 0.1% Triton X-100, pH 7.2). The cells were disrupted by sonication and extracted at 4°C for 30 min. An equivalent amount of protein lysate (20 μg) was electrophoresed on 4–12% NuPAGE gradient gel (Invitrogen Corporation/Novex, Carlsbad, CA, USA) with 3-(N-morpholino)propanesulfonic acid (MOPS) buffer and then electrotransferred to Immobilon-P membranes. Detection of each protein was performed using an ECL Western blotting kit according to the manufacturer’s instructions.

Immunoprecipitation and kinase assay
For immunoprecipitation of p56k, 70 μg of cellular lysate at 1 μg/ml were allowed to react with 2 μg of rabbit polyclonal anti-p56k antibody at 4°C for 2 hr. The immune complexes were recovered by addition of 30 μl of protein G-agarose, and incubation was continued, with rotation, at 4°C for 1 hr. The protein G-agarose was collected by centrifugation (4,000 rpm) for 3 min and washed three times with the lysis buffer. The α-casein kinase activity of immunoprecipitated p56k was assayed as previously described [1,12]. Phosphorylation of α-casein was measured by incubating the protein G-agarose beads with 30 μl of kinase assay cocktail (15 μg of α-casein, 10 μCi [γ-32P]ATP, 100 μM ATP, 1X kinase assay buffer) for 30 min at 30°C. The reaction was stopped by boiling the mixture in 1X SDS sample buffer for 5 min, and the reaction mixture was resolved on an 11% SDS-polyacrylamide gel electrophoresis and the proteins were electrotransferred to an Immobilon-P membrane. The membrane was blotted-dried, and phosphorylation of the casein was detected by autoradiography and quantitated by a phospho-image analyzer. For Western analysis of immunoprecipitated p56k, the membrane was probed with monoclonal anti-p56k antibody and detection was performed using an ECL Western blotting kit.

Statistical analysis
Unless otherwise indicated, each result in this paper is representative of at least three separate experiments. Values represent the mean ± standard deviation (SD) of these experiments. The statistical significance was calculated with Student’s t-test. P values less than 0.05 were considered significant.

Results and Discussion
Apototic effect of L-canavanine on Jurkat T cell clone E6.1 and JCaM1.6
The protein tyrosine kinase p56k is a well-known non-receptor TPK of src-family and is expressed almost exclusively in T cells [1,12]. To understand an involvement of protein tyrosine kinase p56k in L-canavanine-induced apoptotic cell death, the cytotoxic effect of L-canavanine on p56k-positive Jurkat clone E6.1 and p56k-deficient Jurkat clone JCaM1.6 was compared by MTT assay. When the cells were treated with L-canavanine at various concentrations of 1.25–2.5 mM for 36 hr, the decline in cell viability, which occurred in a dose-dependent manner, appeared to be more significant in JCaM1.6 cells than in E6.1 cells (Fig. 1A). Under the same conditions, both apoptotic DNA fragmentation and sub-G1 cells representing apoptotic cells were also enhanced dose-dependently, and these enhancements were more apparent in JCaM1.6 cells than in E6.1 cells (Fig. 1B and IC). These results indicated that the higher susceptibility of p56k-deficient JCaM1.6 cells, as compared with p56k-positive E6.1 cells, toward the cytotoxicity of L-canavanine was attributable to induced apoptosis that could be augmented by the lack of p56k.
Effect of ectopic expression of p56<sup>lk</sup> on hypersensitivity of JCaM1.6 cells toward apoptotic activity of L-canavanine

To further elucidate the involvement of the p56<sup>lk</sup> in L-canavanine-induced apoptosis of Jurkat T cells as a negative modulator, the effect of ectopic expression of p56<sup>lk</sup> on the hypersensitivity of JCaM1.6 cells to the apoptotic activity of L-canavanine was investigated. When the induced apoptotic sub-G<sub>1</sub> peak following treatment with L-canavanine (1.25~2.5 mM) for 38 h was compared between p56<sup>lk</sup>-stable transfectant JCaM1.6/lck cells and p56<sup>lk</sup>-deficient JCaM1.6/vector cells by flow cytometry, the sub-G<sub>1</sub> peak was more obvious in JCaM1.6/vector than in JCaM1.6/lck (Fig. 2A). In order to examine whether mitochondrial membrane potential (Δψm) loss was accompanied by the apoptogenic activity of L-canavanine as well as its modulation by the presence of p56<sup>lk</sup>, JCaM1.6/lck and JCaM1.6/vector, which were treated with 1.25~2.5 mM L-canavanine for 38 hr, were analyzed by 3,3'-dihexyloxacarbocyanine iodide (DiOC<sub>5</sub>) staining. As shown in Fig. 2B, L-canavanine-induced loss of Δψm was more significant in JCaM1.6/vector cells than in JCaM1.6/lck cells, indicating that the treatment with L-canavanine resulted in a decline in the mitochondrial membrane potential in Jurkat T cells, which could be more significant in the absence of p56<sup>lk</sup>. In addition, when the L-canavanine-induced activation of multiple caspases were compared between JCaM1.6/lck cells and JCaM1.6/vector cells by Western blot analysis, the L-canavanine-induced activation of caspase-9, -8, -7, and -3, and cleavage of PARP and PLCγ-1 were more dominantly detected in the absence of p56<sup>lk</sup> (Fig. 3). Previously, upregulation of Fas ligand
Kinetic analysis of p56\textsuperscript{ck} kinase activity following L-canavanine treatment of JCaM1.6/Ick cells

Since current data showed that the absence of p56\textsuperscript{ck} caused sensitization to L-canavanine-induced apoptotic cell death of Jurkat T cells, we tested whether the kinase activity of p56\textsuperscript{ck} was altered following treatment with L-canavanine. When the p56\textsuperscript{ck}-stable transfectant JCaM1.6/Ick cells were treated with 2.5 mM L-canavanine for various time periods, the α-casein kinase activity of p56\textsuperscript{ck} appeared to be enhanced by approximately 1.4~1.6-fold at 15~30 min, and then sustained ~1.3-fold enhanced level until 6 h after exposure to 2.5 mM L-canavanine (Fig. 4A). Under the same conditions, there was no significant change in the level of p56\textsuperscript{ck} protein. While a mobility shift from p56\textsuperscript{ck} to p60\textsuperscript{ck}, which was previously known to reflect a rapid and significant activation of p56\textsuperscript{ck} [29], was not detected upon the L-canavanine-induced elevation in the casein kinase activity of p56\textsuperscript{ck}, there was no detectable p56\textsuperscript{ck} in JCaM1.6 cells (Fig. 4B). These results demonstrated that the kinase activity of p56\textsuperscript{ck} was

(FasL) and/or Fas expression has been implicated as a potential mechanism in the apoptotic cell death induced by antineoplastic drugs [4,7,15,16]. However, there was no alteration the levels of Fas and Fas ligand (FasL) following exposure to 1.25~2.5 mM L-canavanine in both cells, suggesting that Fas-death signaling might not be associated with L-canavanine-induced apoptosis. It is noteworthy that Western blot analysis confirmed that the p56\textsuperscript{ck}-stable transfectant JCaM1.6/Ick cells, but not the p56\textsuperscript{ck}-deficient JCaM1.6/vector cells, could express the p56\textsuperscript{ck} protein. These results demonstrated that although the p56\textsuperscript{ck} was not a prerequisite for L-canavanine-induced apoptosis, it could contribute to suppress the L-canavanine-induced apoptosis by reducing the loss of ΔΨm and the activation of caspase-9, -8, -7, and -3.
enhanced following treatment with L-canavanine in Jurkat T cells, supporting that the p56 
kinase was involved in the L-canavanine-induced apoptotic signaling pathway as a negative modulator.

Comparison of cytotoxic effect of L-canavanine on wild-type Jurkat T cell clone A3, FADD-deficient Jurkat T cell clone I2.1, and caspase-8-deficient Jurkat T cell clone I9.2

Although the expression levels of Fas and FasL in Jurkat T cells were not changed after treatment with 1.25~2.5 mM L-canavanine, we compared the cytotoxic effect of 2.5 mM L-canavanine on FADD- and caspase-8-positive wild-type Jurkat T cells (clone A3) with those on FADD-deficient Jurkat T cells (clone I2.1) and caspase-8-deficient Jurkat T cell (clone I9.2), both of which were previously refractory to Fas-mediated apoptosis [11], in order to further examine an involvement of Fas/FasL system in L-canavanine-induced apoptosis. As shown in Fig. 5, irrespective of the FADD deficiency, both Jurkat clones A3 and I2.1 showed similar sensitivity to the cytotoxicity of 2.5 mM L-canavanine, whereas the caspase-8-deficient Jurkat clone I9.2 appeared to be slightly less sensitive to the cytotoxicity of L-canavanine, as compared with A3 and I2.1. These results confirmed that the L-canavanine-induced apoptosis of Jurkat T cells was not provoked by the interaction of Fas with FasL. These results also suggested that although the caspase-8 was activated, it was not a prerequisite for the L-canavanine-induced apoptotic cell death in Jurkat T cells.

Effect of pan-caspase inhibitor z-VAD-fmk, caspase-9 inhibitor z-LEHD-fmk, caspase-3 inhibitor z-DEVD-fmk, caspase-4 inhibitor z-LEVD-fmk, or caspase-12 inhibitor z-ATAD-fmk on L-canavanine-induced death signaling in Jurkat T cell clone E6.1

To elucidate further the death signaling pathway for L-canavanine-induced apoptosis, we examined the effect of the pan-caspase inhibitor (z-VAD-fmk), which is known to inhibit broad-range caspses [23] on L-canavanine-induced apoptotic events in Jurkat T cell clone E6.1. After the cells were pretreated with z-VAD-fmk at a concentration of 30 mM for 1 h, the cells were exposed to 2.5 mM L-canavanine for 38 h. Although there was a barely detectable apoptotic sub-G1 peak in continuously growing Jurkat T cells, it was elevated to the level of 35.1% in the presence of 2.5 mM L-canavanine for 40 h (Fig. 6A). The sub-G1 peak induced by L-canavanine was completely diminished by pretreatment with 30 mM z-VAD-fmk, as determined by flow cytometry analysis. At the same time, Western blot analysis revealed that the L-canavanine-induced apoptotic events such as the activation of caspase-8, -7, and -3, Bid cleavage, and degradation of PARP and PLC7-1 were completely abrogated by the pretreatment with z-VAD-fmk, whereas L-canavanine-induced caspase-9 activation appeared to be sustained. These results excluded the possible involvement of caspase-8 activation as an initial signal provoking the mitochondrial membrane potential loss as well as the activation of caspase cascade in L-canavanine-induced apoptosis.

On the other hand, pretreatment of the cells with individual caspase inhibitors such as 30 μM caspase-9 inhibitor (z-LEHD-fmk), 30 μM caspase-3 inhibitor (z-DEVD-fmk), and 4 μM caspase-12 inhibitor (z-ATAD-fmk) appeared to partially reduce the L-canavanine-induced sub-G1 peak to the level of 18.8%, 16.6%, and 18.2%, respectively. However, L-canavanine-induced apoptotic sub-G1 peak was not affected by pretreatment with 4 μM caspase-4 inhibitor (z-LEVD-fmk). Under these conditions, the presence of z-LEHD-fmk, z-DEVD-fmk, or z-ATAD-fmk caused not only a complete prevention of L-canavanine-induced generation of 43/41 kDa active forms of caspase-8 and 17 kDa active caspase-3, but also a significant reduction in the level of degradation of PARP. At the same time, 37/35 kDa active forms
of caspase-9 as well as 20 kDa active caspase-7 was detected at the similar level to that of the L-canavanine- treated control cells, and 19 kDa active caspase-3, which was not detected in the L-canavanine- treated control cells, was detected. Recently, it has been reported that the proteolytic cleavage of procaspase-9 (47 kDa) within the apoptosome yields 35/12 kDa active caspase-9 in order to cleave procaspase-3 (32 kDa) into active caspase-3 (19 kDa), and subsequent feedback cleavage of procaspase-9 by 19 kDa active caspase-3 generates 37/10 kDa active caspase-9, which can cleave not only 19 kDa active caspase-3 into 17 kDa active caspase-3 but also 35 kDa procaspase-7 into 20 kDa active caspase-7 [28,32]. These previous and current results demonstrated that the activation of caspase-9 and -3, which was a prerequisite for L-canavanine- induced apoptosis, was upstream of the activation of caspase-7 and -8. However, there was no suppressive effect on the activation of caspase-9, -3, -7, and -8, and degradation of PARP in the presence of caspase-4 inhibitor (z-LEVD-fmk), which failed to suppress L-canavanine-induced sub-G1 peak. In particular, both 17 kDa active form and much smaller amount of 19 kDa active form of caspase-3 were concurrently produced in the presence of z-LEVD-fmk. Since human caspase-4 and -5, possessing a CARD pro-domain at the N-terminal, like human caspase-12, show a high similarity to mouse caspase-12, they have been proposed to play roles in the ER stress-mediated apoptosis of human cells [14]. However, current results suggested that although L-canavanine was previously reported to induce unfolded protein response (UPR) by accumulating structurally aberrant proteins in the ER, and thus over-expression of ubiquitin was able to confer tolerance to the cytotoxicity of L-canavanine [22,27], ER stress-mediated activation of caspase-12 rather than caspase-4 appeared to be involved in L-canavanine-induced apoptotic signaling in order to support the proper activation of caspase-3 mediated by caspase-9. Consequently, current results indicated that the L-canavanine-induced apoptotic signaling pathway was mediated by activation of caspase-9 and -3, where ER stress-mediated caspase-12 activation might be required for its proper progression, leading to the activation of caspase-7 and caspase-8.

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References


초록 : 인체 급성백혈병 Jurkat T 세포에 있어서 L-canavanine에 의해 유도되는 세포자살기전에 미치는 단백질 토신 카니아제 p56^{Lck}의 저해 효과

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L-arginine 구조유사체인 L-canavanine의 인체 급성백혈병 Jurkat T 세포에 대한 apoptosis 유도성이 단백질 토신 카니아제 p56^{Lck}에 어떻게 조절되는지를 규명하기 위해 p56^{Lck}를 발현하는 Jurkat T 세포주 E6.1과 p56^{Lck}-결손 Jurkat T 세포주 CaM1.6이 있어서 L-canavanine의 세포독성을, L-canavanine에 의해 유도되는 apoptotic DNA fragmentation 및 apoptotic sub-G_0 peak 를 비교하여 본 바, p56^{Lck}-negative CaM1.6 세포가 p56^{Lck}-positive E6.1 세포에 비해 L-canavanine의 apoptotics 유도활성에 훨씬 더 민감한 것으로 나타나고, 이러한 p56^{Lck}-negative CaM1.6 세포의 민감성을 CaM1.6 세포에 p56^{Lck} 유전자를 transfection하여 발현시켜 현저히 감소되었다. L-Canavanine에 의해 유도되는 apoptosis현상 현상을 p56^{Lck}-stable transfectant인 CaM1.6/Ick 세포와 empty vector-transfectant인 p56^{Lck}-negative CaM1.6/vector 세포에서 Western blot analysis로 비교한 결과, L-canavanine에 의해 유도되는 mitochondrial membrane potential (Δψm)의 감소, caspase-9, -8, -7 및 -3의 활성화, 그리고 PARP 및 PLOD의 발현이 CaM1.6/vector 세포에서, 더 약하게 나타나였고, CaM1.6/Ick 세포에서 2.5 mM L-canavanine으로 처리한 후 15분간의 p56^{Lck} kinase 활성화의 변화를 α-casein을 기준으로 하여 시각적으로 측정한 결과, L-canavanine의 처리 후 15분간에 p56^{Lck} kinase의 활성화가 약 2.5배 증가된 수준으로 kinase 활성화가 유지되는 것으로 확인되었다. L-Canavanine에 의한 apoptosis의 개시에 Fas/FasL 상호작용이 관련된다는 것을 규명하기 위해 FADD-negative Jurkat T 세포주 I21, caspase-8-negative Jurkat T 세포주 I92 및 wild-type Jurkat T 세포주 A3에 L-canavanine의 세포독성을 비교한 결과, A3와 I21 세포의 경우에 L-canavanine의 세포독성이 동일하게 나타났고, 특히 caspase-8가 결손된 I92 세포의 경우는 L-canavanine의 세포독성에 대한 민감성이 I3와 I21 세포에 비해 낮게 나타나지만 변화되는 것으로 나타나, L-canavanine에 의한 apoptosis에 대한 Fas/FasL 상호작용이 관련되지 않으며, 또한 caspase-8의 역할이 필수적이지 않음을 시사하였다. Jurkat T 세포에 있어서 L-canavanine에 의해 유도되는 sub-G_0 peak 및 caspases 활성화에 미치는 pan-caspase inhibitor (z-VAD-fmk), caspase-9 inhibitor (z-LEHD-fmk), caspase-3 inhibitor (z-DEVD-fmk), caspase-4 inhibitor (z-LEHD-fmk) 및 caspase-12 inhibitor (z-ATAD-fmk)의 영향을 조사한 결과, L-canavanine에 의한 apoptosis는 Δψm의 감소, caspase-9 및 caspase-3의 활성화에 따른 caspase-8 및 caspase-7의 활성화, 그리고 PARP의 발현의 순서로 유도되는 것으로 나타났으며, 아울러 caspase-9의 활성화와 함께 caspase-12의 활성화가 L-canavanine 처리에 따른 caspase-3의 활성화에 요구되는 것으로 확인되었다. 결론적으로, L-canavanine 처리에 의한 Jurkat T 세포의 apoptosis는 Δψm 감소, caspase-9, caspase-3 및 caspase-7의 활성화에 의해 유도되며, 이들 apoptosis 현상들은 p56^{Lck}에 의해 negative regulation 되었다.