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Enhanced Expression of Fas Ligand Is Associated with Aburatubolactam C-Induced Apoptosis in Human Jurkat T Cells

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The mechanism for apoptosis induced by aburatubolactam C was investigated in human Jurkat T cells. When the cells were treated with 3 μg/ml of aburatubolactam C, apoptotic DNA fragmentation was first detectable in 3 hr and then increased time-dependently in accordance with upregulation in the protein level of Fas ligand (FasL). Both the DNA fragmentation and upregulation of FasL expression reached a maximal level in 7-8 hr, at which time a significant increase in the tyrosine phosphorylation of multiple cellular proteins was detected, suggesting that the enhanced tyrosine phosphorylation of cellular proteins may result from activation of Fas-mediated death signaling. However, these aburatubolactam C-induced cellular changes and accompanied apoptosis were completely blocked in the presence of genistein, a known protein tyrosine kinase inhibitor. These results indicate that upregulation of FasL expression dictated by protein tyrosine kinase activation and subsequent mediation of Fas death signaling account for aburatubolactam C-induced apoptosis in Jurkat T cells.

Apoptosis, a programmed cell death, plays an important role in the development and homeostasis of multicellular organisms. During apoptosis, a distinctive series of morphological changes occurs, which involves cell shrinkage, loss of cell-cell adhesion, membrane blebbing, and chromatin condensation. These alterations are commonly associated with the internucleosomal DNA fragmentation resulting from activation of endogenous nucleases (1). Although cells can undergo apoptosis as a response to numerous physiological and nonphysiological signals such as TCR engagement (2), tumor necrosis factor (TNF) (3, 4), Fas ligation (5), oxidative stress (6), growth factor withdrawal (7-9), corticosteroids (10, 11), heat shock (12), irradiation (13), and chemotherapeutic agents (14), Fas-mediated apoptosis upon interaction with Fas ligand (FasL) appears to be more critically involved in elimination of cells *in vivo*. In tumors that can be generally characterized by a dysregulated cell growth, it seems likely that the apoptosis pathway is disrupted and the consequent imbalance between apoptotic and mitotic rate confers a survival advantage causing tumor progression. It has been shown that many tumor cells are capable of escaping Fas-mediated cell death by altering Fas expression (15, 16). Since chemotherapy for tumors is principally based on agents that are toxic to the cells and induction of apoptosis in tumor cells can lead to their own destruction, apoptosis has been currently 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 FasL and Fas expression with subsequent induction of apoptotic cell death through activation of Fas signaling has been implicated (17, 18). However, the biochemical mechanisms underlying the induction of FasL in tumors by chemotherapeutic agents and Fas-mediated apoptotic signaling pathway are still poorly defined.

As a majority of studies on Fas-mediated apoptosis has been focused toward T cells, it has been elucidated that Fas plays an important regulatory role for T-cell homeostasis as well as periphery tolerance and T cell-mediated cytotoxic reactions (19-24). Perturbation of Fas in Jurkat T cells by anti-Fas leads to the tyrosine phosphorylation of a number of cellular proteins and this Fas-mediated apoptotic signal is blocked by tyrosine kinase inhibitors, suggesting that one or more protein tyrosine kinases may be involved in relaying Fas death signal (25). Recently it has been reported that a

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Abbreviations used: FasL, Fas ligand; TCR, T cell receptor complex; TNF, tumor necrosis factor; FBS, fetal bovine serum; MeOH, mercaptoethanol; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; DMSO, dimethylsulfoxide; Da, dalton; kDa, kilodalton; IL-2, interleukin 2; IL-2R, interleukin 2 receptor.

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src-family protein tyrosine kinase $p56^{lck}$ and ZAP-70 tyrosine kinase are required for TCR-mediated FasL expression and thus development of activation-induced T cell apoptosis (26, 27). Subsequently it has also been reported that a src-related protein tyrosine kinase $p59^{fyn}$ is physically associated with Fas and may contribute to Fas signal transduction in activated T cells (28). These results indicate that $p56^{lck}$, $p59^{fyn}$, and ZAP-70 tyrosine kinase may be critically involved in the apoptotic death accomplished by interaction of Fas with FasL in T cells.

Aburatubolactam C that was originally isolated from a marine *Streptomyces* sp. as a novel inhibitor of superoxide anionic generation in human neutrophils (29). Recently we have shown that aburatubolactam C induces apoptotic death in human Jurkat T cells. In the present study, cellular responses underlying aburatubolactam C-induced apoptosis were investigated in Jurkat T cells not only to determine its potency as an antineoplastic agent but also to extend our understanding of mechanisms for chemotherapy-induced apoptotic cell death. The results suggest that aburatubolactam C rapidly induces apoptosis in Jurkat T cells through upregulation of FasL expression and subsequent activation of Fas death signal. Additional results indicate that the drug-induced upregulation of FasL is dictated by protein tyrosine kinase activation which can be blocked by protein tyrosine kinase inhibitor genistein.

MATERIALS AND METHODS

Reagents, antibodies, and cells. Aburatubolactam C was kindly provided by Dr. Kaoru Yamada (Sagami Chemical Research Center, Kanagawa, Japan), and genistein was purchased from Life Technologies (Gaithersburg, MD, USA). Radioactive materials including [^{3}H]-thymidine (2 Ci/mM), ^{125}I-goat anti-rabbit IgG, and ^{125}I-rabbit anti-mouse IgG were from NEN Biotechnology System (Boston, MA, USA), and [γ-^{32}P]ATP ($\approx$3,000 Ci/mM) was from Amersham (Arlington Heights, IL, USA). Anti-phosphotyrosine antibody PY-20 was purchased from ICN (Biomedicals, Inc., Costa Masa, CA, USA). Monoclonal anti-FasL and anti-Fas antibodies were purchased from Transduction Laboratories (Lexington, KY, USA). Human Jurkat T leukemia cells, human myeloid leukemia U937 cells, and murine lymphoma BW5.1.4.7.G.1.4 cells were kindly supplied from Dr. Albert A. Nordin (Gerontology Research Center, NIA/NIH, Baltimore, MD, USA), and murine lymphoid neoplasm P388D1 cells and murine fibroblast NIH3T3 cells were from American Type Culture Collection (Rockville, MD, USA). Jurkat and U937 were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD, USA) containing 10% FBS (UBI, Lake placid, NY, USA), 20 mM HEPES (pH 7.0), 5 x 10^{-5} M β-MeOH, and 100 μg/ml gentamycin. The culture medium used for BW5147.G.1.4, P388D1 and NIH3T3 was Dulbecco's Modified Eagles Medium (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FBS, 20 mM HEPES (pH 7.0), 1 mM sodium pyruvate, 5 x 10^{-5} M β-MeOH, and 100 μg/ml gentamycin.

Cytotoxicity assay. The cytotoxic effect of aburatubolactam C on Jurkat T cells was analyzed by either MTT assay reflecting the cell viability or [^{3}H]thymidine-incorporation assessing cellular replicative capacity. For MTT assay, Jurkat T cells (10^5) were added to the serial dilution of aburatubolactam C in 96-well plates. At the various times after incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 2 hr. To determine IC_{50} values, each leukemia cell line was incubated with various concentrations of aburatubolactam C for 20 hr before addition of MTT solution. 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 plate reader. The incorporation of [^{3}H]thymidine into DNA by Jurkat T cells treated by aburatubolactam C was determined by the addition of 10^5 cells to serial dilutions of aburatubolactam C in 96-well plates. At each time point after incubation, 1 μCi/well of [^{3}H]thymidine was added for 1 hr before the cells were harvested and assayed for the incorporation of [^{3}H]thymidine by liquid scintillation.

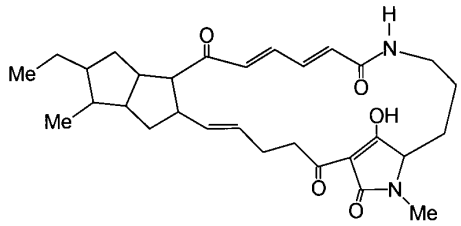

FIG. 1. Structure of aburatubolactam C.

DNA fragmentation analysis. Apoptotic DNA fragmentation induced in Jurkat T cells following the treatment by external stimulants was determined as previously described (30). Briefly, the cells were harvested by centrifugation and then treated with a lysis buffer (1% NP-40, 20 mM EDTA, 50 mM Tris-HCl, pH 7.5). After centrifugation for 5 min at 1600 x g, the supernatant is collected and brought to 1% SDS, and treated for 2 hr at 50°C with RNase A and subsequently with proteinase K for 2 hr at 37°C. The DNA fragments were precipitated with 2.5 volumes of ethanol in the presence of 5 M ammonium acetate and visualized following electrophoresis on a 2% agarose gel as described elsewhere (2).

Western blot analysis. Cellular lysates were prepared by suspending 5 x10^6 Jurkat T cells in 100 μl of lysis buffer (20 mM Tris-HCl, 137 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, 1 mM phenylmethylsulponyl fluoride, 1% NP-40, pH 8.0). The cells were disrupted by sonication and extracted at 4°C for 30 min. Equivalent amounts of cell lysates were subjected to electrophoresis on 8-16% gradient SDS polyacrylamide gels and electrotransferred to Immobilon-P membranes (Millipore Co., Bedford, MA, USA). The membranes were allowed to react with anti-phosphotyrosine PY-20, anti-FasL, anti-Fas, or anti-$p56^{lck}$ and then with ^{125}I-labeled affinity purified rabbit anti-mouse IgG or ^{125}I-labeled affinity purified goat anti-rabbit IgG. Autoradiography was obtained after exposure at -70°C.

RESULTS AND DISCUSSION

Cytotoxic activity of aburatubolactam C. Aburatubolactam C is a natural product that has been isolated from the culture broth of a marine *Streptomyces* sp. as a novel inhibitor of superoxide anionic generation in human neutrophils (29). Aburatubolactam C is a new lactam compound and the molecular formula is deduced to be $C_{30}H_{40}N_2O_5$ with molecular mass of 508 Da (Fig. 1). Aburatubolactam C is cytotoxic for various continuously proliferating tumor cell lines, and the IC_{50} values determined by MTT assay appeared to be 1.9 μg/ml for Jurkat T cells, 1.2 μg/ml for U937 cells, 0.3 μg/ml for BW5.1.4.7.G.1.4 cells, 0.3 μg/ml for P388D1 cells, and 7.9 μg/ml for NIH3T3 cells.

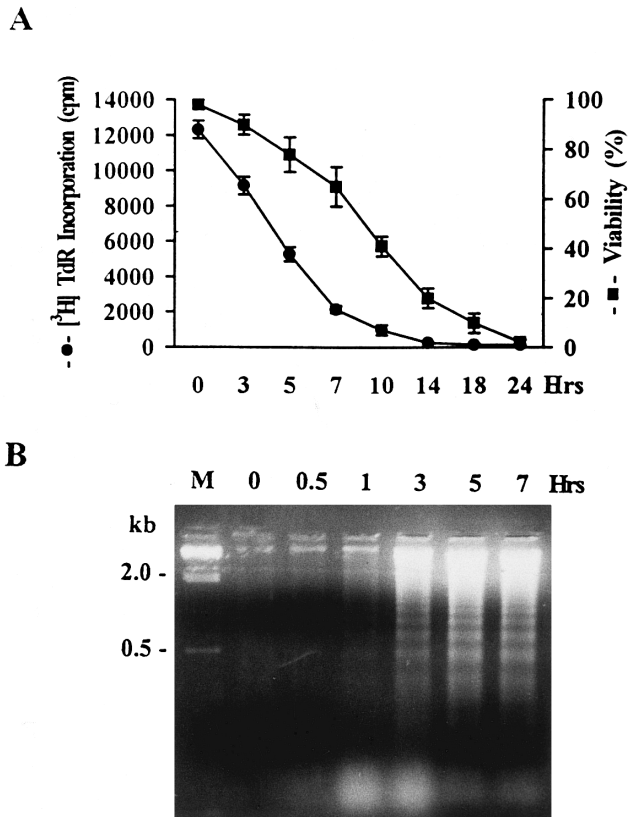

FIG. 2. Effect of aburatubolactam C on [^{3}H]thymidine-incorporation and cell viability (A), as well as apoptotic DNA fragmentation (B) in Jurkat T cells. Continuously growing Jurkat T cells (10^5) were incubated with 3 μg/ml of aburatubolactam C in 96-well plate. For the indicated time the cells were processed to assess either the incorporation of [^{3}H]-thymidine as an index of proliferation or the colored formazan crystal produced from MTT as an index of cell viability. Equivalent cultures were prepared and the cells were collected at the indicated times to analyze apoptotic DNA fragmentation by NP-40 lysis method using 2% agarose gel electrophoresis as described in Materials and Methods.

To understand the mechanisms underlying the cytotoxicity, the effect of aburatubolactam C on Jurkat T cells was investigated. When cells were treated with aburatubolactam C at a concentration of 3 μg/ml, [^{3}H]thymidine-incorporation rapidly declined and only 17% of the proliferative capacity remained at 7 hr and was completely abrogated by 14 hr. Cell viability determined by MTT assay was sustained to the level of 65% in 7 hr and declined to a minimal level in 24 hr (Fig. 2A). Under these conditions, apoptotic DNA fragmentation was detectable by 3 hr and continuously increased time-dependently (Fig. 2B). These results demonstrate that aburatubolactam C is able to rapidly inhibit the incorporation of [^{3}H]-thymidine under conditions that cell viability is maintained, and suggest that the cytotoxic effect of aburatubolactam C is attributable to the induced apoptotic DNA fragmentation.

Upregulation of FasL by aburatubolactam C. Since induction of apoptotic death in Jurkat T cells appeared to be a critical response to aburatubolactam C, it was highly likely that elucidation of the cellular mechanisms underlying aburatubolactam C-induced apoptosis is essential not only to determine its potency as an antineoplastic agent but also to understand the mechanism regulating drug-induced apoptosis. Evidence that autocrine and/or paracrine activation of Fas signaling caused by the upregulation of FasL and Fas expression as a mechanism for chemotherapeutic drug-induced apoptosis has been accumulated (31). In order to investigate whether upregulation of FasL and Fas expression is involved as an underlying mechanism that accounts for aburatubolactam C-induced apoptosis in Jurkat T cells, kinetic expression of FasL and Fas was analyzed by Western blotting. As shown in Fig. 3, both FasL and Fas protein were detected at low levels in untreated Jurkat T cells. After the cells were treated with 3 μg of aburatubolactam C, an increase in the protein level of FasL and Fas was first detected by 3 hr and increased progressively until at least 8 hr. These results indicate that apoptosis mediated by aburatubolactam C in Jurkat T cells correlates with upregulation of FasL as well as Fas expression and subsequent activation of Fas death signal.

Induction of tyrosine phosphorylation of cellular proteins by aburatubolactam C. It is generally accepted that protein tyrosine kinase activation is an early signal in Fas-induced apoptosis, because Fas ligation rapidly induces the tyrosine phosphorylation of multiple cellular proteins (25). In addition, the possible involvement of a src-family protein tyrosine kinase p59^{fyn} has been suggested in Fas-mediated apoptosis in T cells (28). Based on these previous data, we decided to examine whether an increase in the tyrosine phosphorylation of cellular proteins is accompanied with aburatubolactam C-induced apoptosis in Jurkat T cells. When the kinetic analysis of cellular proteins derived from Jurkat T cells treated with 3 μg/ml of aburatubolactam

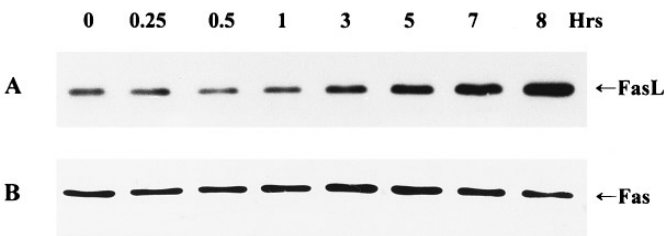

FIG. 3. Upregulation in the protein level of FasL in Jurkat T cells after aburatubolactam C treatment. The cells (6-8 x10^6) were incubated at a concentration of 1 x 10^6/ml with 3 μg/ml of aburatubolactam C for the indicated times and prepared for the cell lysates. Equivalent amounts of cell lysate (100 μg) were electrophoresed on 8-16% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P. The membrane was probed with anti-FasL (A) and anti-Fas (B), and then with ^{125}I-labeled affinity-purified rabbit anti-mouse IgG. The protein FasL and Fas were visualized by autoradiography after exposure at −70°C.

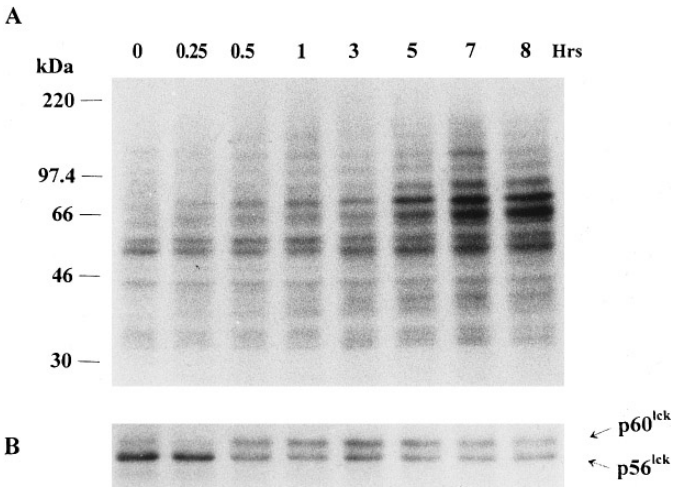

FIG. 4. Kinetic analysis of the tyrosine phosphorylation of the cellular protein (A), and electrophoretic mobility shift of $p56^{lck}$ to $p60^{lck}$ (B) in human Jurkat T cells following addition of aburatubolactam C. The cells were incubated with 3 μg/ml of aburatubolactam C for the indicated times. For each time point an equivalent amount of cell lysate (100 μg) was electrophoresed on 8-16% SDS gradient polyacrylamide gels and electrotransferred to Immobilon-P. The tyrosine-phosphorylated protein and $p56^{lck}$ were detected by probing with anti-phosphotyrosine antibody PY-20 and rabbit antiserum to $p56^{lck}$, and then with ^{125}I-labeled affinity-purified rabbit anti-mouse IgG or ^{125}I-labeled affinity-purified goat anti-mouse IgG. Autoradiography was obtained after exposure at −70°C.

C was performed by anti-phosphotyrosine immunoblotting, a significant increase of tyrosine phosphorylation of several cellular proteins ranging in molecular mass from 32 to 120 kDa was detected at 5 hr and a maximal level was attained within 7 hr (Fig. 4A). The time-dependency of tyrosine phosphorylation of multiple cellular proteins in Jurkat T cells after addition of aburatubolactam C appeared to be consistent with that of induced apoptotic DNA fragmentation as well as enhanced expression of FasL (Figs. 2B and 3A). These results suggest that an increased level of tyrosine phosphorylation of multiple cellular proteins accompanied with aburatubolactam C-induced apoptosis in Jurkat T cells is due to protein tyrosine kinase activation that occurs proximal to induction of Fas signal transduction through upregulation of FasL expression.

Although $p56^{lck}$ kinase activation is known to be a proximal signaling event dependent on the presence of CD45 tyrosine phosphatase for T-cell activation through TCR stimulation and triggers several signaling pathways that drive induction of cytokine gene expression and clonal expansion, it has been also reported that $p56^{lck}$ kinase is required for FasL expression and thus induction of apoptotic death in response to anti-CD3 (26). To investigate the involvement of $p56^{lck}$ in upregulation of FasL expression upon aburatubolactam C-treatment, alteration of $p56^{lck}$ was investigated based on electrophoretic mobility shift that can be detectable by Western blot analysis. Previously it was reported that ligand binding to either TCR or IL-2R alters the phosphorylation state of $p56^{lck}$, resulting in a change in electrophoretic mobility from $p56^{lck}$ to $p60^{lck}$ on polyacrylamide gels, and that the IL-2-induced appearance of $p60^{lck}$ form was preceded by a rapid and significant activation of $p56^{lck}$ (32-34). As shown in Fig. 4B, a characteristic shift in $p56^{lck}$ mobility was detectable in 30 min and sustained until 8 hr after addition of aburatubolactam C, indicating that $p56^{lck}$ was rapidly activated well before the significant increase in tyrosine phosphorylation of the cellular proteins. These results suggest that a potential candidate for the tyrosine protein kinase involved in the early stage of aburatubolactam C-induced apoptosis may be the src-related $p56^{lck}$, and that a rapid activation of $p56^{lck}$ and subsequent change in electrophoretic mobility may be required for the upregulation of FasL and Fas expression in Jurkat T cells after treatment of aburatubolactam C.

Effect of genistein on aburatubolactam C-induced apoptosis. The effect of genistein, a known protein tyrosine kinase inhibitor (35), on aburatubolactam C-induced cellular changes and apoptosis in Jurkat T cells was investigated in order to elucidate further the requirement of protein tyrosine kinase activation in the aburatubolactam C-induced upregulation of FasL expression accompanying apoptosis. The induced apoptotic DNA fragmentation by aburatubolactam was significantly inhibited in the presence of 200 μM of genistein (Fig. 5). The effect of genistein was also examined on aburatubolactam C-induced tyrosine phosphorylation of cellular proteins and alteration in electrophoretic mobility of $p56^{lck}$. As shown in Fig. 6A, there was no detectable increase in tyrosine phosphorylation of cellular proteins in the presence of 200 μM of genistein. Under these conditions, upregulation of FasL expres-

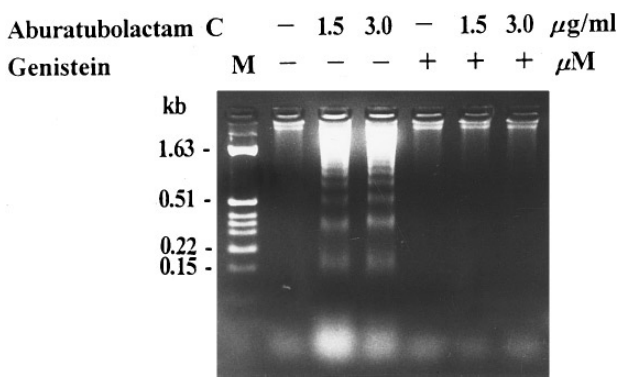

FIG. 5. Inhibitory effect of genistein on aburatubolactam C-induced apoptotic DNA fragmentation in Jurkat T cells. The cells were treated with 200 μM of genistein for 1 hr prior to the incubation with 3 μg/ml of aburatubolactam C for 5 hr, and then processed to analyze the apoptotic DNA fragmentation as described in Materials and Methods.

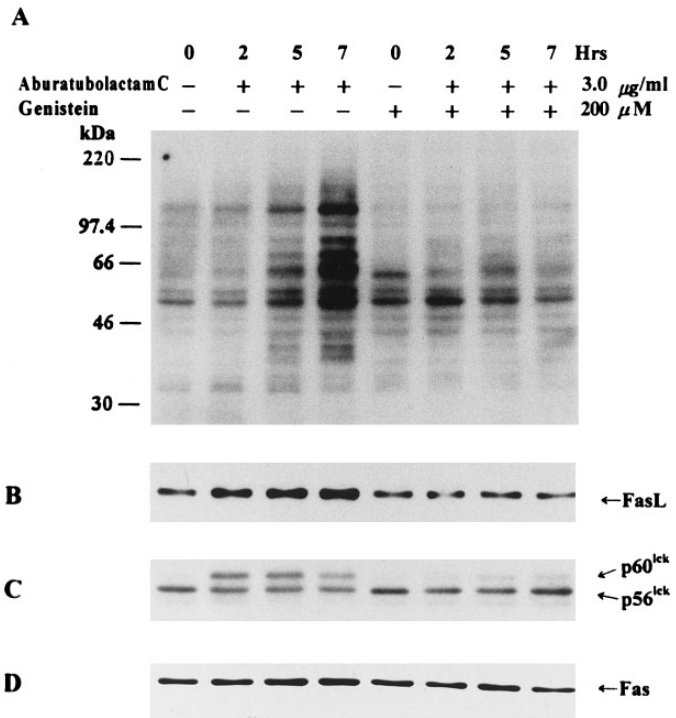

FIG. 6. Effect of genistein on aburatubolactam C-induced tyrosine phosphorylation of cellular proteins (A), upregulation in the protein level of FasL (B), and electrophoretic mobility retardation of $p56^{lck}$ (C), and on expression in the protein level of Fas (D) in Jurkat T cells. After pretreatment of the cells with 200 μM of genistein for 1 hr, the treated cells and untreated cells were incubated with 3 μg/ml of genistein for the indicated times. The cells were processed to assess the tyrosine phosphorylation of cellular proteins, and the alteration of FasL and Fas expression as well as electrophoretic mobility of $p56^{lck}$ in gels by Western analysis.

sion appeared to be completely blocked, and the pronounced mobility shift of $p56^{lck}$ in gel was significantly reduced to nearly detectable level (Fig. 6B and 6C). However, under the same conditions the protein level of Fas was relatively constant regardless of the presence of genistein (Fig. 6D). These results confirm that the activation of $p56^{lck}$ and subsequent appearance of $p60^{lck}$ form are critical for the induced apoptosis, and precedes the upregulation of FasL expression as well as the elevated tyrosine phosphorylation of multiple cellular proteins in Jurkat T cells following aburatubolactam C-treatment.

Together these results demonstrate that protein tyrosine kinase activation is required for aburatubolactam C-induced upregulation of FasL expression and tyrosine phosphorylation of several cellular proteins may be sequentially associated with aburatubolactam C-induced apoptosis in human Jurkat T cells. A potential candidate for the tyrosine protein kinase involved with aburatubolactam C-induced apoptosis is thought to be the src-related protein tyrosine kinase $p56^{lck}$, which is abundantly expressed in T cells and plays an important role in mediating signals downstream from TCR/CD3 and IL-2 receptor signaling pathway (32-34). Investigations of cellular signaling pathway underlying upregulation of FasL expression in Jurkat T cells following aburatubolactam C-treatment is currently being undertaken.

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