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Protein tyrosine kinase p56^{lck}-deficiency confers hypersusceptibility to ρ-fluorophenylalanine (pFPhe)-induced apoptosis by augmenting mitochondrial apoptotic pathway in human Jurkat T cells

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ABSTRACT

Phenylalanine analog, ρ-fluorophenylalanine (pFPhe)-mediated cytotoxicity and several apoptotic events including mitochondrial cytochrome *c* release, activation of caspase-9, -3, and -8, Bid cleavage, degradation of PARP and PLCγ-1, and DNA fragmentation were more significant in p56^{lck}-deficient Jurkat T cells (JCaM1.6) than in wild-type Jurkat T cells (E6.1). The susceptibility of JCaM1.6 toward apoptogenic activity of pFPhe decreased after acquisition of p56^{lck} by transfection. The p56^{lck} kinase activity increased 1.6-fold at 15–30 min after pFPhe treatment. The pan-caspase inhibitor (z-VAD-fmk) completely blocked the pFPhe-mediated apoptotic changes except caspase-9 activation. The caspase-8 inhibitor (z-IETD-fmk), which failed to influence pFPhe-induced caspase-9 activation, completely blocked caspase-8 activation and PLCγ-1 degradation with a marked reduction in caspase-3 activation and PARP degradation, indicating pFPhe-induced caspase-8 activation as a downstream event of mitochondria-dependent activation of caspase-9. These results indicate that the deficiency of p56^{lck} augments pFPhe-induced mitochondrial cytochrome *c* release and resultant apoptotic cell death in Jurkat T cells.

A protein tyrosine kinase (PTK) p56^{lck} is a typical non-receptor TPK of src-family and is expressed almost exclusively in T cells [1]. The p56^{lck} interacts with cytoplasmic regions of CD4 and CD8 coreceptor molecules, and thus plays an important role in relaying TCR-mediated activation signal. In addition, p56^{lck} is known to relay signals from β chain of the IL-2 receptor, indicating critical roles of p56^{lck} for T-cell activation and proliferation. The importance of p56^{lck} for T-cell propagation is initially indicated by virtue of its overexpression, resulting from retroviral insertion into the lck locus, in two Molony murine leukemia virus-induced lymphoid tumors [2]. Besides the typical roles of p56^{lck} directly associated with T-cell propagation, it has also been shown that p56^{lck} is required for FasL expression during activation-induced T cell apoptosis [3]. Although these previous studies have indicated that p56^{lck} is associated with activation-induced T-cell apoptosis mainly via upregulating FasL expression, several recent evidences have indicated a direct requirement of p56^{lck} for certain types of apoptosis induced by ionizing radiation, ceramide or rosmarinic acid, through modulating mitochondria-dependent apoptotic signaling pathway [4–6]. On the other hand, p56^{lck}-deficient murine helper T cell clone resulting from p56^{lck}-specific antisense RNA expression was hypersusceptible to apoptosis when activated through TCR [7]. Although the previous data have suggested that the role of p56^{lck} involved in T-cell apoptosis might be distinctive depending on initial triggers provoking apoptosis, the precise mechanism remains obscure.

Several studies have reported that amino acid analogs possess growth inhibitory activity toward tumor cells in culture and experimental tumors in vivo, and are able to enhance chemo- or radiosensitivity of tumors [8,9]. Induction of apoptosis has also been implicated in the inhibitory activity of amino acid analogs on the growth of tumor cells [10,11]. These previous results have proposed the possible application of amino acid analogs to pharmacological treatment of malignant conditions including cancers. A primary mechanism underlying these antitumor activities of amino acid analogs, leading to cell damage, is thought to be their incorporation into cellular proteins in substitution for the intact forms and induction of structurally aberrant proteins with impaired function or degradation [12,13], which can be more significant in tumor cells than in normal cells due to the difference of their mitotic rates. However, the involvement of apoptotic cell death in the inhibitory activity of amino acid analogs against tumor cells, which is requisite for evaluating their potency as a chemotherapeutic agent, remains largely unknown.

Recently we have found that ρ-fluorophenylalanine (pFPhe), the phenylalanine analog, which possesses enough structural similarity to compete with phenylalanine for phenylalanyl-tRNA

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activation and for its incorporation into cellular proteins, can exert cytotoxicity through apoptotic cell death in human acute leukemia Jurkat T cells. In addition, we have found that the lack of $p56^{lck}$ in Jurkat T cells potentiate the pFPhe-induced apoptosis, suggesting an inhibitory effect of $p56^{lck}$ on pFPhe-induced apoptosis. In order to understand further not only the mechanism by which pFPhe induces apoptosis, but also the component of pFPhe-mediated apoptotic signaling pathway, which is augmented in the absence of $p56^{lck}$, in the present study, we have investigated pFPhe-induced apoptotic signaling pathway and its modulation by $p56^{lck}$ using wild-type Jurkat T cells (clone E6.1), $p56^{lck}$-deficient Jurkat T cells (clone JCaM1.6) and $p56^{lck}$-transfected JCaM1.6 (clone JCaM1.6/lck). The results demonstrate that pFPhe induces apoptosis in Jurkat T cells via mitochondria-dependent death signaling pathway including mitochondrial cytochrome *c* release and resultant activation of caspase-9 and -3, which is negatively regulated by $p56^{lck}$, suggesting that a novel suppressive effect of $p56^{lck}$ on apoptosis mediated by pFPhe in human acute leukemia Jurkat T cells.

Materials and methods

Reagents, antibodies, and cells. ρ-Fluorophenylalanine (pFPhe) was purchased from Sigma Chemical (St. Louis, MO). ECL Western blotting kit was from Amersham (Arlington Heights, IL). Anti-cytochrome *c*, anti-Fas and anti-FasL were purchased from Pharmingen (San Diego, CA). Anti-caspase-3, anti- poly (ADP-ribose) polymerase (PARP), anti-Bid, anti-PLCγ-1, anti-$p56^{lck}$, and anti-β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-caspase-8, anti-caspase-9, anti-caspase-7, and anti-Bid were from Cell Signaling Technology (Beverly, MA). A broad-range caspase inhibitor z-VAD-fmk and caspase-8 inhibitor z-IETD-fmk were obtained from Calbiochem (San Diego, CA). Annexin V-FITC apoptosis kit was from Clontech (Takara Bio Inc., Shiga, Japan). Human acute leukemia Jurkat T cell line E6.1, $p56^{lck}$-deficient Jurkat T cell clone JCaM1.6, Jurkat T cell clone A3, and FADD-deficient Jurkat T cell clone I2.1 were purchased from ATCC (Manassas, VA). Stable transfectants of Jurkat JCaM1.6 (JCaM1.6/vector and JCaM1.6/lck) were supplied from Dr. Arthur Weiss (University of California, San Francisco, CA). Jurkat T cells were maintained in RPMI 1640 (Life Technologies, Gaithersburg, MD) containing 10% FBS, 20 mM HEPES (pH 7.0), 5×10^{-5}M β-mercaptoethanol, and 100 μg/ml gentamycin.

Cytotoxicity assay. The cytotoxic effect of pFPhe on Jurkat T cells was analyzed by MTT assay reflecting the cell viability. Briefly, Jurkat T cells (5×10^4) were added to the serial dilution of pFPhe in 96-well plates. At 20 h after incubation, 50 μl of MTT solution (1.1 mg/ml) was added to each well and incubated for an additional 4 h. 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 pFPhe treatment was determined by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis as previously described [14].

Flow cytometric analysis. Cell cycle progression of Jurkat T cells following pFPhe treatment was analyzed by Flow cytometry as described elsewhere [15]. The extent of necrosis was detected with Annexin V-FITC apoptosis kit. The cells (1×10^6) were washed with 1X binding buffer and then incubated with Annexin V-FITC and PI for 15 min before being analyzed by flow cytometry according to the manufacturer's instructions.

Preparation of cell lysate and Western blot analysis. Cellular lysates were prepared by suspending 5×10^6 Jurkat T cells in 200 μl

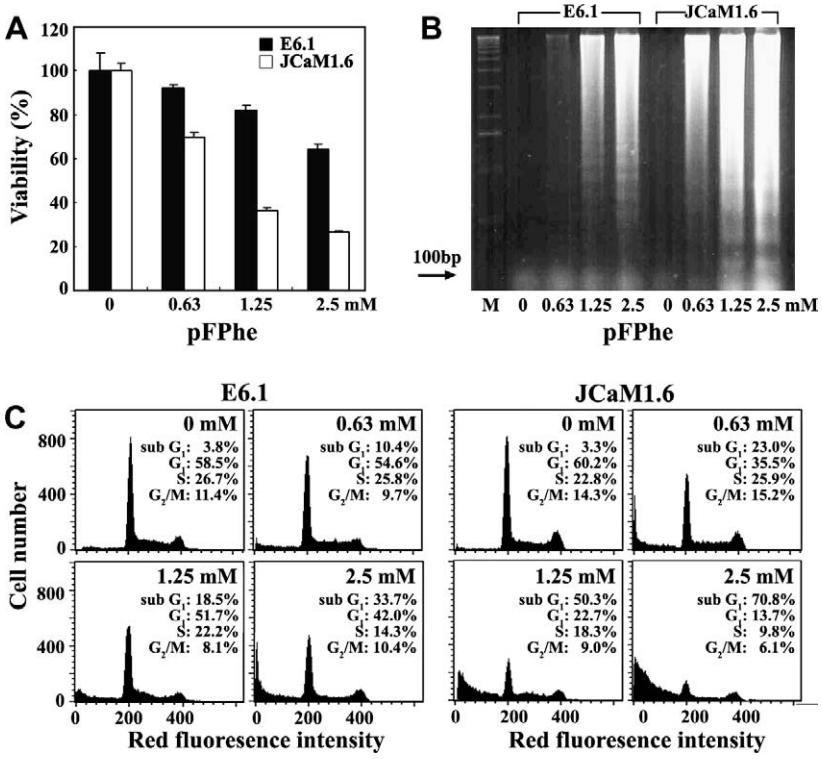

Fig. 1. Effect of pFPhe on cell viability (A), apoptotic DNA fragmentation (B), and cell cycle distribution (C) in wild-type Jurkat T cells (clone E6.1) and $p56^{lck}$-deficient Jurkat T cells (clone JCaM1.6). Individual cells (5×10^4) were incubated with indicated concentrations of pFPhe in a 96-well plate for 20 h and the final 4 h were incubated with MTT. The cells were sequentially processed to assess the colored formazan crystal produced from MTT as an index of cell viability. Each value is expressed as means ± SD ($n = 3$). $P < 0.05$ compared to control. Equivalent cultures were prepared to analysis apoptotic DNA fragmentation by Triton X-100 lysis methods using 1.2% agarose gel electrophoresis. The analysis of cell cycle distribution was performed on an equal number of cells (2×10^4) by Flow cytometry after staining of DNA by propidium iodide (PI).

of lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM sodium orthovanadate, 15 mM $MgCl_2$, 0.1% Triton X-100, 25 mM MOPS, 2.5 μg/ml proteinase inhibitor E-64, 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% SDS gradient polyacrylamide gel with 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.

Detection of mitochondrial cytochrome c in cytosolic protein extracts. To assess mitochondrial cytochrome *c* release in Jurkat T cells following pFPhe treatment, cytosolic protein extracts were obtained as described elsewhere [14]. The cytosolic extracts free of mitochondria were analyzed for cytochrome *c* by Western blotting.

Immunoprecipitation and kinase assay. For immunoprecipitation of $p56^{lck}$, 70 μg of cellular lysate at 1 μg/μl were allowed to react with 2 μg of rabbit polyclonal anti-$p56^{lck}$ antibody at 4 °C for 2 h. The immune complexes were recovered by addition of 30 μl of protein G-agarose. The α-casein kinase activity of immunoprecipitated $p56^{lck}$ was assayed as previously described [15]. 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-^{32}P]ATP, 100 μM ATP, 1X kinase assay buffer) for 30 min at 30 °C. The reaction mixture was electrophoresed on an 11% SDS–polyacrylamide gel and then electrotransferred to an Immobilon-P membrane. The membrane was blot-dried, and phosphorylation of the casein was detected by autoradiography and quantitated by a phosphoimage analyzer. For Western analysis of immunoprecipitated $p56^{lck}$, the membrane was probed with monoclonal anti-$p56^{lck}$ antibody and detection was performed using an ECL Western blotting kit.

Results and discussion

Apoptogenic effect of ρ-fluorophenylalanine (pFPhe) on wild-type Jurkat T cells (clone E6.1) and $p56^{lck}$-deficient Jurkat T cells (clone JCaM1.6)

To understand apoptogenic effect of ρ-fluorophenylalanine (pFPhe) as well as its modulation by a src-family protein tyrosine kinase (PTK) $p56^{lck}$, the cytotoxic effect of pFPhe on $p56^{lck}$-positive Jurkat T cells (clone E6.1) and $p56^{lck}$-deficient Jurkat T cells (clone JCaM1.6) was compared by MTT assay. When the cells were treated with pFPhe at various concentrations of 0.63–2.5 mM for 20 h, the decline in the cell viability, which occurred in a dose-dependent manner, appeared to be more significant in JCaM1.6 than in E6.1 (Fig. 1A). Under the same conditions, the apoptotic DNA fragmentation as well as the ratio of sub-G_1 phase representing apoptotic cells was more apparent in JCaM1.6 than in E6.1 (Fig. 1B and C). These results indicate that the higher susceptibility of $p56^{lck}$-deficient JCaM1.6, as compared with $p56^{lck}$-positive E6.1, toward the cytotoxicity of pFPhe is attributable to induced apoptotic cell death that can be enhanced in the absence of $p56^{lck}$.

pFPhe-induced apoptotic signaling pathway in E6.1 and JCaM1.6

To examine the modulation mechanism of pFPhe-mediated apoptosis by $p56^{lck}$ in Jurkat T cells, we compared pFPhe-induced apoptotic signaling pathway between $p56^{lck}$-positive E6.1 and $p56^{lck}$-negative JCaM1.6. Since mitochondrial cytochrome *c* release plays an important role in the commitment of apoptosis provoked by many physiological and nonphysiological signals, and since the released cytochrome *c* together with apoptotic protease activating factor-1 (Apaf-1) activates caspase-9 in the presence of dATP, and the latter then activates caspase-3 [16,17], we first examined, by Western blot analysis, that whether pFPhe-mediated apoptosis accompanied mitochondrial release of cytochrome *c* and this phenomenon occurred more significantly in JCaM1.6 than in E6.1. As shown in Fig. 2A, the level of the cytosolic cytochrome *c* increased in the presence of pFPhe (0.63–2.5 mM) in a dose-dependent manner, and the mitochondrial cytochrome *c* release was more apparent in JCaM1.6 than in E6.1. In addition, pFPhe-induced activation of caspase-9 and -3, and resultant degradation of PARP into two fragments appeared to be more dominant in JCaM1.6 than in E6.1 (Fig. 2B). The caspase-8 activation via the proteolytic degradation of a 57-kDa proenzyme into 41-/43-kDa activated form was detected in both E6.1 and JCaM1.6 following treatment with pFPhe, and the level of 26-kDa Bid protein, which was previously cleaved by caspase-8 to produce the truncated Bid (tBid) causing the mitochondrial cytochrome *c* release [18,19], appeared to decline in accordance with pFPhe-induced caspase-8 activation. Again, the pFPhe-induced caspase-8 activation as well as Bid cleavage was more significant in JCaM1.6 than in E6.1. As a potential mechanism underlying the apoptosis induced by antineoplastic drugs,

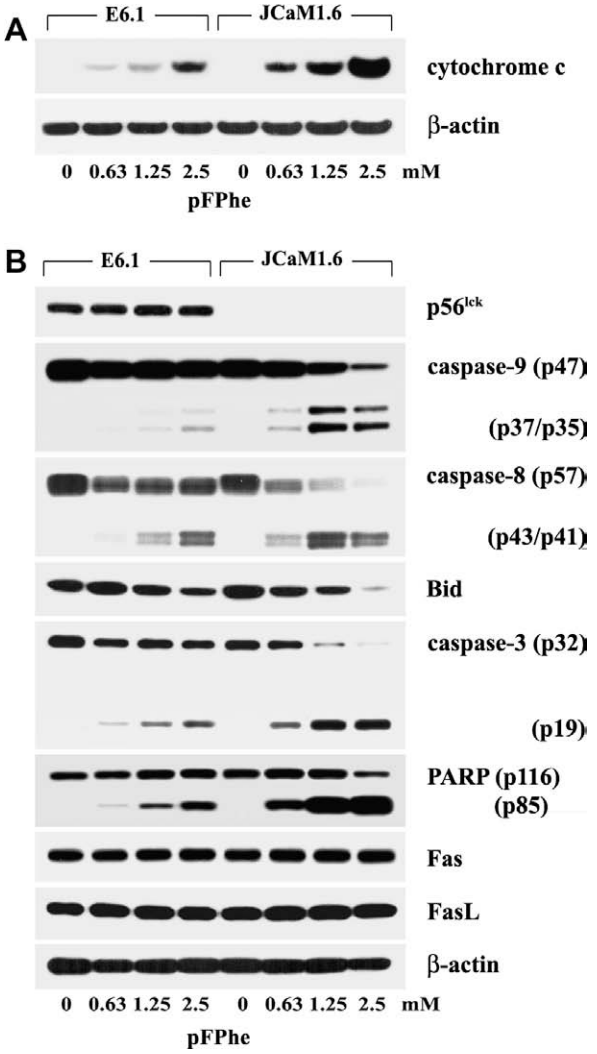

Fig. 2. Western blot analysis of cytochrome *c* release and β-actin (A), and $p56^{lck}$, caspase-9 activation, caspase-8 activation, Bid, caspase-3 activation, PARP cleavage, FasL, Fas, and β-actin (B) in E6.1 and JCaM1.6 after treatment with various concentrations of pFPhe. The cells ($\sim 5 \times 10^6$ cells) were incubated with indicated concentrations of pFPhe for 20 h and prepared for the cell lysates. Equivalent amounts (20 μg) of cell lysates were electrophoresed on 4–12% SDS gradient polyacrylamide gels with MOPS buffer and electrotransferred to Immobilon-P membrane. Western analysis was performed as described in Materials and methods.

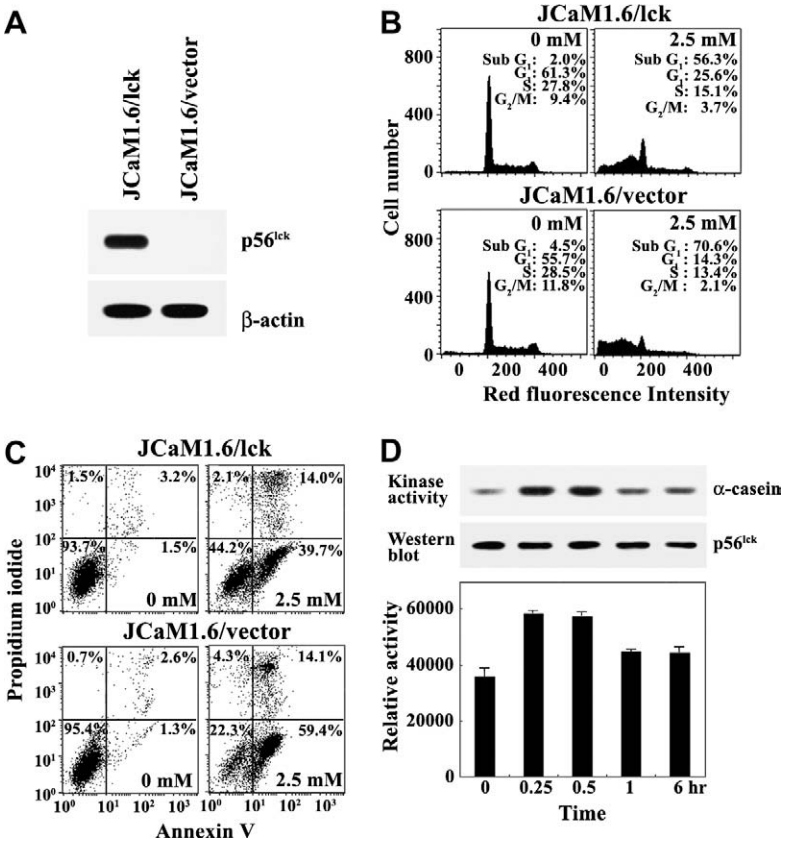

Fig. 3. Western analysis of $p56^{lck}$ expressed in JCaM1.6/lck (A), comparison of pFPhe-mediated apoptotic sub-G_1 peak (B) and apoptotic cell death (C) between JCaM1.6/lck and JCaM1.6/vector, and kinetic analysis of pFPhe-induced alteration in $p56^{lck}$ kinase activity in JCaM1.6/lck (D). After JCaM1.6/lck and JCaM1.6/vector were incubated with 2.5 mM pFPhe for 20 h, the cells were harvested. The analysis of apoptotic sub-G_1 peak was performed on an equal number of cells (2×10^4) by flow cytometry after staining of DNA by PI. To examine an occurrence of necrosis with pFPhe-mediated apoptosis, the equivalent cells were stained and analyzed by flow cytometry to detect Annexin V-FITC positive and/or PI positive cells. For the time course of pFPhe-induced alteration in the kinase activity of $p56^{lck}$, JCaM1.6/lck were incubated with 2.5 mM pFPhe for indicated time periods and processed to analyze the α-casein kinase activity as described in Materials and methods.

upregulation of Fas ligand (FasL) and/or Fas expression has been implicated [20]. There was, however, no change in the level of FasL and Fas in both E6.1 and JCaM1.6 following exposure to 0.63–2.5 mM pFPhe, suggesting that Fas-death signaling might not be associated with pFPhe-induced apoptosis. To examine further an involvement of Fas/FasL system in pFPhe-mediated apoptosis, we compared cytotoxic effect of pFPhe on FADD-positive Jurkat T cells (clone A3) with that on FADD-deficient Jurkat T cells (clone I2.1), which was previously refractory to Fas-mediated apoptosis [21]. Irrespective of the FADD deficiency, both Jurkat T cell clones showed similar sensitivity to the cytotoxicity of pFPhe (data not shown), excluding the involvement of Fas/FasL system in the pFPhe-mediated apoptosis. Since it has been reported that caspase-8 activation can play a role in drug-induced apoptotic signaling pathway either as an upstream event [14,19,22] or as a downstream event of mitochondrial cytochrome *c* release [23], current data suggest that $p56^{lck}$ kinase might negatively influence either pFPhe-induced caspase-8 activation and subsequent generation of tBid triggering mitochondrial cytochrome *c* release or pFPhe-induced mitochondrial cytochrome *c* release and resultant activation of caspase cascade.

Effect of ectopic expression of $p56^{lck}$ on hypersusceptibility of JCaM1.6 toward apoptogenic activity of pFPhe

To confirm the involvement of the $p56^{lck}$ in pFPhe-induced apoptosis of Jurkat T cells as a negative modulator, we decided to examine whether the hypersusceptibility of $p56^{lck}$-deficient JCaM1.6 to the apoptogenic activity of pFPhe is reduced by ectopic expression of $p56^{lck}$ in JCaM1.6. As shown in Fig. 3A, Western blot analysis demonstrated that the stable transfectants of JCaM1.6 with the $p56^{lck}$ gene was able to express the $p56^{lck}$ protein. When the induced apoptotic sub-G_1 peak after treatment with 2.5 mM pFPhe for 20 h was compared between $p56^{lck}$-stable transfectants JCaM1.6/lck and $p56^{lck}$-deficient JCaM1.6/vector by flow cytometry, the sub-G_1 peak was more obvious in JCaM1.6/vector than in JCaM1.6/lck (Fig. 3B). In order to examine whether necrosis was accompanied by the apoptogenic activity of pFPhe as well as its modulation by the presence of $p56^{lck}$, JCaM1.6/lck and JCaM1.6/vector, which were treated with 2.5 mM pFPhe for 20 h, were analyzed by Annexin V staining. As shown in Fig. 3C, the treatment of JCaM1.6/lck and JCaM1.6/vector with pFPhe caused an enhancement in the levels of early apoptotic cells stained only with Annexin V-FITC, and late apoptotic cells stained with both Annexin V-FITC and PI, and these apoptotic changes appeared to be more dominant in JCaM1.6/vector than in JCaM1.6/lck. Under these conditions, however, the necrotic cells stained only with PI were barely detected. These results confirmed that although the $p56^{lck}$ was not a prerequisite for pFPhe-mediated apoptotic signaling pathway, it could contribute to suppressing the pFPhe-induced apoptosis. Since current data showed that the presence of $p56^{lck}$ reduced the susceptibility to pFPhe-mediated apoptotic cell death of Jurkat T cells, we tested if the kinase activity of $p56^{lck}$ in JCaM1.6/lck was altered after exposure to pFPhe. When the kinetic alteration of $p56^{lck}$ activity in JCaM1.6/lck following treatment with 2.5 mM pFPhe was assessed by in vitro kinase assay using α-casein as the substrate, the kinase activity of $p56^{lck}$ appeared to increase by approximately 1.6-fold at 15–30 min, and

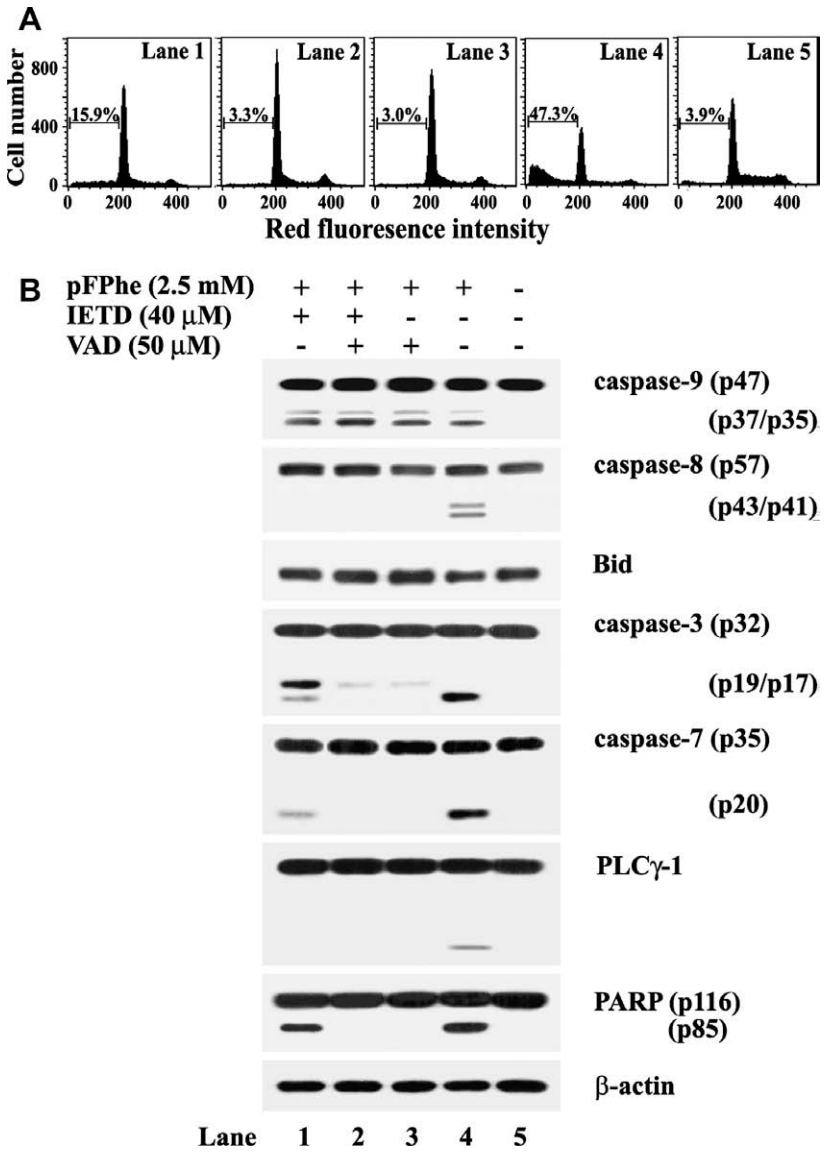

Fig. 4. Apoptotic change in the cell cycle distribution (A) and Western blot analysis of activation of caspase-9, -8, -3, and -7, Bid, and cleavage of PLCγ-1 and PARP (B) in JCaM1.6/lck after treatment with 2.5 mM pFPhe in the presence of z-VAD-fmk and/or z-IETD-fmk (B). JCaM1.6/lck (4×10^5/ml) was preincubated in the individual or simultaneous presence of z-VAD-fmk (50 μM) or z-IETD-fmk (40 μM) for 1 h and then treated with 2.5 mM pFPhe for 24 h. Flow cytometric analysis of cell cycle distribution and Western analysis were performed as described in Materials and methods.

sustained to 1.2-fold-increased level at 1–6 h after treatment with pFPhe (Fig. 3D). At the same time, there was no significant change in the level of $p56^{lck}$ protein. These results demonstrated that the kinase activity of $p56^{lck}$ was enhanced following treatment with pFPhe in Jurkat T cells, supporting the involvement of 56^{lck} kinase in the pFPhe-induced apoptotic signaling pathway as a negative modulator.

Effect of pan-caspase inhibitor z-VAD-fmk or caspase-8 inhibitor z-IETD-fmk on pFPhe-induced death signaling in JCaM1.6/lck

To elucidate further the death signaling pathway for pFPhe-induced apoptosis, which can be modulated by $p56^{lck}$, we examined the effect of the pan-caspase inhibitor (z-VAD-fmk), which is known to inhibit broad-range caspases [24], and the caspase-8 inhibitor (z-IETD-fmk) [25] on pFPhe-induced apoptotic events in JCaM1.6/lck. After cells were pretreated either with each caspase inhibitor or with both caspase inhibitors for 1 h, the cells were exposed to 2.5 mM pFPhe for 24 h. Although continuously growing JCaM1.6/lck showed a barely detectable apoptotic sub-G_1 peak, it increased to the level of 47.2% in the presence of 2.5 mM pFPhe for 24 h (Fig. 4A). The pFPhe-induced sub-G_1 peak was reduced to the basal level by pretreatment with z-VAD-fmk or with both z-VAD-fmk and z-IETD-fmk, whereas the sub-G_1 peak was partially reduced to the level of 15.9% by pretreatment of z-IETD-fmk. Under the same conditions, Western blot analysis revealed that pFPhe-induced apoptotic events such as activation of caspase-8, -3 and -7, Bid cleavage, and cleavage of PARP and PLCγ-1 were completely abrogated by z-VAD-fmk, whereas caspase-9 activation was sustained (Fig. 4B). In the presence of z-IETD-fmk, pFPhe-mediated caspase-9 activation was not influenced, but caspase-8 activation and PLCγ-1 degradation were completely abrogated with a partial reduction in the activation of caspase-3 and -7, and PARP cleavage. These results indicated that pFPhe-induced caspase-8 activation were a downstream event of caspase-9 activation, and suggested that caspase-8 together with caspase-9 might contribute to activation of caspase-3 and -7, leading to cleavage of PLCγ-1 and PARP.

In summary, these results demonstrate that the pFPhe-induced apoptotic signaling pathway leading to apoptotic DNA fragmentation was provoked by mitochondrial cytochrome *c* release and resultant activation of caspase cascade, which could be negatively regulated by the protein tyrosine kinase $p56^{lck}$ in Jurkat T cells. These results first provide the apoptogenic mechanism of an amino acid analog, ρ-fluorophenylalanine (pFPhe), along with its suppressive modulation by the protein tyrosine kinase $p56^{lck}$ in human acute leukemia Jurkat T cells.

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