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Platycodin D-induced apoptosis through nuclear factor-κB activation in immortalized keratinocytes

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Abstract

Platycodi Radix is the root of *Platycodon grandiflorum* and it is widely used in the traditional Oriental medicine as an expectorant for pulmonary diseases and a remedy for respiratory disorders. Platycodin D is the major constituent of triterpene saponins in the root. This study investigates apoptosis by platycodin D in immortalized human keratinocytes (HaCaT). Platycodin D-induced apoptosis in HaCaT cells was confirmed by DNA fragmentation, caspase-3 activation, and caspase-8 activation. Platycodin D could activate inhibitor of nuclear factor-κB kinase (IKK)-β in the nuclear factor-kappaB (NF-κB) activation of upstream level, but not IKK-α. Pretreated-*N*-*tosyl*-L-phenylalanine chloromethyl ketone (TPCK), a potent NF-κB inhibitor, could suppress the induction of apoptosis and activation of NF-κB of HaCaT cells by platycodin D. We also demonstrated that platycodin D-mediated apoptosis of HaCaT cells upregulates Fas receptor and Fas ligand (FasL) expression, but did not exhibit p53 activation. HaCaT cells were also transfected with pFLF1, which preserves the promoter region of Fas receptor gene containing NF-κB binding site. On incubation with platycodin D, the NF-κB activity related to Fas receptor increased in a dose-dependent manner. Among the major transcription elements on Fas receptor and FasL promoter, NF-κB activation was shown to have an essential role in the expression of the death receptor such as FasL. These results suggest that platycodin D has the ability to induce apoptosis in HaCaT cells through the upregulation of Fas receptor and FasL expression via to NF-κB activation in the transcriptional level. These results demonstrate that the NF-κB activation plays a crucial role in the induction of apoptosis in human HaCaT cells on treatment with platycodin D.

Keywords: Platycodin D; NF-κB activation; HaCaT cells; Apoptosis; Caspase-3 and 8; Fas and Fas ligand

1. Introduction

Apoptosis (programmed cell death) is a common physiological process that occurs actively during the embryonic development as well as in the maintenance of tissue homeostasis (Kerr et al., 1972). The molecular mechanism of apoptosis has been recently established and there are many reviews in the literature (Chen et al., 2001; Dunn et al., 2002; Schuler and Green, 2001; Somasundaram, 2000; Wajant, 2002). The signal-induced apoptosis can be roughly divided into either the receptor-mediated extrinsic apoptosis or the mitochondrial-mediated intrinsic apoptosis (Ashkenazi and Dixit, 1998; Green and Reed, 1998). In particular, the extrinsic apoptotic pathway is triggered as a consequence of ligand binding to a death receptor, such as the Fas receptor and FasL. FasL induces apoptosis through the Fas receptor protein. The cytoplasmic tail of each receptor contains the so-called death domain receptor (DD) that interacts with a cytoplasm, and the cytoplasmic portions of the Fas trimer that interacts with Fas-associated death domain protein (FADD) have a direct pathway to apoptosis through the recruitment and activation of procaspase-8. Activated caspase-8 subsequently activates downstream effector caspases, such as caspases-3, -6, and -7 (Israels and Israels, 1999). The Fas system has proven to be essential in contributing to homeostasis of the epidermis. Other groups have recently investigated that the apoptosis of Fas-induced keratinocytes in response to UV

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radiation prevents the accumulation of procarcinogenic p53 mutations by deleting UVR-mutated keratinocytes (Hill et al., 1999). Furthermore, strong evidence illustrates that dysfunction of Fas expression and/or signaling contributes to the pathogenesis of diseases, such as toxic epidermal necrosis (Viard et al., 1998) and UVB induced fragmentation of DNA in human HaCaT cells. These diseases increase the levels of apoptosis as indicated by the increased levels of fragmented DNA, Fas receptor, poly ADP-ribose polymerase (PARP), and FasL protein (Mammone et al., 2000).

One important transcription factor that can impact keratinocytes proliferation is NF-κB, which is an inducible transcription factor that is activated in response to a variety of environmental stimuli (Baeuerle, 1991; Baeuerle and Baichwal, 1997; Wulczyn et al., 1996). The regulation of NF-κB activation is one of the important factors for controlling apoptosis. Several studies indicate that NF-κB activity can, in some cases, prevent oncogenesis and promote apoptosis (Kasibhatla et al., 1998, 1999). NF-κB is involved in the transcriptional regulation of both Fas receptor and FasL (Kasibhatla et al., 1999). These protein expressions are regulated by the activation of the NF-κB complex, which is essential for FasL promoter activity when conserved in the human FasL promoter (−150 bp) (Matsui et al., 1998).

Platycodin D was isolated from the root of *Platycodon grandiflorum* (Platycodi Radix), a perennial herb that is distributed in East Asia. Its roots have been widely used for the treatment of several kinds of chronic inflammatory diseases in Oriental medicine (Lee, 1973; Ozaki, 1995; Takagi and Lee, 1972). These facts indicate that the extracts containing platycodin saponins possess anti-inflammatory activity and platycodin D is a major anti-inflammatory constituent (Ahn et al., 2005; Choi et al., 2001; Kim et al., 2001; Shin et al., 2002; Tada et al., 1975). Contrary to the previous concept of anti-inflammation, the HaCaT cells showed drastic increase of the NF-κB activity on exposure platycodin D, which thought to be an anti-inflammatory agent by our cell-based assay system (Ahn et al., 2003; Moon et al., 2001b). This finding suggests that platycodin D might have proapoptotic activity as well as anti-inflammatory activity, depending on cell types. The apoptotic effect of platycodin D in immortalized keratinocytes has not been well defined. In these studies, we focused on how platycodin D regulates NF-κB in HaCaT cells during the process of apoptosis.

2. Materials and methods

2.1. Chemicals and cell lines

Platycodin saponins were purified from the methanol extract of Platycodi Radix according to the previous reports (Tada et al., 1975; Zhao et al., 2005) and their structures were identified using thin-layer chromatography (TLC) with authentic samples and by the nuclear magnetic resonance (NMR) data. The Great EscAPe Fluorescence detection kit was obtained from Clontech Laboratories (Palo Alto, CA, U.S.A.). Geneticin (antibiotic G-418) and cell culture media were from Gibco BRL (Grand Island, NY, U.S.A.). TPCK, trypsin–EDTA, protease inhibitors cocktails, Dulbecco's phosphate buffer saline (D-PBS), and dimethyl sulfoxide were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Other chemicals and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Human HaCaT cells were originally obtained from Dr. Norbert E. Fusenig (German Cancer Research Center, Heidelberg, Germany) (Boukamp et al., 1988). KM1214, Caki, and Sk-mel-2 cell lines were obtained from the Korean Cell Line Bank in Seoul National University Cancer Center. Lewis lung carcinoma (LLC), SCC-13, and RAW 264.7 cell lines were obtained from the American Type Culture Collection (Rockville, MD, U.S.A.).

2.2. Cell culture

Transfectant human HaCaT cells were cultured with 500 μg/ml of geneticin (100 μg/ml) for the selection and maintenance of stable transformants (Moon et al., 2001a,b). Reporter (SEAP) gene assay, preparation of nuclear extracts, and electrophoretic mobility shift assays (EMSA) were performed as previously described (Ahn et al., 2003; Moon et al., 2001b).

2.3. Cytotoxicity assay

Cytotoxicity of platycodin D was evaluated by the {3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide} (MTT) assay (Scudiero et al., 1988) using the Cell Counting Kit (CCK-8) purchased from Dojindo Laboratories (Tokyo, Japan). In brief, immortalized HaCaT (1×10^4) cells were plated on 96 wells of an enzyme-linked immunoassay (ELISA) plate, incubated for 24 h, and washed twice with 100 μl of phosphate-buffered saline (PBS). They were incubated with various concentrations of platycodin D for 3 h. To assess the effect of the potent NF-κB inhibitor on cell death, HaCaT cells were pretreated for 30 min with TPCK (100 μM) before exposure to platycodin D. The extension of incubation for another 3 h in the incubator at 37 °C was made with 10 μl of the CCK-8 solution. The resulting color was measured at 450 nm using a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, U.S.A.) (Ishiyama et al., 1996).

2.4. Analysis of DNA fragmentation

The 3×10^6 cells were seeded in T-25 plates, and after 24 h, they were treated with platycodin D at various times. The cells were harvested and washed twice with DNA extraction washing solution. The cells were centrifuged at 600 ×*g* for 10 min at 4 °C, lysed in a 200 μl lysis buffer {20 mM EDTA, Tris, pH 8.0, 0.8% (w/v) sodium dodecyl sulfate (SDS)}. The supernatant was incubated at 37 °C for 2 h in 400 μg/ml RNase A and 240 μg/ml proteinase K was added. Then, the cells were mixed by flipping the tip of the tube and were incubated at 50 °C for at least 3 h or, if preferred, overnight (Bush et al., 2001). DNA was extracted with phenol: chloroform and precipitated with ethanol. DNA samples were electrophoresed using a 2% agarose gel containing ethidium bromide at a low voltage

(i.e., 40 V for 4 h or until the loading dye was migrated to two-thirds of the way down the gel), and the resulting DNA bands were visualized under the UV light. We also performed the same experiment to assess the effect of the potent NF-κB inhibitor on apoptosis, HaCaT cells were pretreated for 30 min with TPCK (100 μM) before exposure to platycodin D.

2.5. Terminal dUTP nick-end labeling (TUNEL) assay

HaCaT cells were grown in 10% fetal bovine serum (FBS)-supplemented Dulbecco's modified Eagle's medium (DMEM) for 48 h and were exposed to various concentrations of platycodin D that was dissolved in medium for 3 h. The percentage of cells floating in the medium increased over time during the treatment. Adherent cells were washed by D-PBS twice and the floating cells were recovered through centrifugation at 2800 ×*g* for 15 min. The cells were washed in D-PBS twice, collected by centrifugation at 14,000 ×*g* for 10 min, and were fixed in a phosphate-buffered paraformaldehyde solution (4%, pH 7.4) for 1 h at RT with gentle shaking. The cells were resuspended in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min on ice. The cells were washed again with D-PBS and terminal dUTP nick-end labeling was stained with the in situ cell death detection kit, TMR red (Roche, Mannheim, Germany), following the manufacturer's protocol.

2.6. RNA extraction and reverse transcriptase (RT)-polymerase chain reaction (PCR)

Both unstimulated and stimulated cells were washed and RNA was extracted using TRI REAGENT™ according to the manufacturer's instructions (Sigma Chemical Co., St. Louis, MO). Reverse transcription was performed at 37 °C in the presence of 200 nM oligo(dT), 0.1 M dithiothreitiol (DTT), 0.5 U RNAguard, 10 mM dNTP, 0.5 U MMTV, and 10 μg tRNA. Semi-quantitative PCR was conducted with *Taq* polymerase for 20 cycles with each cycle consisting of 1 min at 94 °C, 1 min at 53 °C, and 1 min at 72 °C. The primers used for β-actin amplification were: sense 5′-AATCTGGCACCA-CACC-TTCTACA-3′ and antisense 5′-CGACGTAGCA-CAGCTTCTCCTTA-3′, the primers for FasL were: sense 5′-CAACTCAAGGTCCATGCCTC-3′ and antisense 5′-AGATT-CCTCAAAATTGACCAG-3′, the primers for Fas receptor were: sense 5′-GACAA-AGCCCATTTTTCTTCC-3′ and antisense 5′-ATTTATTGCCACTGTTTCAGG-3′. The PCR products were separated by 1% agarose gel electrophoresis, and the gels were viewed under UV transillumination.

2.7. Western blot analysis

The platycodin D-treated cells were washed three times with D-PBS, lysed {10 mM Tris–Cl (pH 7.4), 3 mM $CaCl_2$, 2 mM $MgCl_2$, 1% NP-40, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and protease inhibitors cocktails} for 30 min on ice. The lysate was centrifuged at 14,000 ×*g* for 15 min and the supernatant was collected. The protein concentration was determined with Bio-Rad Protein Assay Reagent (Bio-Rad, Vancouver, BC, Canada). The protein (10 μg) was separated on SDS-polyacrylamide gels, electroblotted onto polyvinyllidene fluoride (PVDF) membranes. The transblotted membranes were washed twice with TBST (Tris-buffered saline with Tween-20). After blocking with TBST containing 5% skim milk for 1 h, membranes were incubated with primary antisera overnight at 4 °C with shaking. Blots were washed three times in TBST for 20 min, and then, they were incubated with horseradish peroxidase (HRP)-conjugated secondary antisera for 1 h at room temperature. Blots were developed for visualization using the ECL Plus detection kit (Amersham, UK). Antibodies used for Western blotting were IκBα (BD Pharmingen, Mississauga, ON, Canada), caspase-3, IKK-α, IKK-β, and α-tubulin (Santa Cruz, CA, U.S.A.).

2.8. Fas receptor and FasL expression

HaCaT cells were grown in 10% FBS-supplemented Dulbecco's modified Eagle's medium (DMEM) medium for 48 h and were exposed to various concentrations of platycodin D that was dissolved in medium for 3 h. During the treatment, the percentage of cells floating in the medium increased over time. Adherent cells were washed by D-PBS twice and the floating cells were recovered through centrifugation at 2800 ×*g* for 15 min. Immunostaining for Fas receptor and FasL expressions was performed by incubating the cells with fluorescein isothiocyanate (FITC)-coupled anti-human Fas receptor/FasL antibodies and was proved a specific antibody binding through exposed-cells treated with FITC-coupled anti-mouse IgG. The cells were analyzed by flow cytometry using the Cell Quest program from Becton Dickinson Co.

2.9. Fluorescence microscopy

HaCaT cells were grown on sterile 25-mm round glass coverslips (poly-L-lysine coated, Iwaki, Japan) that were placed in a 6-well plate, and they were cultured for the desired period of time. The subconfluent cells were treated with 15 μM platycodin D for 1 h, washed three times with PBS, fixed with 4% formaldehyde in PBS for 10 min at ambient temperature, and washed with 20 mM glycine-PBS three times. Nonspecific binding sites were blocked with the solution containing 2% BSA in PBS, which was incubated at 37 °C for 1 h. After the blocking solution was removed, the coverslips were incubated with anti-p65 antibody that was diluted to a ratio of 1 : 100 in blocking solution. After three washes with the 0.1% Tween-20 in PBS over a 15 min period, they were incubated for another 30 min with FITC-conjugated goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, MO, U.S.A.), which was diluted to a ratio of 1 : 100 in 2% BSA. After three washes with PBS-Tween 20, the coverslips were mounted with Gel/Mount aqueous mounting medium (Biomega, Foster City, CA, U.S.A.). The cells were examined under an Olympus VANOX AHBT3 microscope that was equipped with epifluorescence illumination (AH3-RFCA) and images were taken with a digital camera (Qimaging, CA, U.S.A.).

2.10. Immunoprecipitation and kinase assay in vitro

To analyze the IKK activity, 500 μg of cytoplasmic extracts that were prepared using the procedures described for Western blotting were incubated for 3 h or overnight at 4 °C with 5 μg of IKK-β and IKK-α polyclonal antibodies (Santa Cruz Biotechnology) and 60 μg of Protein G agarose-conjugated beads. After washing with washing buffer [50 mM HEPES (pH 7.0), 250 mM NaCl, 5 mM EDTA, and 0.1% NP-40] twice and kinase buffer once, the beads were incubated with 20 μl of kinase buffer [20 mM HEPES (pH 7.4), 10 mM $MgCl_2$, 2 mM $MnCl_2$, 25 mM β-glycerophosphate, 4 mM NaF, 0.1 mM Na_3VO_4, and 1 mM DTT] containing 10 mM ATP, 5 μCi of [γ^{32}-P] ATP, and 1 μg of full-length IκBα (amino acids 1–317, Santa Cruz Biotechnology, Inc.) at 30 °C for 20 min. The reaction was stopped by the addition of an equal volume of Laemmli buffer and the sample was subjected to 10% SDS-PAGE. The phosphorylated IκBα (1–317) was visualized by autoradiography.

2.11. DNA transfection and luciferase assay

The luciferase construct, pFLF1 containing the promoter region between −1435 and +236 of the Fas receptor gene with the NF-κB binding site was a gift from Dr. Nakanishi (Fujita et al., 2004). This promoter region included 18-base pairs upstream of the translation start codon of the firefly luciferase gene in the pGV-B vector. HaCaT cells were transfected using the lipofectamine reagent (Gibco BRL, Grand Island, NY). The luciferase assay was performed with the Dual-Luciferase Reporter Assay System (Promega, WI). As an internal control, the cells were cotransfected with the *Renilla* luciferase gene expression vector (pRL-TK, Promega, WI). Luciferase assay was performed according to the manufacturer's instructions. The luciferase levels were normalized to the level of *Renilla* luciferase activity in each transfect.

3. Results

3.1. Platycodin D led to activation of NF-κB in a short time during induced cell death in HaCaT cells

Until recently, the property of platycodin D inducing apoptosis has not been studied in HaCaT cells. Much of the variable results about triterpenoid saponins that are cited in the literature appeared to depend on chemical structure (Fig. 1). The cytotoxicity of platycodin D showed highest sensitivity to HaCaT cells among several cancer cell lines tested in the experiment (Table 1) (Fig. 2A). We observed that platycodin D induces, in a dose- and time-dependent manner, the cell death of immortalized human HaCaT cells. In order to measure the activation of NF-κB, the cell-based assay system was studied in

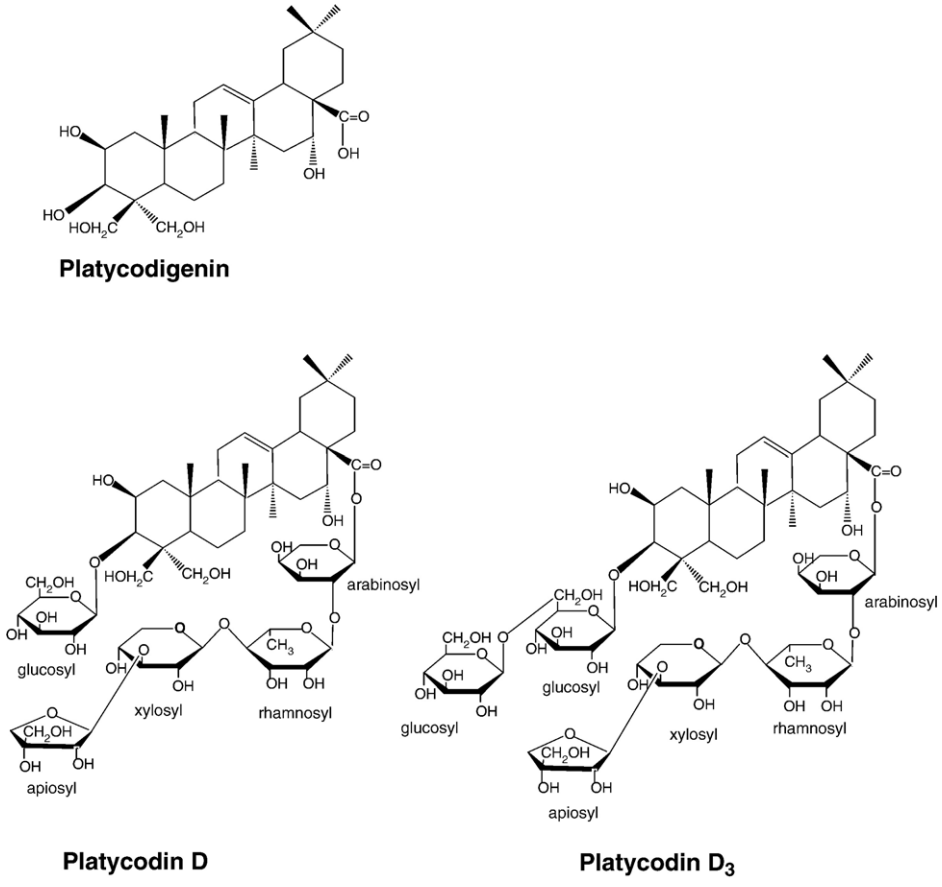

Fig. 1. Structures of platycodigenin, platycodin D, and platycodin D_3.

Table 1
Cytotoxic activity of platycodin D against various cancer cells

Cell lines	IC_{50} (μM)
HaCaT (skin)	18.2±2.1
LLC (lung)	41.3±1.3
KM1214 (colon)	53.4±2.4
SCC-13 (skin)	51.0±1.4
Caki (kidney)	38.2±2.1
RAW 264.7 (monocytes)	68.3±3.2
Sk-mel-2 (skin)	50.3±2.4

our established in vitro model (Moon et al., 2001b). The effects of platycodin saponins on the activation of NF-κB were also examined (Fig. 2B, C). Of the three compounds tested, platycodin D enhanced the NF-κB activity most, followed by platycodin D_3 and platycodigenin. Fig. 2C also depicts the dose- and time-dependent effects of platycodin D on NF-κB activity of HaCaT cells, which indicates that NF-κB activity strongly and rapidly (at least 10 min) was increased from the basal level, and this elevated level was sustained throughout the culture.

3.2. Platycodin D activated NF-κB via degradation of IκBα in HaCaT cells

The DNA-binding activity of IκB-binding proteins was performed by EMSA, using a nuclear protein extract from 30 μM platycodin D-treated cells and a DNA probe with a consensus IκB-binding sequence. DNA–protein complexes clearly and rapidly increased in a time-dependent manner from the basal level (Fig. 3A). Because the NF-κB activity is known to be inhibited through the blocking of IκB degradation in certain type of cells (Wahl et al., 1998; Weber et al., 2000), we confirmed whether the same inhibitory mechanism functions in the human HaCaT cells. Western blot analysis of the IκBα protein in the platycodin D-treated cells showed that, in agreement with the rapid increase in NF-κB DNA-binding activity, IκBα was readily degraded within 30 min after platycodin D treatment (Fig. 3B). The lowest band was detected at 30 min, after which the expression of IκBα gradually increased in minute amounts. This phenomenon is likely to be explained by the known ability of the activated NF-κB stimulating the process of IκBα gene transcription. As demonstrated in Fig. 3C, NF-κB binding activities were significantly retarded by the anti-p50 and anti-p65 antibodies, which indicates that the inducible NF-κB complex in HaCaT cells is composed of the p50 and p65 subunits. Competition experiments shown in Fig. 3D also confirmed the specificity of binding to the κB oligonucleotide. Therefore, under the present conditions, platycodin D definitely seems to activate NF-κB at the level of DNA binding in the nucleus.

3.3. Platycodin D caused an increase in oligonucleosomal DNA fragmentation

Alternatively, we could demonstrate that apoptosis occurs in platycodin D-treated HaCaT cells using a TUNEL assay, which is used to label DNA strand breaks generated during apoptosis. At a dose of 30 μM, platycodin D induced apoptosis in virtually almost of the cells after a 3 h treatment (Fig. 4A). The analysis

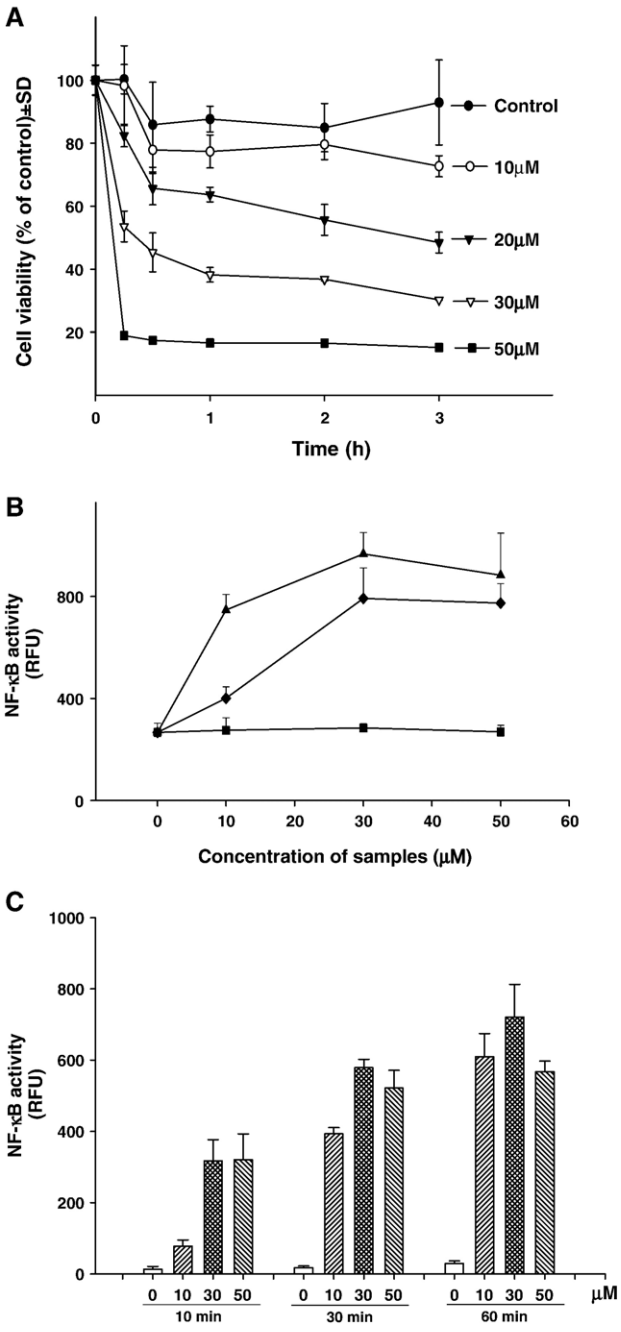

Fig. 2. Effect of cell viability and NF-κB activity in transfectant HaCaT cells treated with platycodin D using a cell-based assay system. Cell death was determined by using the Cell Counting Kit (CCK-8). The data represents the mean of three independent determinations (A). Transfectant HaCaT cells (3×10^6) were treated with platycodigenin, platycodin D, and platycodin D_3 at 37 °C for the given time (B). Platycodin D and platycodin D_3 stimulated the NF-κB activation measured by production of SEAP (secreted alkaline phosphatase) as a reporter gene, which includes the NF-κB binding site. Dose- and time-responses of platycodin D-treated in transfectant HaCaT cell showed highly increase of the NF-κB activation (C). Mean values and standard deviations from three independent experiments are shown. Significant differences in NF-κB activities between untreated and platycodin are indicated by $^*p<0.05$ and $^{**}p<0.01$.

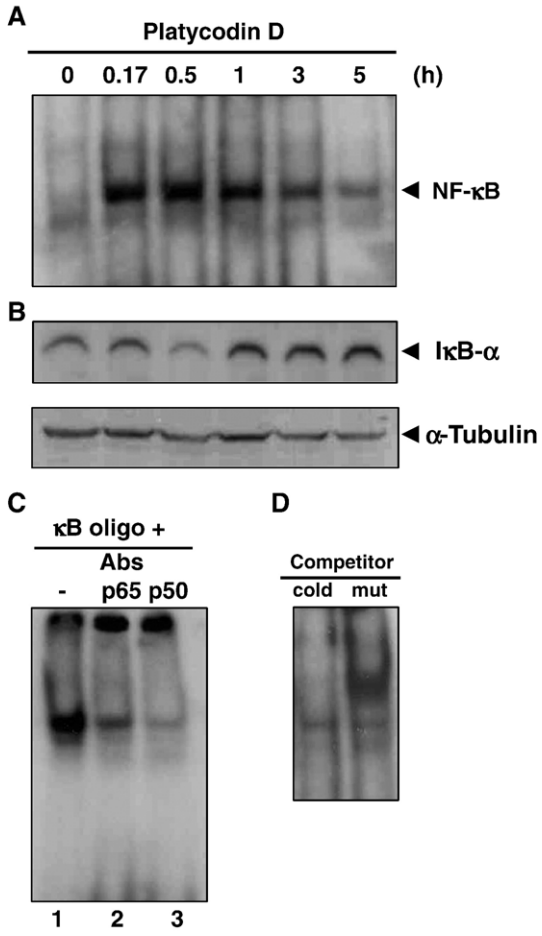

Fig. 3. Time-dependency of NF-κB activation by platycodin D in HaCaT cells. The EMSA demonstrated the active NF-κB DNA-binding activity in HaCaT cells. (A) Shows the time-dependency of NF-κB activation in response to 30 μM platycodin D. A filled arrow indicates the position of the platycodin D-inducible NF-κB·DNA complex. The cytoplasmic extracts of these cells were used to study the level of IκBα by Western blot analysis probed with anti-IκBα antibody as described in Methods (B). Total cell extracts were treated with platycodin D (lane 1) and then they were incubated with the specific antibodies against the NF-κB subunits p65 (lane 2) and p50 (lane 3) (C). For demonstrating the specificity of protein binding and the identity of the proteins, 100-fold molar excess of unlabeled NF-κB consensus oligonucleotide was added to the nuclear extracts of HaCaT cells treated for 30 min with 30 μM platycodin D (D).

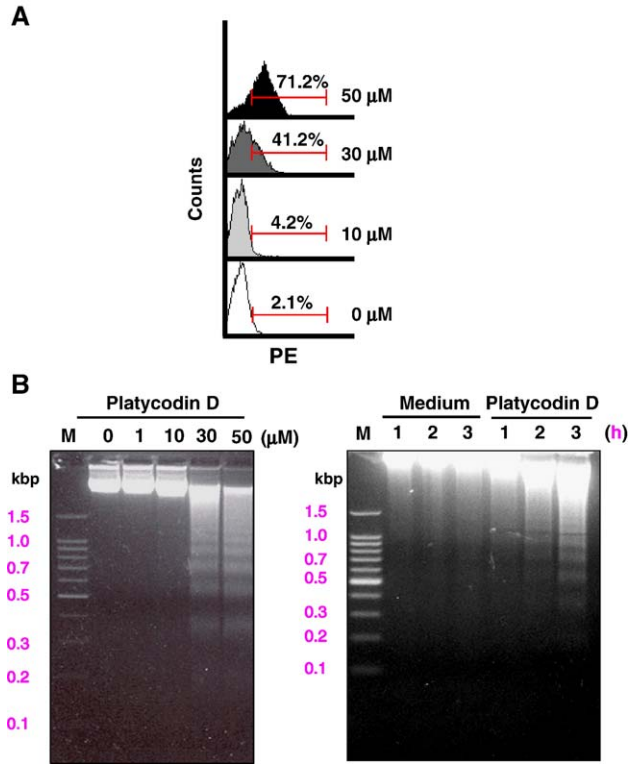

Fig. 4. Induction of apoptosis by platycodin D in HaCaT cells. In the TUNEL assay, cells were treated with 0 (control), 10, 30, 50 μM platycodin D for 3 h. The percentage shown in each histogram was the percentage of apoptotic cells measured by positive cells (A). The histograms are from one representative set of three independent experiments. Dose- and time-dependent effect of platycodin D on DNA fragmentation (B). The left lane (M) is a 100-base pair DNA ladder.

of DNA extracted from platycodin D-treated HaCaT cells showed the dose- and time-dependent generation of nucleosomal-sized ladders of DNA fragments (Fig. 4B). At a platycodin D concentration above 30 μM, DNA fragmentation was significantly induced. The DNA ladder assay indicated that platycodin D-induced cell death proceeds through an apoptotic pathway, but not necrosis.

3.4. Platycodin D induced apoptosis by activation of caspase-3 and -8

During apoptosis, a series of proteolytic cleavages of various intracellular polypeptides is initiated (Earnshaw et al., 1999). Because cells undergoing apoptosis execute the death cascade by activating caspases (Thornberry and Lazebnik, 1998), we analyzed whether the treatment of platycodin D gave rise to the activation of caspase-3, a key enzyme of apoptosis (Nicholson et al., 1995). As shown in Fig. 5, a clear reduction of procaspase-3 zymogen could be observed in a time-dependency

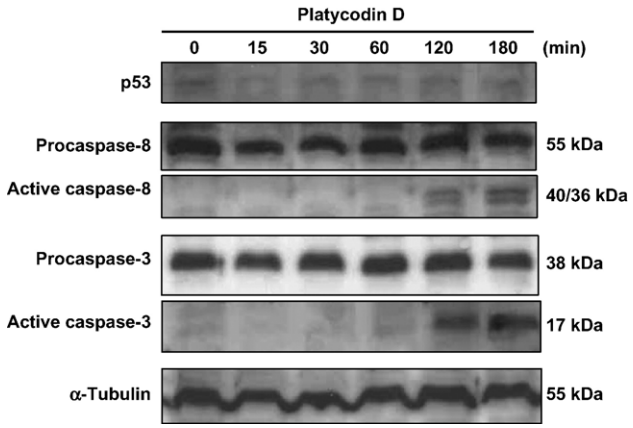

Fig. 5. The effects of platycodin D on caspase-3 and -8 activation. HaCaT cells were treated with 30 μM platycodin D for 0, 15, 30, 60, 120, or 180 min. The key apoptotic proteins, caspases-8 and -3 were activated by platycodin D within 2–3 h. Proteins were extracted and subjected to Western blot analysis.

on treatment of cells with 30 μM of platycodin D. Furthermore, a distinct decrease of procaspase-8 expression was demonstrated by converting a 56-kDa precursor to an active 36-kDa proteolytic cleavage product with an increase in the 40-kDa fragment. The results occurred as little as 30 μM platycodin D in a dose-dependent manner. It is clear that platycodin D selectively induces apoptosis through the caspases-8-mediated pathway. The contemporary model of caspases-8 function illustrates a downstream function of the death receptor-mediated pathways involving CD95/Fas/APO-1 and tumor necrosis factor α receptor (TNFR), while caspase-9 acts downstream of the mitochondrial-mediated pathway components, such as Apaf-1 and cytochrome c (Ashkenazi and Dixit, 1998; Green and Reed, 1998).

3.5. IKK was activated in response to platycodin D in HaCaT cells

Signal-induced serine phosphorylation and degradation of IκB proteins are primarily mediated by IKK. In order to demonstrate the role of IKK in NF-κB activation, the HaCaT cell extract prepared at various time intervals after the treatment of platycodin D were immunoprecipitated using a monoclonal antibody against IKKβ. The extract was also subjected to an immune-complex kinase assay using a full-length IκBα subunit (amino acids 1–317) as a substrate. Platycodin D stimulated IKK activity, which led to reaching a maximum 4-fold within 30 min compared to the untreated cells, and the kinetics of IKK activation elicited by platycodin D was transient after 1 h (Fig. 6A).

3.6. Platycodin D caused nuclear translocation of the NF-κB proteins in HaCaT cells

To examine visually whether treatment with platycodin D can move the NF-κB of cytoplasm into nuclei, the cells with 15 μM platycodin D were incubated for 1 h and the protein was detected with anti-p65 (NF-κB subunit) antibody using the immunofluorescent technique. The staining was restricted mostly to the cytoplasm, and only a weak reaction was observed in the nuclei of the control (Fig. 6A, B, left). In contrast, intense fluorescence was mainly observed in the nuclei of cells that had been treated with platycodin D (Fig. 6A, B, right).

3.7. NF-κB inhibitor (TPCK) suppressed platycodin D-induced apoptosis and activation of NF-κB

To demonstrate whether a potent NF-κB inhibitor (TPCK) can suppress platycodin D-induced apoptosis, the cells were pretreated for 30 min with TPCK prior to platycodin D stimulation. Treatment with TPCK had a marked inhibitory effect on NF-κB activation by platycodin D (Fig. 7A). Pretreatment with TPCK also resulted in the marked reduction of the percentages of platycodin D-induced cell death, indicating that NF-κB activation was required for apoptosis on HaCaT cells (Fig. 7B and C).

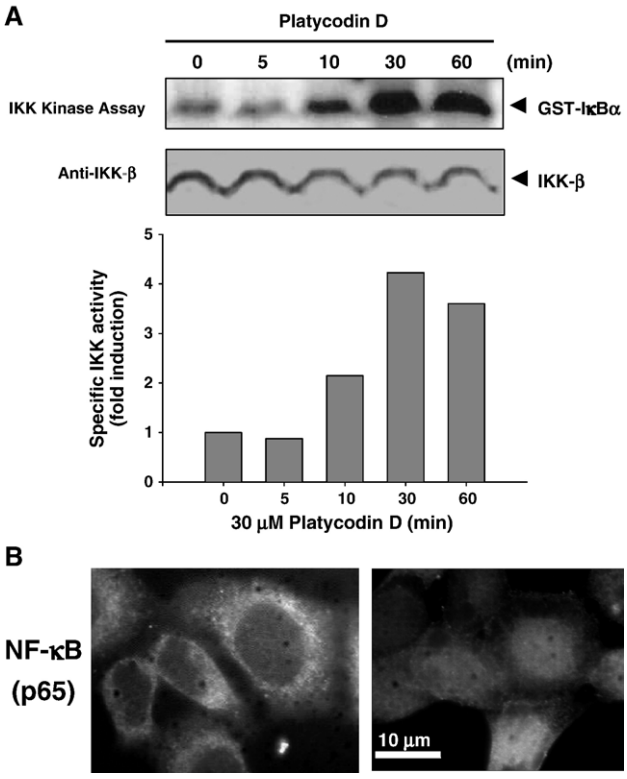

Fig. 6. Activation of IKK in response to platycodin D and immunocytochemical identification of NF-κB translocation in HaCaT cells treated with platycodin D. HaCaT cells were treated with platycodin D (30 μM) for the indicated times. IKK complex was immunoprecipitated with an anti-IKKβ monoclonal antibody, and the immune complex was subjected to an in vitro assay using a full-length IκBα (amino acids 1–317) as a substrate. Following the SDS-PAGE, the portion of the gel containing the substrate was dried and processed for autoradiography. The portion containing IKK was analyzed by Western blotting for IKKβ protein (A). The cells treated with 15 μM platycodin D for 1 h was stained with the anti-p65 antibody. The staining method is described in the text. Untreated cells (left), 15 μM platycodin D treated cells (right). Bar represents 10 μm (B).

3.8. Induction of apoptosis by platycodin D is dependent on Fas receptor and FasL proteins

Possible roles for platycodin D include the regulation of the cellular apoptosis threshold and the expression of receptors of ligands that induce apoptosis. We determined that membrane-mediated death receptors play crucial roles in transducing the apoptotic signals. Western blotting was performed to examine whether platycodin D induces Fas receptor and FasL proteins (Fig. 8A).

To confirm this data, flow cytometry was also applied to prove more correctly. Platycodin D stimulated the expression of Fas receptor and Fas ligand protein in HaCaT cells in a dose- and time-dependent fashion, with the optimized concentration being 30 μM (Supplementary data). The surface FasL protein was absent in resting HaCaT cells, and a significant induction of surface FasL expression was observed following a 20 μM platycodin D treatment. First, the constitutive Fas receptor expression in HaCaT cells, as determined by flow cytometry, increased a little in resting

HaCaT cells, but platycodin D also stimulated the expression of Fas receptor compared with those that were not treated. The increase of activation-induced cell death by platycodin D was due to the preferential stimulation of Fas receptor and FasL expression.

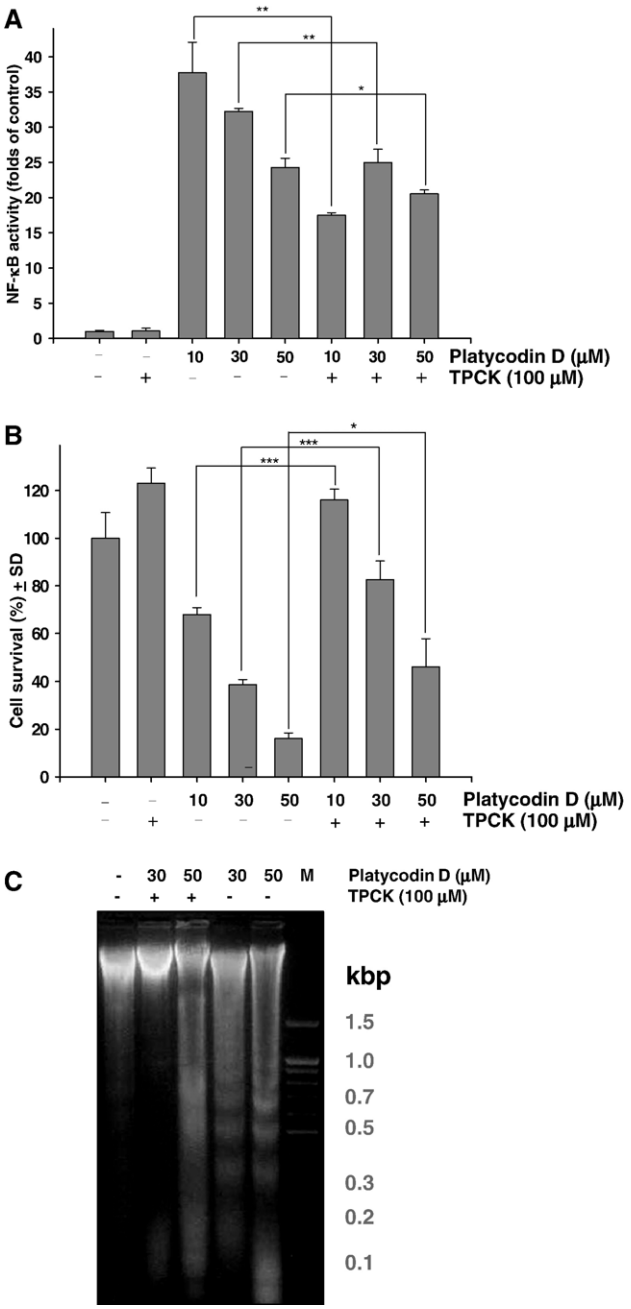

Fig. 7. Inhibitory effect of the potent NF-κB inhibitor in the process of platycodin D-induced apoptosis. HaCaT cells were pre-incubated for 30 min with TPCK (100 μM) before exposure to various concentrations of platycodin D. NF-κB activity was measured using our cell-based assay system (A). After 3 h of platycodin D treatment, cell viability was measured by CCK-8 (B). Percentages of survival cells are expressed as means±SD of three independent experiments. *$p<0.05$ and **$p<0.01$ (by Student's t test). Total DNA extracted from the cells was pre-incubated for 30 min with TPCK (100 μM) before exposure to various concentrations of platycodin D (C).

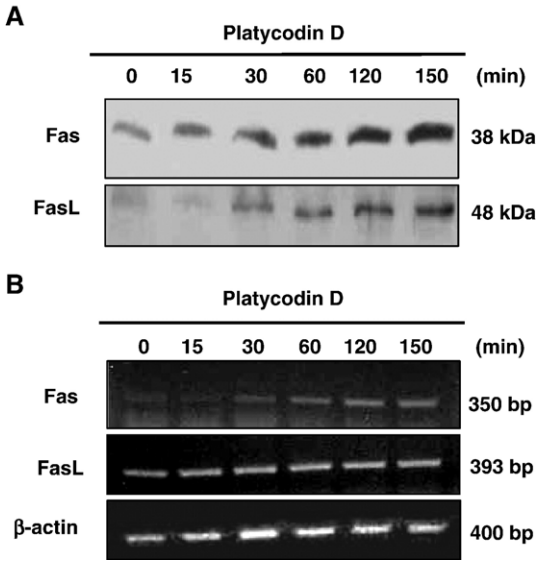

Fig. 8. Expression of proteins and mRNAs of Fas receptor and Fas ligand in HaCaT cells treated with platycodin D. Proteins were extracted from HaCaT cells treated with 30 μM platycodin D at various indicated times and subjected to Western blot analysis (A). Total RNA was prepared and the cDNA from the mRNA was amplified through 20 cycles. The resulting products of semi-quantitative RT-PCR were eletrophoresed in 1% agarose gels and were stained with ethidium bromide (B).

3.9. Activation of NF-κB induced Fas receptor and FasL mRNA expression in HaCaT cells

Because of the profound activation of the Fas receptor and FasL promoters by NF-κB signal transduction, the Fas receptor

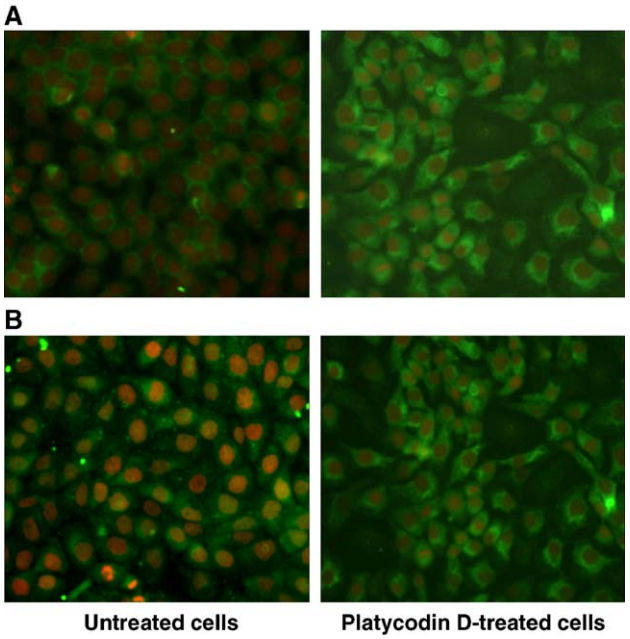

Fig. 9. platycodin D induces cell death by activating Fas receptor and FasL aggregation. Microscopic images of Fas receptor (A) and Fas aggregation (B) showing HaCaT cells that were untreated (left) or treated with 15 μM platycodin D for 3 h (right), then stained with FITC-conjugated anti-Fas and anti-FasL antibodies and visualized under fluorescence. Cells were viewed and photographed using a fluorescent microscope with 200× magnification.

and FasL expressions were further examined in terms of when NF-κB was activated by platycodin D. The activation by platycodin D triggered a significant increase of the cell surface FasL mRNA (Fig. 8B). But Fas mRNA levels monitored by RT-PCR were low or almost undetectable in normal conditions and were weakly increased after being in the presence of 30 μM platycodin D for 1 h, which suggests that a significant FasL upregulation occurred at the transcription level. Moreover, Fas receptor mRNA levels that were monitored illustrated a weak expression after being in the presence of 30 μM platycodin D for 2 h (Fig. 8B). Taken together, these results showed that early NF-κB activation induces the increase of FasL mRNA and protein levels together with the increase in Fas receptor expression, which may be responsible for the induction of apoptosis in human HaCaT cells.

3.10. Platycodin D induces apoptosis by activating Fas receptor and FasL aggregation

To observe whether the Fas receptor and FasL clustering exist on the cell surfaces, immunofluorescence microscopy was performed. Using a FITC-conjugated anti-Fas receptor and anti-FasL antibodies, it was demonstrated that the Fas receptor and FasL aggregated and most likely initiated the death signals. In the control cells (without platycodin D treatment), only a weak staining was observed (Fig. 9A, B, left). However, in the platycodin D-treated cells, a strong fluorescence staining was observed in a Fas receptor and FasL aggregates of the HaCaT cells (Fig. 9A, B, right).

3.11. Stimulation of Fas promoter activity by platycodin D

The HaCaT cells were transiently transfected with pFLF1 and were cultured for 18 h. Then, the cells were treated with 10–50 μM platycodin D for 3 h and the lysates were prepared from the transfected cells for the determination of luciferase activity. The luciferase activity increased up to three-fold compared to that of the control at 30 μM platycodin D, showing a dose-dependency and then decreased slightly at 50 μM platycodin D (Fig. 10). These results suggest that NF-κB is involved in the positive regulation of Fas receptor promoter. They are consistent with those of the EMSA and the cell-based assay as described above.

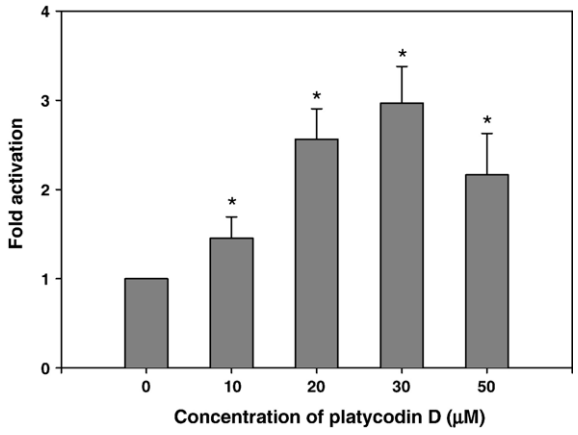

Fig. 10. Analysis of Fas receptor promoter activity by luciferase assay in HaCaT cells treated with platycodin D. HaCaT cells were transfected with the pFLF1 plasmid as described in Materials and methods and cells were cultured for 18 h. The luciferase activity level was measured in the HaCaT cells that were treated with 10−50 μM platycodin D according to the given time. The luciferase levels were normalized to the level of *Renilla* luciferase activity in each transfectant. Mean values and standard deviations from three independent experiments are shown. Significant differences in luciferase activity between untreated and platycodin D-treated are indicated by $*p<0.05$ (by Student's *t* test).

4. Discussion

In Oriental medicine, Platycodi Radix has been widely used for the treatment of several kinds of chronic inflammatory diseases (Ozaki, 1995). We investigated the mechanism of platycodin saponins, platycodin D, platycodin D_3, and platycodigenin (Fig. 1) on the induction of apoptosis in HaCaT cells. First, the NF-κB activity was measured using the cell-based assay system. Of the three compounds, platycodin D showed the highest tendency to activate the NF-κB level compared with platycodin D_3 and platycodigenin in HaCaT cells (Fig. 2B).

We previously confirmed that our cell-based assay system could monitor the NF-κB activation induced by lipopolysaccharides and ultraviolet rays in skin keratinocytes and macrophages (Ahn et al., 2003; Moon et al., 2001b). In the present study, several evidence was presented that platycodin D mediates the inflammatory response by activating NF-κB in immortalized skin keratinocytes. Contrary to the inhibition of NF-κB activation, which is usually observed in anti-inflammatory agents, platycodin D enhanced the level of NF-κB activity initially. We found that NF-κB was induced and activated in the early stage of the platycodin D treatment, and it activated the regulation of caspase-3. Therefore, in contrast to the anti-apoptotic activity of NF-κB, a new proapoptotic role for NF-κB was defined by platycodin D-treated in HaCaT cells. The apoptotic mechanism of platycodin D was reversely suppressed by a NF-κB inhibitor, TPCK (serine protease inhibitor) and decreased the apoptosis in HaCaT cells. From these results, the NF-κB activation is an important factor involving apoptosis in HaCaT cells. As an approach to investigate the effect of platycodin D on the activity of the IκB kinase complex that is composed of at least three subunits: two catalystic subunits, IKK-α and IKK-β, and a regulatory protein, IKK-γ, we carried out an immunoprecipitation of IKC with IKK-β antibody. This process was confirmed by Western blotting with an Antibody against IKK-β. In the subsequent in vitro kinase assay, the greatest activation was observed at the concentration of 30 μM for a 30 min treatment with platycodin D, whereas platycodin D slightly inhibited the DNA binding of NF-κB when it was treated for 1 h. These results suggest that the mechanism of NF-κB activation by platycodin D involves the activation of IKK-β but not IKK-α activity.

To demonstrate whether NF-κB plays a role in the promoter activity of the Fas receptor gene, cells were transfected with pFLF1 and luciferase reporter gene assay was performed. We confirmed that NF-κB is involved in the positive regulation of Fas receptor promoter activity in HaCaT cells stimulated with

platycodin D. This is consistent with the action of okadaic acid (Fujita et al., 2004; Goto et al., 2002). Accordingly, platycodin D is a potent inducer of NF-κB-dependent expression of Fas receptor. For the study of mechanism we could demonstrate that Fas receptor and FasL proteins were regulated in HaCaT cells and their levels were enhanced by the treatment of platycodin D. The expression of the Fas receptor and FasL mRNAs in HaCaT cells were also increased by this treatment, as determined by RT-PCR. These findings suggest that platycodin D upregulates the expression of the Fas receptor and the Fas ligand in HaCaT cells. The aggregated receptors recruit adapter proteins from the cytoplasm, which, in turn, activate caspases and their proapoptotic signals. According to the downstream mechanism, there was a clear time-dependent reduction in pro-caspase-8, following the reduction in pro-caspase-3, which is a key enzyme under apoptotic processes. But there was no change in the expression of p53, Bcl-2 or Bax expression, which is associated with mitochondrial-mediated intrinsic apoptosis (data not shown). We found that NF-κB was induced and activated in the early stage of the platycodin D treatment, and it upregulated the expression of the Fas receptor and FasL through the transcriptional mechanism. Therefore, in contrast to the anti-apoptotic activity of NF-κB, a new proapoptotic role for NF-κB was defined by mediating Fas-dependent apoptosis in HaCaT cells.

Although many compounds were isolated from the root of *Platycodon grandiflorum,* platycodin D showed more potent biological activities than any other platycodin saponins (Kim et al., 2001; Shin et al., 2002). From these reasons, we speculated that the chemical structure of platycodin D maintains biologically active form, which might be interacting with the surface of HaCaT cells. This phenomenon might affect HaCaT cells to activate NF-κB at the transcription level during apoptosis.

In conclusion, platycodin D is a class of triterpenoid saponins that induces apoptosis in HaCaT cells. Further studies on the apoptotic mechanism and potential applications to human health such as treatment of skin cancer are required in the near future.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejphar.2006.03.012.

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