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Cytochrome C-dependent Fas-independent apoptotic pathway in HeLa cells induced by Δ^{12}-prostaglandin J_2

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Abbreviations: Δ^{12}-PGJ_2, 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2; NF-κB, Nuclear factor κB; PG, prostaglandin; PPARγ, peroxisome proliferator activated receptor γ; Q-VD (OMe)-OPH, Q-Val-Asp (OMe)-CH_2-OPH; z-VAD-fmk, z-Val-Ala-Asp (OMe) fluoromethylketone

Abstract

Cyclopentenone prostaglandins (PGs) have antiproliferative activity on various tumor cell growth *in vitro*. Particularly, 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2 (Δ^{12}-PGJ_2) was reported for its antineoplastic and apoptotic effects on various cancer cells, but its mechanism inducing apoptosis is still not clear. In this study, we have characterized apoptosis induced by Δ^{12}-PGJ_2 in HeLa cells. Treatment of Δ^{12}-PGJ_2 induced apoptosis as indicated by DNA fragmentation, chromatin condensation, and formation of apoptotic body. We also observed release of cytochrome c from mitochondria and activation of caspase cascade including caspase-3, -8, and -9. And the pan-caspase inhibitor z-Val-Ala-Asp (OMe) fluoromethyl-ketone (z-VAD-fmk) and Q-Val-Asp (OMe)-CH_2-OPH (Q-VD (OMe)-OPH) prevented cell death induced by Δ^{12}-PGJ_2 showing participation of caspases in this process. However, protein expression level of Bcl-2 family was not altered by Δ^{12}-PGJ_2, seems to have no effect on HeLa cell apoptosis. And ZB4, an antagonistic Fas-antibody, exerted no effect on the activation of caspase 8 indicating that Fas receptor-ligand interaction was not involved in this pathway. Treatment of Δ^{12}-PGJ_2 also leads to suppression of nuclear factor κB (NF-κB) as indicated by nuclear translocation of p65/RelA and c-Rel and its DNA binding ability analyzed by EMSA. Taken together, our results suggest that Δ^{12}-PGJ_2-induced apoptosis in HeLa cell utilized caspase cascade without Fas receptor-ligand interaction and accompanied with NF-κB inactivation.

Keywords: Δ^{12}-prostaglandin J_2; apoptosis; cytochrome c; Fas

Introduction

Apoptosis is an evolutionary conserved process that removes damaged or unwanted cells. It is essential for animal development and tissue homeostasis, and when dysregulated, can result in cancer, neurodegenerative diseases, or autoimmune diseases (Thompson, 1995). Apoptosis is induced by a wide variety of cellular stresses such as DNA damage, UV radiation, ionizing radiation and oxidative stress (Nagata, 1997). Antitumor agents also induce apoptosis in some cancer cells both *in vitro* and *in vivo*, indicating that apoptosis plays a very important role in cancer chemotherapy (Kaufmann, 1989; Meyn *et al.*, 1995).

Cyclopentenone prostaglandin (PG) such as PGA_2 and 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2 (Δ^{12}-PGJ_2), enzymatic dehydration products of PGE_2 and PGD_2 respectively, have antineoplastic effects on the growth of various cancer cells (Fukushima *et al.*, 1982; Fukushima *et al.*, 1994). Previously, we described that when human hepatocarcinoma cells were exposed to Δ^{12}-PGJ_2, they showed characteristic morphological features of apoptosis such as chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. And intracellular glutathione level modulates the induction of apoptosis by Δ^{12}-PGJ_2 (Kim *et al.*, 1996). The expression of genes and their products, including p53, c-myc, heat shock protein 70, Sox-4 (Sry-HMG box gene) and SSF-1 (a novel splicing factor), are up- and down-regulated by presence of Δ^{12}-PGJ_2 in human hepatocarcinoma cells. And these genes and their products serve as a positive or negative regulator in Δ^{12}-PGJ_2-induced apoptotic pathway in tumor cells (Lee *et al.*, 1995; Ahn *et al.*, 1998; Ahn *et al.*, 1999; Ahn *et al.*, 2002). But the precise mechanism by which Δ^{12}-PGJ_2-initiates the cellular events resulting in cell death is not fully understood.

It was reported that Bcl-2 family protein regulate

the release of apoptotic cytochrome C by mitochondrial channel voltage dependent anion channel (VDAC) (Shimizu *et al*., 1999). In response to a variety of apoptotic stimuli, cytochrome C is released from its normal position within the intermembrane space of mitochondria to cytosol. Once released in the cytosol, cytochrome C binds with Apaf-1 and triggers oligomerization of Apaf-1/cytochrome C complex that activates procaspase 9. Activated caspase 9 in turn cleaves and activates caspase 3, caspase 6 and caspase 7, which function as downstream effector of cell death program (Thornberry and Lazebnick, 1998).

NF-κB is a transcription factor involved in the immune response as important regulators. NF-κB presents in most cell types in an inactive cytoplasmic form bound to its inhibitor protein IκB. A variety of stimuli such as TNFα and IL-1β release NF-κB from IκB inhibition (Baldwin, 1996; Paul, 1999), allowing it to translocate to the nucleus and exert transcriptional control on its target genes. NF-κB is known to be involved in several physiological processes, including the regulation of proliferation and apoptosis (Barkett and Gilmore, 1999; Baldwin, 2001). There is large evidence that NF-κB is related to anti-apoptotic effects. However, some reports have shown that NF-κB is also involved in the induction of apoptosis (Bernard *et al*., 2002), and relationship between c-Rel component overexpression and apoptosis is reported in numerous cell types (Abbadie *et al*., 1993). Also activation of Rel/NF-κB factors by cytokine withdrawal (Sohur *et al*., 2000), by paclitaxel (Huang *et al*., 2000), or by p53 (Ryan *et al*., 2000) are reported to promote apoptosis.

Although the potent apoptosis inducing effect of Δ^{12}-PGJ_2 highlights the possibility that Δ^{12}-PGJ_2 might be applied for the treatment of human cancer, the mechanism based on molecular events has not been fully elucidated. Here, we have evaluated possible apoptotic mechanism(s) of Δ^{12}-PGJ_2 in human cervical cancer HeLa cells by determining alteration of apoptosis-related molecules including bcl-2, bax, caspase 9, caspase 8, caspase 3, cytochrome C and NF-κB.

Materials and Methods

Reagents

Δ^{12}-PGJ_2 was obtained from BioMol (Plymouth Meeting, PA) and caspase inhibitors were from Enzyme Systems Products (Livermore, CA). Protease inhibitor cocktail was from Roche Molecular Biochemicals (Mannheim, Germany). Agonistic anti-Fas antibody (CH11) and antagonistic anti-Fas antibody (ZB4) were from Upstate Biotechnology (Lexington, KY). 3-(4,5-Dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and Hoechst 33342 were from Sigma (St. Louis, MO). Mouse anti-poly (ADP-ribose) polymerase was obtained from Serotec (Oxford, UK), anti-caspase 3, -8, cytochrome c, porin A were from Pharmingen (San Diego, CA) and anti-caspase 9, anti-NF-κB p65, anti-NF-κB p50, anti-NF-κB c-Rel, Bax, Bcl-2, Bcl-xL and NF-κB consensus oligomucleotide were from Santa Cruz (Santa Cruz, CA). Horseradish peroxidase-conjugated secondary antibody against rabbit and mouse IgG and anti-β-actin were from Sigma. Unless specified otherwise, all reagents were purchased from Sigma.

Cell culture and viability assay

Human cervical carcinoma HeLa cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 media supplemented with 10% heat-inactivated (56°C for 30 min) fetal bovine serum (Hyclone Laboratories Inc., Logan, UT) and 100 units/ml of penicillin/ streptomycin (Gibco BRL, Grand Island, NY) at 37°C in a humidified atmosphere of 5% CO_2.

To observe the effect of Δ^{12}-PGJ_2 on the growth of HeLa cells, cell viability was determined by MTT assay (Mossmann, 1983). In brief, cells were plated in 96-well plates and then treated with indicated concentrations of Δ^{12}-PGJ_2. After incubation of indicated times, MTT reagent was added to final concentration of 500 g/ml. Three hours later, MTT crystals were collected and dissolved with isopropanol containing 0.04 N of HCl and the absorbance was measured at 570 nm.

Evaluation of apoptosis

Induction of apoptosis was determined by fragmentation of genomic DNA and fluorescence microscopic examination of cells stained with Hoechst 33342. For genomic DNA extraction, cells were treated with Δ^{12}-PGJ_2 or with caspase inhibitors for indicated times, and then genomic DNA was extracted using DNA extraction buffer [5 mM Tris-Cl pH 8.0, 20 mM EDTA pH 8.0, 1% sodium dodecyl sulfate (SDS), 50 g/ml proteinase K] and extraction with phenol-ethanol was followed. DNA pellets were dissolved in TE buffer (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) containing 20 g/ml of RNase A and incubated at 37°C for 1 h. 5 g of DNA was separated on 1.8% agarose gels with 1 × TBE (20 mM Tris-acetate, 1 mM EDTA). DNA in the gel was stained with ethidium bromide and visualized under UV light and photographed. To observe morphological changes of nuclei, HeLa cells were treated with Δ^{12}-PGJ_2 or with caspase inhibitors for indicated times, and further incubated in the presence of Hoechst 33342 (final 10 M) for 10 min at 37°C. Cells were examined under fluorescence microscope (Carl Zeiss, Oberkochen, Germany).

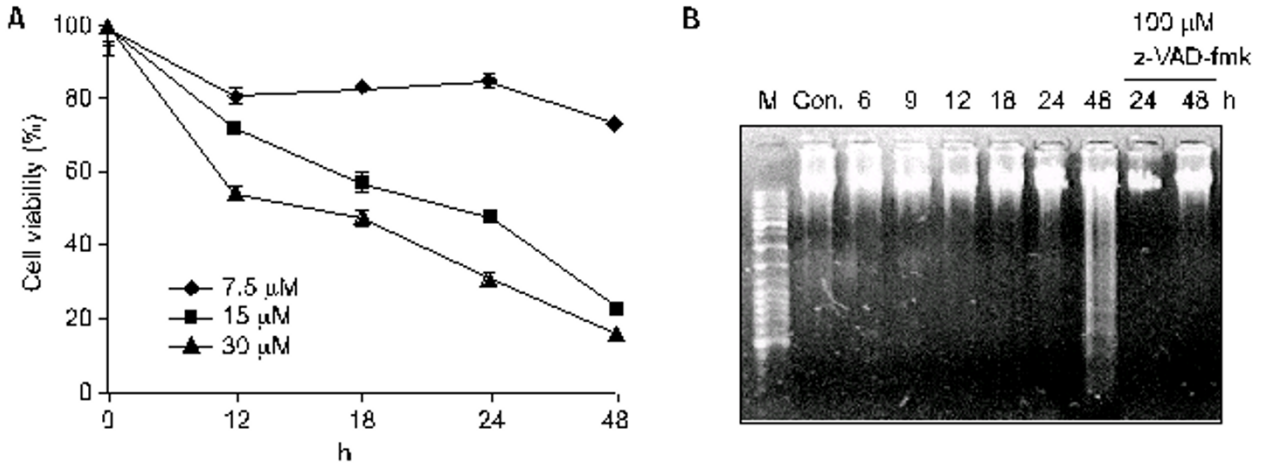

Figure 1. Δ^{12}-PGJ_2 induced growth inhibition and DNA fragmentation in HeLa cells. (A) HeLa cells were treated with 7.5 M (◆), 15 M (■) and 30 M (▲) of Δ^{12}-PGJ_2 in time-dependent manner and cell viability was measured by MTT assay. (B) 30 M of Δ^{12}-PGJ_2 were treated for indicated time and genomic DNAs were extracted and separated in 1.8% agarose gel. Last two lanes were preincubated with 100 M of z-VAD-fmk for 1 h, and then treated with 30 M of Δ^{12}-PGJ_2 for indicated time. All these results were represented from three independent experiments. M: 10 kb Marker.

Western blot analysis

Cells were harvested and lysed with RIPA buffer [1% Triton X-100, 20 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 1 mM EDTA] containing protease inhibitor cocktail. 30 g of each protein was separated in 12% SDS-polyacrylamide gel (PAGE), and transferred to a nitrocellulose membrane in transfer buffer [25 mM Tris base, 193 mM glycine, 20% methanol]. The membrane was blocked in 5% nonfat dried milk in phosphate-buffered saline (PBS) and then incubated with primary antibody in blocking solution at room temperature, followed by extensive washing in PBS containing 0.1% Tween-20. The blot was then incubated with peroxidase-conjugated secondary antibody in blocking solution at room temperature, followed by washing in PBS containing 0.1% Tween-20. Antigen was detected using the enhanced chemiluminescence western blotting detection system (Amersham-Pharmarcia, Buckinhamshire, UK).

Electrophoretic mobility shift assay (EMSA)

HeLa cells were treated with Δ^{12}-PGJ_2 for indicated time, washed twice with PBS. Nuclear extract for EMSA were prepared as described by Dignam *et al*. 10 g of nuclear extract and 1 g of poly (dI-dC) were incubated for 15 min in binding buffer [10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 0.5 mM EDTA, 1 mM $MgCl_2$, 0.5 mM DTT, 4% glycerol, 0.05 mg/ml poly (dI-dC)]. For supershift assay, antibodies of NF-κB p50 and p65 were added and incubated for 30 min. And then NF-κB consensus sequence, which was 3'-end labeled with ^{32}P were incubated for another 30 min. After binding reaction, samples were analyzed by 6% native polyacrylamide gel (PAGE), dried for 1 h and subjected to autoradiography.

Results

Induction of HeLa cell apoptosis by Δ^{12}-PGJ_2

To determine the apoptotic effect of Δ^{12}-PGJ_2, HeLa cells were treated with 7.5, 15 and 30 M of Δ^{12}-PGJ_2 in indicated time points. Δ^{12}-PGJ_2 treatment caused inhibition of HeLa cell growth in concentration- and time-dependent manner (Figure 1A). In case of high concentration of Δ^{12}-PGJ_2 (15 and 30 M), cell viability was decreased to 50% at least 18 h. Also after 48 h treatment, fragmentation of genomic DNA indicated by DNA ladder, were observed (Figure 1B). As shown in Figure 2, Δ^{12}-PGJ_2 treated HeLa cells also showed typical apoptotic morphologies including chromatin condensation, membrane blebbing, vacuolization, etc. These results do support that Δ^{12}-PGJ_2 induces apoptosis in HeLa cells.

Release of cytochrome c and activation of caspase cascade

We investigated whether caspase cascade should be involved in Δ^{12}-PGJ_2-induced apoptosis in HeLa cells. Cytochrome c was known to be released from mitochondria to cytosol response to death signal and activate caspase cascade, we examined the release of cytochrome c from mitochondria by Western blot analysis. As shown in Figure 3A, Δ^{12}-PGJ_2 released the cytochrome c to cytosol in time-dependent manner. Porin was detected as a reference mitochondrial protein for subcellular fractionation. Also, activation of

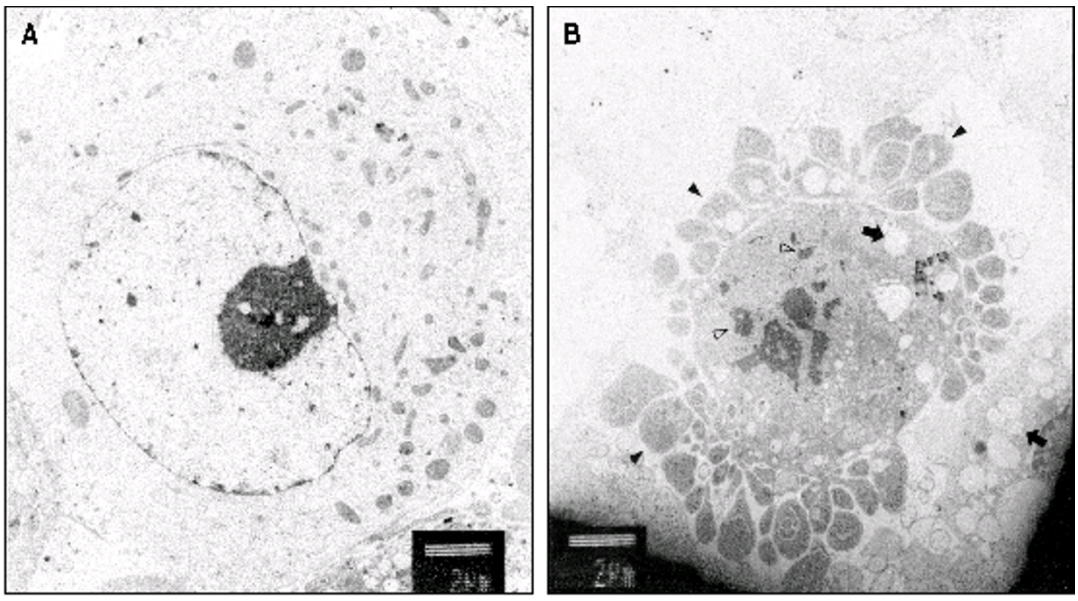

Figure 2. Δ^{12}-PGJ_2 induced apoptosis in HeLa cells. Electron microscopic images of control(A) and 30 M of Δ^{12}-PGJ_2 treated (B) HeLa cells (48 h; ×3,000 magnification). Δ^{12}-PGJ_2 induced the characteristic apoptotic features such as cell shrinkage, chromatin condensation (△), vacuolization (⬆) and formation of apoptotic bodies (▲) were shown above.

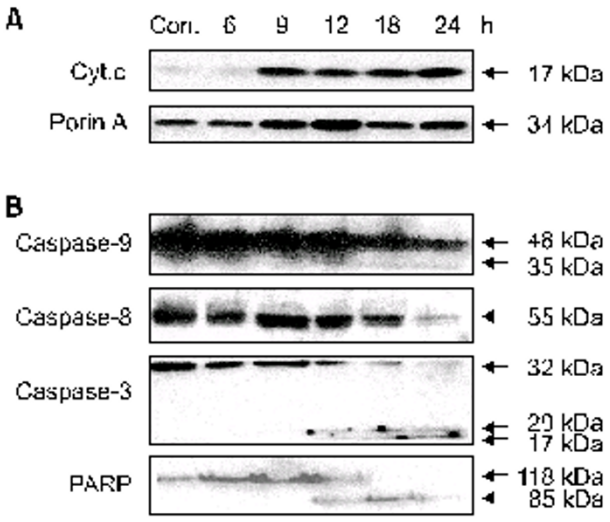

Figure 3. Release of cytochrome C from mitochondria and activation of caspase cascade induced by Δ^{12}-PGJ_2. (A) Cellular fractionation study of Δ^{12}-PGJ_2 treated HeLa cells. Lysate of Δ^{12}-PGJ_2 treated cells was fractionated and immunoblot assay was performed as described in Materials & Methods. Porin is a marker protein to confirm the mitochondrial fractionation. (B) 30 M of Δ^{12}-PGJ_2 activated caspase cascade in time-dependent manner.

casapse 9, 8 and 3 was observed and poly (ADP-ribose) polymerase (PARP), substrate of caspase 3 underwent degradation as caspase 3 was activated (Figure 3B).

To confirm the involvement of caspases in this pathway, the effects of specific caspase inhibitors were tested. HeLa cells were pretreated with 100 M of z-VAD-fmk and Q-VD (OMe)-OPH for 1h and treated with 30 M of Δ^{12}-PGJ_2 in succession for 24 h. Stained with Hoechst 33342, HeLa cells were observed under fluorescence microscope. Pretreatment with pan-caspase inhibitors abolished nuclear fragmentation and chromosome condensation induced by Δ^{12}-PGJ_2 (Figure 4). Also, pretreatment of pan-caspase inhibitor, z-VAD-fmk, blocked genomic DNA fragmentation in concentration of 100 M as shown in Figure 1B (the last two lanes). These results indicate that release of cytochrome c and the activation of caspase cascade play important role in Δ^{12}-PGJ_2-induced apoptosis in HeLa cells.

Expression levels of Bcl-2 family

Since bcl-2 and its homologues are important regulators downstream of mitochondria, changes in the expression of either pro-apoptotic or anti-apoptotic bcl-2 family members can affect apoptosis. We have measured the protein expression levels of Bcl-2, Bax, Bcl-xL by Western blot analysis. Figure 5 shows the changes in response to Δ^{12}-PGJ_2 of Bcl-2, bax and bcl-xL protein level in HeLa cells. Bcl-2 was almost undetectable in HeLa cells and Bax did not show any changes of protein levels. Although slight decrease of Bcl-xL by Δ^{12}-PGJ_2 occurred, no change in the relative level of Bcl-2 and Bax was observed as indicated by the protein levels remained largely unaltered.

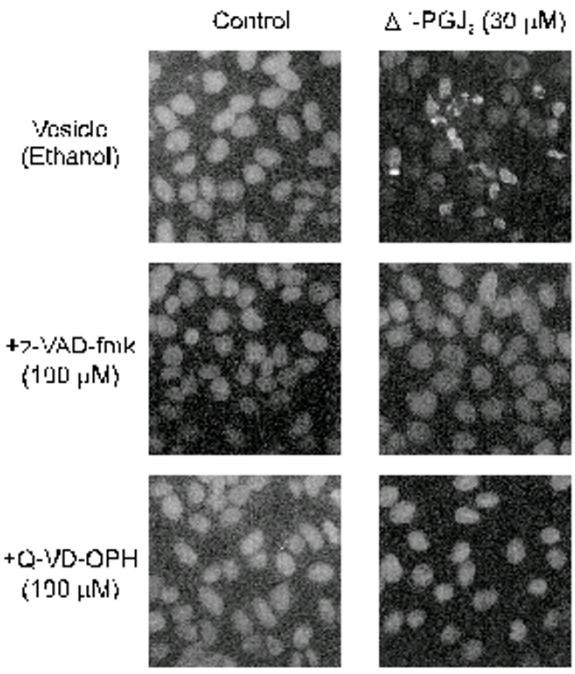

Figure 4. Effects of general caspase inhibitors, z-VAD-fmk and Q-VD(OMe)-OPH. Cells were pretreated with 100 M of z-VAD-fmk and Q-VD(OMe)-OPH for 1 h and then treated with 30 M of Δ^{12}-PGJ_2 for 24 h. Cells were stained with 10 M of Hoechst 33342 and observed under fluorescence microscope in ×40 magnification.

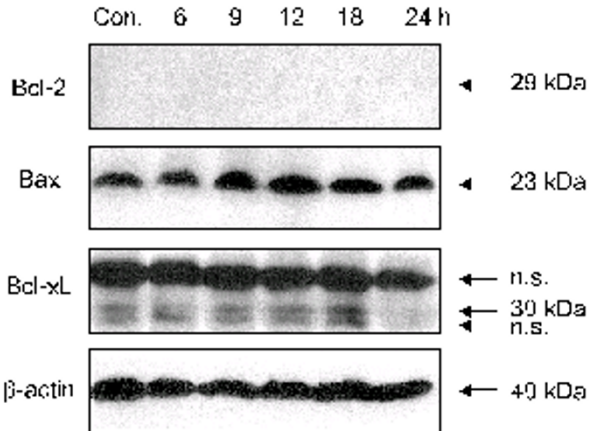

Figure 5. Change of the expression level in Bcl-2 family. HeLa cells were treated with 30 M of Δ^{12}-PGJ_2 for indicated time, and whole lysates were separated in 10% SDS-PAGE. Immunoblot assay was followed with Bcl-2, Bax and Bcl-xL antibodies. β-Actin was shown as control. n.s. indicates non-specific signals.

Effect of antagonistic Fas-antibody on Δ^{12}-PGJ_2-induced caspase 8 activation

Apoptosis can also be led by Fas receptor-ligand interaction through activation of caspase 8. To investigate whether the Fas receptor-ligand interaction is involved in HeLa cell apoptosis induced by Δ^{12}-PGJ_2,

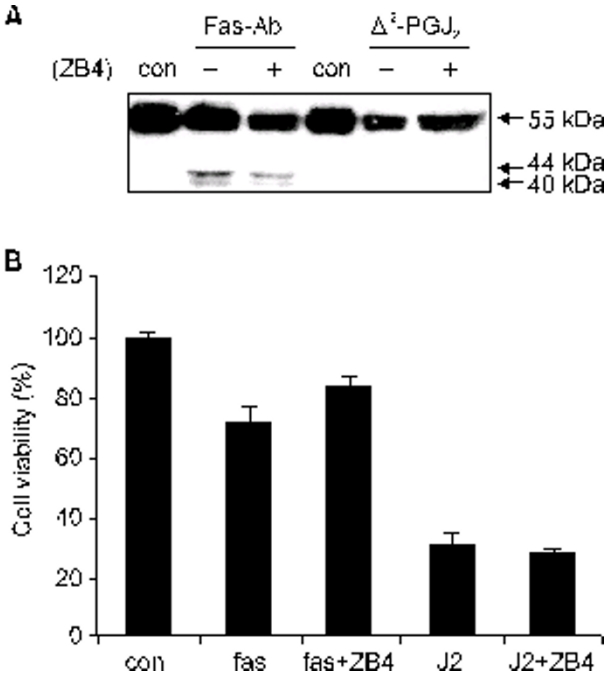

Figure 6. The effect of antagonistic Fas-Ab (ZB4) on the activation of caspase 8 induced by Δ^{12}-PGJ_2. (A) HeLa cells pretreated with or without 500 ng/ml of ZB4 were incubated with 30 M of Δ^{12}-PGJ_2 or CH11 (agonistic Fas-antibody; 1 g/ml) for 24 h. Cell lysates were subjected to 10% SDS-PAGE, and Western blot analysis was performed with caspase 8 antibody. (B) HeLa cells were treated as above (Figure 6A), and cell viability was determined by MTT assay. This experiment was performed in triplicate. Fas indicates for agonistic Fas-antibody CH11, J2 for Δ^{12}-PGJ_2 and ZB4 for anta-gonistic Fas-Ab, ZB4.

HeLa cells were pretreated with or without antagonistic Fas antibody ZB4 and then treatment of Δ^{12}-PGJ_2 and agonistic Fas antibody CH11 was followed. After 24 h, we observed activation of caspase 8 by Western blot assay. In Figure 6A, ZB4 inhibited the caspase 8 activation and reduced active fragment of caspase 8 by CH11. In Figure 6B, ZB4 pretreatment also recovered cell viability induced by CH11 up to 90% compared to 70% that of Fas-only treated group. But ZB4 pretreatment did not show any effects on Δ^{12}-PGJ_2 both in activation of caspase 8 and cell viability (Figure 6A, B). Thus, Fas receptor-ligand system does not appear to be involved in the activation of caspase 8 and activation of caspase 8 in Figure 3B seems to be induced by caspase 8-caspase 3 activation loop.

Suppression of NF-κB during Δ^{12}-PGJ_2-induced apoptosis

NF-κB is an ubiquitous transcription factor that translocates to nucleus and binds to a specific DNA sequence as a dimeric complex. Activation of NF-κB serves as a survival signal in response to various

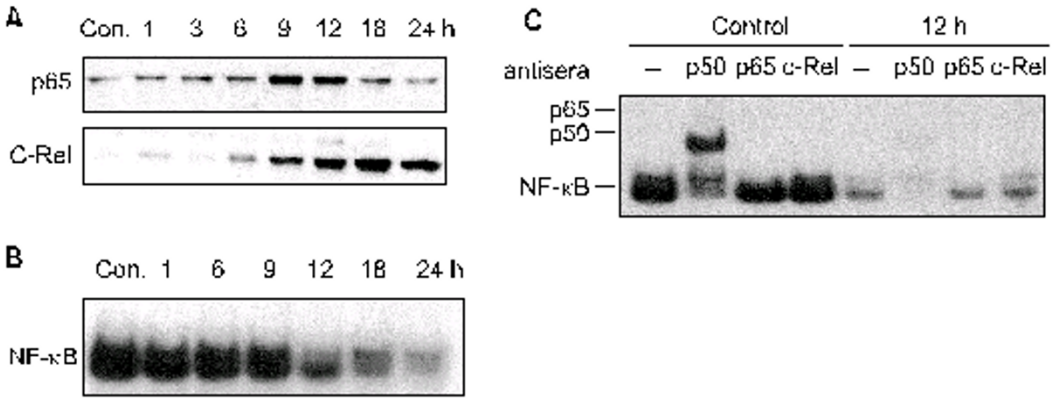

Figure 7. Translocation and DNA binding of NF-κB components. (A) Nuclear extracts of Δ^{12}-PGJ_2-treated HeLa cells were subjected to 10% SDS-PAGE, and Western blot analysis was performed using p65 and c-Rel antibodies. (B) Nuclear extracts (5 g) were prepared as described above (Figure 7A), and their DNA binding ability were examined by EMSA. (C) Supershift assay with antisera to NF-κB p50, p65 and c-Rel.

apoptotic stimuli. To investigate the effect of Δ^{12}-PGJ_2 on NF-κB activation, nuclear extracts were obtained and analyzed by Western blot assay. As shown in Figure 7A, Δ^{12}-PGJ_2 treatment of HeLa cells decreased expression of p65 in nuclear extracts after 12 h. On the contrary, c-Rel component resulted in a significant increment after 12 h. Treatment of Δ^{12}-PGJ_2 increased nuclear translocation of c-Rel and decreased that of RelA/p65 in time-dependent manner.

To test whether the nuclear translocation of NF-κB coincides with its DNA binding ability, κB-binding activity was analyzed by EMSA (Figure 7B). Δ^{12}-PGJ_2 significantly decreased NF-κB binding activity in time-dependent manner after 12 h of Δ^{12}-PGJ_2 treatment. The tendency of decreasing NF-κB activity corresponded with that of decreasing of p65 in nuclear extract after 12 h, and its tendency also agreed in reverse with c-Rel translocation to the nucleus.

In Figure 7C, supershift experiments were performed to determine the specific subunit constituents of the NF-κB complex. P65 and p50 subunits of NF-κB were shifted at control group, and those binding activity were decreased at 12 h, according with its EMSA results showing that major components of NF-κB are p50-p50 dimer and p50-p65 heterodimer.

Discussion

Our data demonstrated that loss of cell viability treated with Δ^{12}-PGJ_2 was due to induction of apoptotic cell death. They exhibited many of the typical characteristics of apoptosis including fragmentation of genomic DNA, cell shrinkage, membrane blebbing, chromatin condensation, and appearance of apoptotic bodies as shown in electron microscopic study (Figure 2). Recently apoptotic pathway is described in two major pathways. One is the old well-known caspase-dependent apoptotic pathway and the other is the apoptosis-inducing factor (AIF)-dependent apoptotic pathway (Hunot and Flavell, 2001). Former is started at Fas receptor-ligand interaction that activates caspase 8 and in turn, activates downstream caspases, or with release of mitochondrial cytochrome c leading to formation of apoptosome and activation of caspase cascades. Caspases are crucial effectors in apoptotic cell death that contribute to the morphological and biochemical changes. The latter employs a caspase-independent death effector AIF, which can leads to apoptotic pathway showing large-scale DNA degradation. In certain conditions, AIF can also act upstream of cytochrome c release, regulating the apoptosome-based apoptotic pathway, but this signaling pathway is still not clearly understood. In our study, we confirmed that cytochrome c release and subsequent activation of caspase cascade occurred when Δ^{12}-PGJ_2 is treated in HeLa cells (Figure 3), and these effects were blocked by pan-caspase inhibitor, z-VAD-fmk and Q-VD (OMe)-OPH, indicating that represented apoptotic characteristics of HeLa cells with Δ^{12}-PGJ_2 are endowed with caspase-dependent pathway. However, AIF also can regulate upstream of caspase cascade in ambiguous conditions, and the possibility that AIF is responsible to Δ^{12}-PGJ_2-induced apoptosis in HeLa cells should not be eliminated.

The pattern of caspase activation during Δ^{12}-PGJ_2-induced apoptosis was typical. First cytochrome c was released from mitochondria, and then caspase 9, 3, and -8 was activated in turn. Generally, caspase 8 is activated *via* Fas receptor-ligand interaction and acts as an upstream caspase of caspase 3 directly activating procaspase 3 or *via* truncation of Bid (Li *et al*., 1998; Breitschopf *et al*., 2000; Lutter *et al*., 2001). However, Δ^{12}-PGJ_2-induced apoptosis in HeLa cells, Fas receptor-ligand interaction turned out not to

be concerned in this process as shown in Figure 6. Caspase 3 is activated prior to the activation of caspase 8 and this situation can be explained by caspase 3-caspase 8 amplification loop (Slee *et al*., 1999). The activated caspase 9 cleaves procaspase 3, and in turn, caspase 3 activates caspase 8 by proteolysis. And caspase 8 acts as executionor caspase rather than initiator.

The Bcl-2 family of anti-(Bcl-2, Bcl-xL, Mcl-1) and pro-(Bax, Bad, Bid) apoptotic proteins are also important in the regulation of cell death. But in this process, bcl-2 family protein expression was not altered by Δ^{12}-PGJ_2, and it seems that bcl-2 family does not appeared to be linked in Δ^{12}-PGJ_2-mediated apoptosis in HeLa cells.

Many chemotherapeutic reagents have been shown to induce apoptosis in various cancer cells. It is important to understand how each drug act to induce apoptosis on certain cancer cells. Cyclopentenone prostaglandins (PGs) were reported as its anti-inflammatory effects and apoptosis-inducing activity in various tumor cells. Δ^{12}-PGJ_2 was reported as effective apoptosis inducing agent (Lee J-H *et al*., 1995; Kim H-S *et al*., 1996), and also reported as natural ligand of peroxisome proliferator activated receptor γ (PPARγ), inducing apoptosis via PPARγ-dependent pathway in certain conditions (Nishida *et al*., 2002; Zander *et al*., 2002). But in this study, we did not considered about the possibility of PPARγ-activated pathway, because HeLa cells turned out not to express PPARγ (data not shown). Our western blot data of PPARγ also coincided with previous studies, and we did not carry out further experiment to check PPARγ activity.

Another specific aspect of this apoptosis mechanism is suppression of NF-κB. NF-κB is a ubiquitous transcription factor that is involved in the immune response. NF-κB remain sequestered in the cytoplasm in an inactive form bound to IκBα, an inhibitory protein of NF-κB. Activation and nuclear translocation of NF-κB regulates expression of various genes which play important role in the immune and inflammatory responses. In Δ^{12}-PGJ_2-induced apoptosis in HeLa cells, translocation of p65/RelA component to nucleus was decreased after 12 h, and at the same time DNA binding activity of NF-κB also decreased. These results show that p50-p50 dimer and p50-p65 heterodimer maybe exist as major form at the basal levels of HeLa cells. This result also agrees with supershift assay results in Figure 7C which show supershifted bands on p50 and p65 antisera-treated lanes both in control and 12 h treated groups, and justifies p50-p50 dimer and p50-p65 heterodimer might be major form in this conditions.

As shown in Figure 7B, treatment of Δ^{12}-PGJ_2 suppressed DNA binding activity of NF-κB, and we assumed that NF-κB plays an important role as survival signal in HeLa cells, and Δ^{12}-PGJ_2 suppresses it's signaling pathway somehow. One possibility that we can consider about is the role of translocation of c-Rel component. Translocation of c-Rel to nucleus increases as Δ^{12}-PGJ_2-induced apoptosis proceed, and this is opposite phenomenon to that of p65 component (Barkett M and Gilmore TD, 1999; Huang Y *et al*., 2000; Sohur US *et al*., 2000). NF-κB can, under some circumstances, also promote cell death. The precise mechanisms that can determine the anti- or pro-apoptotic role for NF-κB have not been clearly defined yet. Maybe changes in the transcription factors that function cooperatively with NF-κB at different promoters would have the effect of switching its activity from one set of genes to another. It has recently shown that activation of the tumor suppressor and transcription factor p53 can lead to nuclear localization of NF-κB (Ryan KM *et al*., 2000). In these experiments, NF-κB was required for p53-mediated apoptosis. Also it is possible that p53 modulation of NF-κB function through such a mechanism might determine the pro- or anti-apoptotic function of NF-κB. Also anti- and pro-apoptotic effects of NF-κB, specially c-Rel component, occur in the same cell via the up-regulation of manganese superoxide dismutase (Bernard *et al*., 2002). It is possible that Δ^{12}-PGJ_2 can also act as p53 in this case. But we can not prove the role of c-Rel in Δ^{12}-PGJ_2 induced apoptosis in HeLa cells yet.

Present study was aimed at investigating the intracellular apoptotic signaling mechanisms in HeLa cells response to Δ^{12}-PGJ_2. We found that in HeLa cells, Δ^{12}-PGJ_2 mediated the release of cytochrome c prior to the activation of caspase cascade and presented the possibility of NF-κB involved in the Δ^{12}-PGJ_2-induced apoptosis. But pro-apoptotic effects of c-Rel in Δ^{12}-PGJ_2-induced apoptotic process should be studied further.

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