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Induction of p53 and apoptosis by Δ^{12}-PGJ_2 in human hepatocarcinoma SK-HEP-1 cells

Jeong-Hwa Lee, Ho-Shik Kim, Seong-Yun Jeong, In-Kyung Kim*

Department of Biochemistry, Catholic University Medical College, 505 Banpo-Dong, Socho-Ku, Seoul 137-701, South Korea

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Abstract **Human hepatocarcinoma cells (SK-HEP-1) were induced to die through apoptosis by treatment with Δ^{12}-prostaglandin (PG)J_2, as characterized by the appearance of a typical DNA ladder. The induction of apoptosis by Δ^{12}-PGJ_2 was specifically blocked by cycloheximide (CHX). Western analysis using anti-p53 or anti-WAF1 monoclonal antibodies demonstrated that these two protein levels were increased 3 h after Δ^{12}-PGJ_2 treatment, and accumulated for up to 12 h. The induction of p53 protein seemed to be dependent on the increase of p53 mRNA level, which was inhibited by CHX treatment. However, delayed addition of CHX after Δ^{12}-PGJ_2 treatment failed to affect both p53 mRNA levels and DNA fragmentation following Δ^{12}-PGJ_2 treatment, indicating that the inhibition of p53 synthesis may contribute to the protective effect of CHX against Δ^{12}-PGJ_2-mediated cytotoxicity. Therefore, our results suggest that the initial events caused by Δ^{12}-PGJ_2, leading ultimately to SK-HEP-1 cell death, involve a certain process required for p53 induction. However, the finding that Δ^{12}-PGJ_2 is also active against Hep 3B cells which are devoid of a functional p53 indicates that p53 may not be the critical requirement for inducing apoptosis by Δ^{12}-PGJ_2.**

Key words: Δ^{12}-PGJ_2; Apoptosis; p53

1. Introduction

Δ^{12}-PGJ_2, one of the cyclopentenone PGs containing an unsaturated carbonyl group, has been reported to have potent growth inhibitory and differentiation inducing activities in some tumor cells [1–3]. It has been shown that Δ^{12}-PGJ_2 is actively transported to nuclei and binds to nuclear proteins [4,5], and binding of the PG molecule to nuclear proteins appears to regulate the expression of specific genes which are responsible for growth inhibition [6]. Recently, we have reported that cyclopentenone PGs cause cell death via an apoptotic pathway in murine leukemia L1210 cells, which was preceded by cell cycle arrest in G2/M and dependent upon protein synthesis [7]. Thus, DNA damage and specific gene expression seemed to be involved in Δ^{12}-PGJ_2-mediated apoptosis. However, the molecular mechanisms by which Δ^{12}-PGJ_2 lead cells to apoptotic cell death has not yet been determined.

p53, a nuclear phosphoprotein, is the product of a tumor suppressor gene which is related to cellular response to DNA damage resulting in cell cycle arrest or induction of apoptosis [8–10]. Thus, loss of normal p53 function may abolish the ability to repair or delete the DNA-damaged cells, which contributes to the development and progression of cancer [11,12]. In addition, p53 status is expected to affect the outcome of cellular response after exposure to cytotoxic agents [10,13]. The growth inhibitory activity of p53 is apparently mediated by transcriptional activation of several genes involved in cell proliferation such as WAF1/CIP1 and GADD [14–17].

To determine the involvement of p53 in Δ^{12}-PGJ_2-induced apoptosis, we investigated the change of p53 as well as WAF1 levels following Δ^{12}-PGJ_2 treatment in relation to the induction of apoptosis in human hepatocarcinoma SK-HEP-1 cells which are reported to have wild-type p53 [18]. We also examined the effects of cycloheximide (CHX) on the induction of apoptosis and p53 accumulation by Δ^{12}-PGJ_2 treatment.

2. Materials and methods

2.1. Reagents

Δ^{12}-PGJ_2 was kindly supplied by Ono Pharmaceutical Co. (Osaka, Japan) and a stock solution (10 mg/ml) was prepared in 95% ethanol. Cycloheximide (CHX), actinomycin D (Act-D), proteinase K and RNase A were purchased from Sigma (St. Louis, MO) and DNA size markers were obtained from Gibco BRL (Gaithersburg, MD). All other chemicals used were of reagent grade.

2.2. Cells and treatment protocols

Human hepatocarcinoma SK-HEP-1 cells and Hep 3B cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and maintained in RPMI 1640 medium containing 10% heat-inactivated fetal bovine serum and 50 μg/ml of gentamicin. Cells were plated at a density of 1×10^5/ml on a 96-well microtiter plate or a 6-well culture plate in 100 μl or 1 ml of medium, respectively, and incubated with various agents for the indicated times. In some experiments, CHX was added to each well at the specified times after Δ^{12}-PGJ_2 treatment, and incubation was continued for the remaining period before the assessment of DNA fragmentation or p53 induction.

2.3. Cell viability and assessment of apoptotic DNA fragmentation

Cell viability was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method [19]. DNA integrity in SK-HEP-1 cells undergoing apoptosis was evaluated by agarose gel electrophoresis followed by ethidium bromide staining. Cells were lysed and digested overnight at 48°C in lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM EDTA, 10 mM NaCl and 0.5% SDS) containing proteinase K (100 μg/ml). A 1/5 volume of 5 M NaCl and an equal volume of isoamyl alcohol were added to precipitate DNA. The DNA pellet was redissolved in TE buffer (10 mM Tris-HCl, pH 7.8, 1 mM EDTA) and treated with RNase A (0.1 mg/ml) for 4 h at 37°C prior to 1.5% agarose gel electrophoresis.

Quantification of DNA fragmentation was determined by detection of 5-bromo-2′-deoxy-uridine (BrdU)-labeled DNA fragments in the cytoplasm of SK-HEP-1 cells [20] using a cellular DNA fragmentation ELISA kit (Boehringer-Mannheim, Mannheim, Germany). Morphological analysis of the cells undergoing apoptosis was performed by Giemsa staining.

2.4. Western blot analysis

For immunoblots, 1×10^5 cells were lysed in Laemmli lysis buffer [21]

*Corresponding author. Fax: (82) (2) 596-4435.

Abbreviations: PG, prostaglandin; CHX, cycloheximide; Act-D, actinomycin D.

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and cellular proteins were separated by 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then electroblotted onto nitrocellulose papers. After blocking with 5% non-fat milk in phosphate buffered saline, the papers were incubated with a mouse monoclonal antibody against human p53 (Ab-2; Oncogene Science, Cambridge, MA) or human WAF1/CIP1 (Ab-1; Oncogene Science) for 1 h at room temperature. Proteins were revealed by enhanced chemiluminescence system (Amersham, Buckinghamshire, UK) using horseradish peroxidase-conjugated goat anti-mouse immunoglobulin G as a secondary antibody.

2.5. Northern blot analysis

Total RNA was extracted from cells using a commercial system provided by Amresco (Solon, OH). Equal amounts of RNA were electrophoresed through 1% formaldehyde agarose gels and then transferred onto nylon filters. The blots were hybridized with digoxigenin (DIG)-labeled specific DNA probes overnight at 50°C. p53 and β-actin cDNA probes were prepared by polymerase chain reaction with DIG-11-UTP (Boehringer-Mannheim) using specific primers provided by Clontech (Palo Alto, CA). The blots were washed and incubated with alkaline phosphatase-conjugated anti-DIG antibody and then the hybridized probes were immunodetected with chemiluminescent substrate supplied by Boehringer-Mannheim.

3. Results

When SK-HEP-1 cells were incubated with 5 μg/ml of Δ^{12}-PGJ_2, cells began to lose viability after 24 h as determined by the MTT method (Fig. 1A), whereas morphological changes representative of apoptosis such as loss of cell-cell contact, chromatin condensation and reduction of cytoplasmic volume appeared as early as 6 h after treatment (data not shown). The quantitative assay of BrdU-labeled DNA fragments demonstrated that DNA fragmentation significantly increased within 24 h of Δ^{12}-PGJ_2 incubation (Fig. 1B), concurrent with the loss of viability.

Electrophoretic analysis of total cellular DNA isolated from Δ^{12}-PGJ_2-treated SK-HEP-1 cells revealed a pattern of internucleosomal DNA cleavage, which is specific for apoptosis (Fig. 2). Fig. 2 also shows that the induction of ladder DNA was completely blocked by a protein synthesis inhibitor, CHX.

To assess the involvement of p53 in Δ^{12}-PGJ_2-mediated apoptosis, p53 expression in the SK-HEP-1 cells following Δ^{12}-PGJ_2 treatment was examined. Western blot analysis demonstrated that p53 levels began to increase at 3 h and remained high for up to 12 h after treatment (Fig. 3A). We also observed the induction of WAF1, a critical downstream mediator of p53-dependent apoptosis, with the increase in the levels of p53 (Fig. 3A). It appears that the accumulation of p53 protein after Δ^{12}-PGJ_2 treatment is dependent on the increase of p53 mRNA levels as seen in Fig. 3B, rather than on a post-transcriptional mechanism which was suggested in other cells treated with Act-D or UV [8,22]. We then examined the effect of CHX on the induction of p53 , WAF1 and p53 mRNA, since CHX was shown to completely block DNA fragmentation caused by Δ^{12}-PGJ_2 in Fig. 2. Fig. 4 shows that the elevated expression of p53 (and subsequently WAF1) as well as p53 mRNA, which were induced by Δ^{12}-PGJ_2 treatment, were completely prevented by CHX. These results indicate that the protective effect of CHX on Δ^{12}-PGJ_2-induced apoptosis may be due to the inhibition of

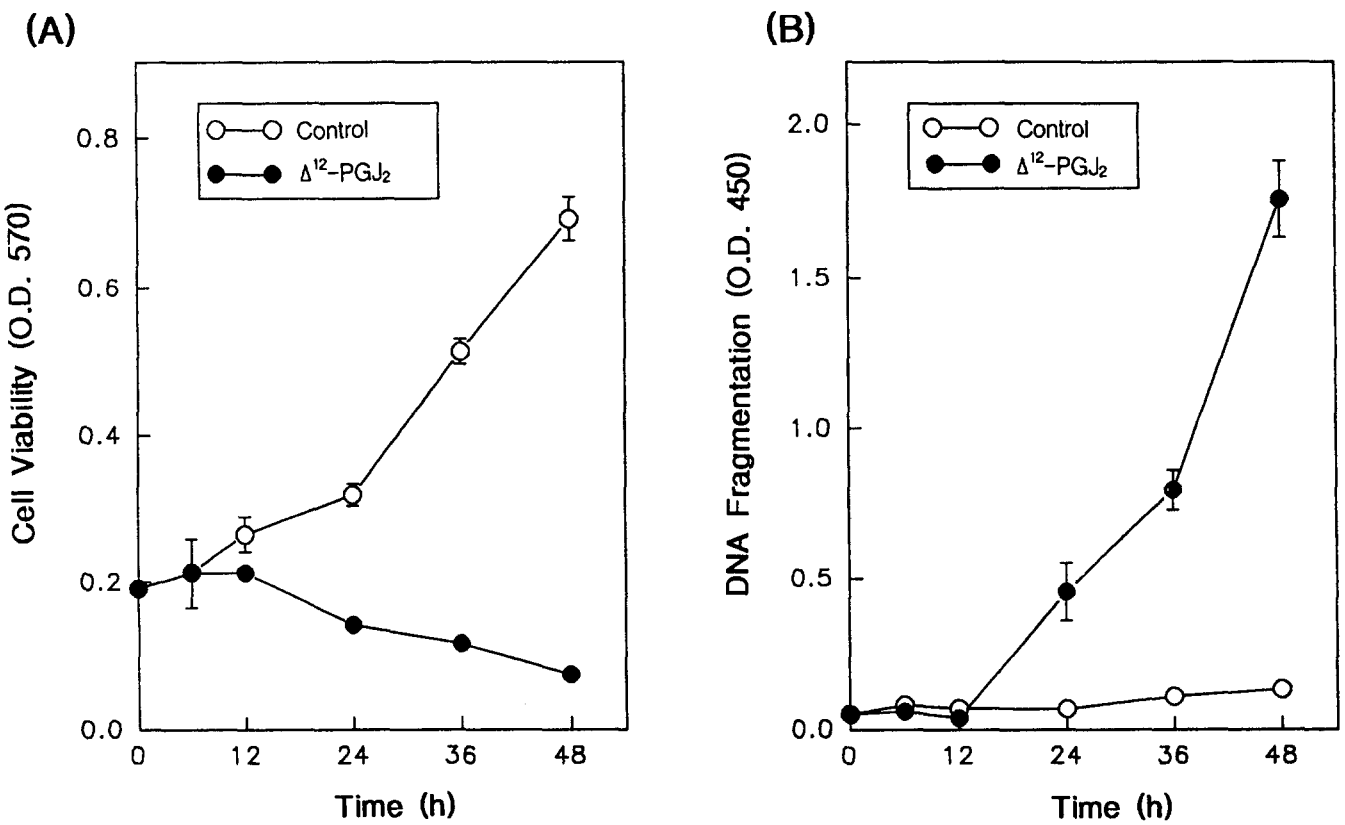

Fig. 1. Kinetics of cell death (A) and DNA fragmentation (B) in SK-HEP-1 cells following Δ^{12}-PGJ_2 treatment. SK-HEP-1 cells were incubated with 5 μg/ml of Δ^{12}-PGJ_2 in a 96-well microtiter plate in triplicate for the indicated times and the viability was assessed by the MTT methods (A). DNA fragmentation was analyzed by evaluating the presence of BrdU-labeled DNA fragments as described in section 2 (B).

p53 induction, and that p53 mRNA accumulation after Δ^{12}-PGJ_2 treatment is evidently an active process requiring the synthesis of new proteins.

To investigate further the relationship between p53 expression and the apoptotic response following Δ^{12}-PGJ_2 treatment, CHX was added at various times. Incubation was continued for a total of 9 h for assessment of p53 mRNA expression or for 48 h for analysis of DNA fragmentation respectively. As shown in Fig. 5, only when added simultaneously or 1 h after Δ^{12}-PGJ_2 treatment, CHX was able to block both the apoptosis and p53 mRNA accumulation. Addition of CHX 3 h after Δ^{12}-PGJ_2 treatment, at which time p53 mRNA levels were not shown to be affected (Fig. 5A), failed to prevent subsequent apoptotic cell death (Fig. 5B). These results, taken together, suggest that p53 may play a role in initiating the apoptotic response of SK-HEP-1 cells exposed to Δ^{12}-PGJ_2, and that the increase in p53 mRNA is regulated by certain proteins which were induced sufficiently within 3 h after Δ^{12}-PGJ_2 treatment.

In Fig. 6 we observed that Act-D also induced p53 and WAF1 protein expression as well as a clear ladder pattern in SK-HEP-1 cells, all of which were suppressed by CHX. In contrast, northern blot analysis of total RNA from Act-D treated SK-HEP-1 cells revealed no significant change in p53 mRNA levels (data not shown) indicating that Δ^{12}-PGJ_2 and Act-D induce p53 expression by different mechanisms.

To determine whether Δ^{12}-PGJ_2 induces apoptosis only through a p53-dependent pathway as suggested above, we examined DNA integrity after Δ^{12}-PGJ_2 treatment in Hep 3B cells in which the p53 transcripts were not detected due to a large deletion of p53 gene [18]. In Fig. 7 DNA from Hep 3B cells treated with a higher concentration (10 μg/ml) of Δ^{12}-PGJ_2 presented a typical ladder pattern, which was also blocked by CHX treatment, suggesting that Δ^{12}-PGJ_2-induced apoptosis is not wholly dependent upon p53 induction.

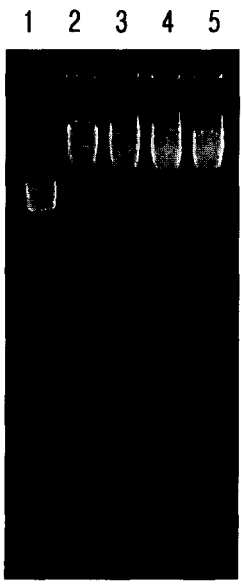

Fig. 2. Electrophoretic analysis of DNA from SK-HEP-1 cells. DNA was analyzed on a 1.5% agarose gel and stained with ethidium bromide. Cells were treated as follows: Lanes: 1 = 123 DNA size marker; 2 = untreated control cells; 3 = cells incubated with 1 μg/ml of CHX; 4 = cells incubated with 5 μg/ml of Δ^{12}-PGJ_2; 5 = cells incubated with 5 μg/ml of Δ^{12}-PGJ_2 in the presence of 1 μg/ml of CHX for 48 h.

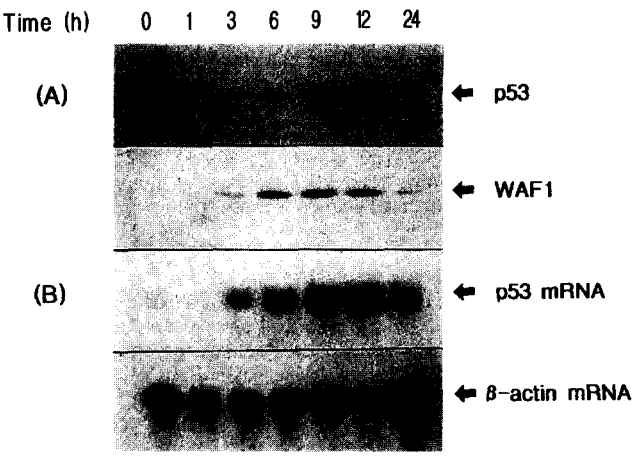

Fig. 3. Levels of p53 and WAF1 proteins (A) and p53 mRNA (B) following Δ^{12}-PGJ_2 treatment. (A) Immunoblot analysis of p53 and WAF1 proteins. Total cellular proteins from 1×10^5 cells were subjected to SDS-PAGE at the indicated times after exposure to 5 μg/ml of Δ^{12}-PGJ_2. p53 positive bands were revealed by an indirect immunoperoxidase method using enhanced chemiluminescence detection reagents as described in section 2. (B) Northern blot analysis of p53 mRNA. RNA was isolated from each preparation and electrophoresed through a formaldehyde agarose gel. The transferred RNA was hybridized with DIG-labeled probes for p53 and β-actin and processed for detection as described in section 2.

4. Discussion

It has been shown that expression of wt p53 leads cells to G1 arrest or apoptotic cell death in response to a variety of DNA damaging agents including UV irradiation, Act-D and topoisomerase inhibitors [8,23]. The results presented in this paper raised the possibility that p53 induction is involved in Δ^{12}-PGJ_2-mediated apoptosis in SK-HEP-1 cells. Our results show that p53 and WAF1/CIP1 levels were increased by Δ^{12}-PGJ_2 with incubation time and attenuated by CHX, correlating with the protective activity of CHX against apoptotic death. But the molecular details of the induction of DNA damage and subsequent p53 expression by Δ^{12}-PGJ_2 remain to be elucidated, although the α, β-unsaturated carbonyl moiety was suggested to be required for the biological activities of the cyclopentenone PGs [24–26]. However, the manner in which Δ^{12}-PGJ_2 increased p53 levels did not seem to be shared with Act-D or UV damage in that Δ^{12}-PGJ_2 primarily increased p53 mRNA levels, whereas Act-D or UV caused an accumulation of p53 protein without detectable changes in p53 mRNA expression, as shown in our experiments and previous reports [8,22].

Our results also demonstrated that active synthesis of certain proteins is necessary to elevate the level of p53 mRNA, thereby initiating apoptosis in SK-HEP-1 cells treated with Δ^{12}-PGJ_2. The requirement of protein synthesis for PG cytotoxicity has been suggested by several investigators [27,28] and it was also shown that the cyclopentenone PGs altered protein synthesis patterns showing induction or suppression of specific proteins in some cells [29,30]. However, any associations between these PGs-induced proteins and the cytotoxic effect of PGs are not yet established. Here we have shown that the proteins which were induced and accumulated before 3 h following Δ^{12}-PGJ_2 treatment may be essential for inducing apoptosis in our system, probably by participating in the increase in p53 mRNA,

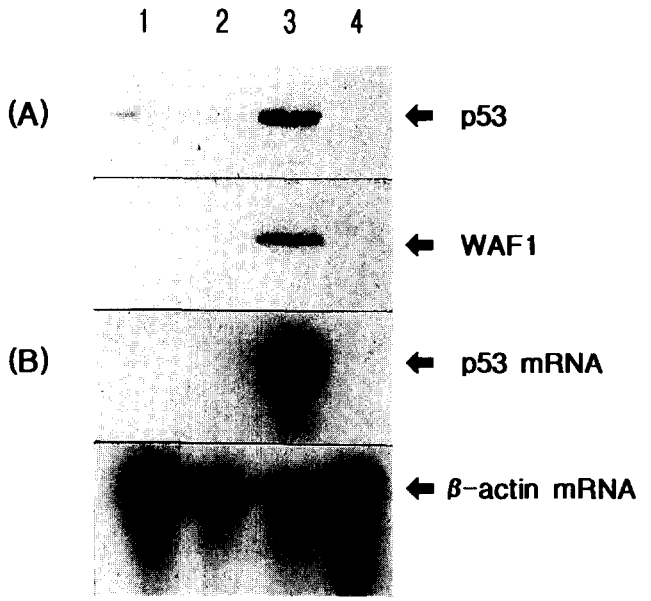

Fig. 4. Inhibition of Δ^{12}-PGJ_2-induced p53/WAF1 expression by CHX. SK-Hep-1 cells were incubated with 1 μg/ml of CHX (lane 2), 5 μg/ml of Δ^{12}-PGJ_2 alone (lane 3), or both agents (lane 4) for 9 h and then processed for detection of p53 and WAF1 proteins (A) or p53 and β-actin mRNA (B) as in Fig. 3. Lane 1 represents the results from control cells.

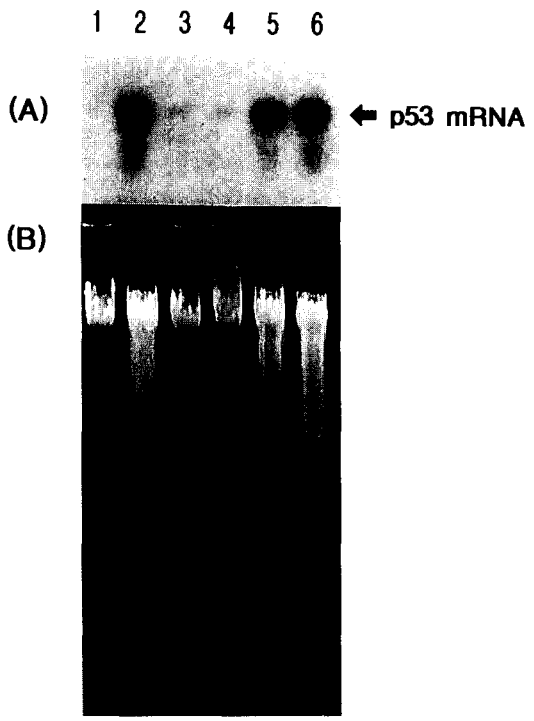

Fig. 5. Delayed addition of CHX after Δ^{12}-PGJ_2 treatment failed to prevent the induction of apoptosis and p53 mRNA. SK-HEP-1 cells were incubated without (lane 1) or with 5 μg/ml of Δ^{12}-PGJ_2 (lane 2). CHX (1 μg/ml) was added at 0, 1, 3, 6 and 9 h (lane 3–7, respectively) after Δ^{12}-PGJ_2 treatment. After 9 h of total incubation, the p53 mRNA level was determined by Northern analysis (A) and DNA fragmentation was also assessed by agarose gel electrophoresis after 48 h of total incubation (B).

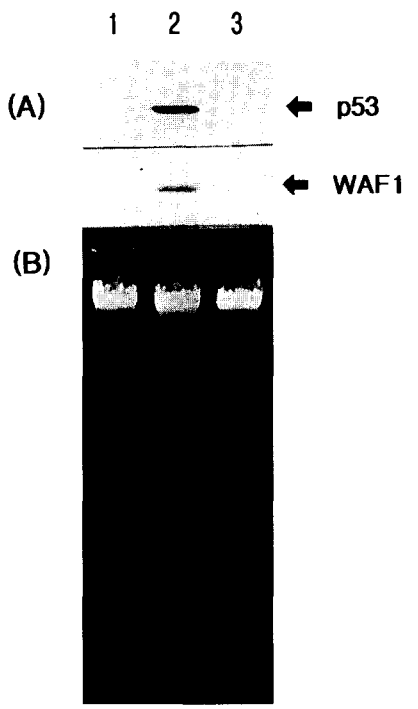

Fig. 6. Induction of DNA fragmentation and expression of p53 and WAF1 proteins in SK-HEP-1 cells by Act-D. Sk-HEP-1 cells were treated Act-D alone (lane 2) or Act-D plus CHX (lane 3) for 48 h and then analyzed for DNA fragmentation as in Fig. 2. (A) The expression of p53 and WAF1 proteins were determined by western blotting after 9 h of incubation (B). Lane 1 shows the results from untreated control cells.

since CHX addition later than 3 h could neither protect cells from entering apoptotic death nor prevent the increase in p53 mRNA.

It is likely, therefore, that the Δ^{12}-PGJ_2-induced proteins may function as transcriptional activators of p53. It has been shown that the p53 promoter is activated by several proteins such as NF-κB, c-myc and p53 itself [31–34]. In addition, NF-κB and c-myc were reported to be induced by DNA damage [35,36]. Thus, we can postulate that one of these p53 transactivator proteins may be a candidate for the Δ^{12}-PGJ_2-induced protein, or may be activated indirectly by a newly synthesized protein after Δ^{12}-PGJ_2 treatment. However, the possibility that the Δ^{12}-PGJ_2-induced proteins may be involved in the stabilization of p53 mRNA, rather than in transactivation, can not be excluded at present.

Despite the correlation of induction between p53 and apoptosis in SK-HEP-1 cells, p53 seems not to be the critical determinant of Δ^{12}-PGJ_2-induced apoptosis, considering that Δ^{12}-PGJ_2 also induced apoptosis in Hep 3B cells devoid of a functional p53. An alternative apoptotic pathway, therefore, may be also activated by Δ^{12}-PGJ_2 to initiate the cell death program, where continued protein synthesis is still indispensable. Otherwise, p53 induction may be an accompanying phenomenon of some early events induced by Δ^{12}-PGJ_2, which involve specific protein synthesis and eventually lead to apoptotic cell death irrespective of p53 status. Either p53 expressing or p53 deficient cells, anyhow, obviously require specific protein synthesis for

Fig. 7. Analysis of DNA fragmentation in Hep 3B cells exposed to Δ^{12}-PGJ_2. Hep 3B cells were exposed to 10 μg/ml of Δ^{12}-PGJ_2 alone (lane 2) or in the presence of 1 μg/ml of CHX (lane 3) for 48 h prior to assessment of DNA by 1.5% agarose gel electrophoresis. Lane 1 is DNA from untreated control cells.

apoptosis induction, although it is not certain whether the same protein is involved in both cells. Therefore, further investigations aimed at identifying a newly synthesized protein by Δ^{12}-PGJ_2 and its subsequent targets, if present, will provide more comprehensive understanding of the exact mechanism through which Δ^{12}-PGJ_2 induced apoptosis.

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