Volume 321, number 2,3, 209–214 FEBS 12353 April 1993

Prostaglandin A_2 and Δ^{12}-prostaglandin J_2 induce apoptosis in L1210 cells

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Received 12 February 1993

Treatment of L1210 cells with prostaglandin A_2 (PGA_2) or 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2 (Δ^{12}-PGJ_2) resulted in significant G2/M arrest and subsequent DNA fragmentation at concentrations that are cytotoxic to the cells. On agarose gel electrophoresis, DNA ladder formation was evident 24 h after the addition of Δ^{12}-PGJ_2 and remained apparent through 72 h, whereas G2/M accumulation was observed 6 h after the treatment. When the morphology of cells was examined by electron microscopy, L1210 cells incubated with a cytotoxic dose of PGA_2 or Δ^{12}-PGJ_2 for 24 h showed the characteristic morphological features of apoptosis such as chromatin condensation, nuclear fragmentation and formation of apoptotic body. Cycloheximide blocked the DNA fragmentation and morphological changes induced by Δ^{12}-PGJ_2. Our results suggest that these cyclopentenone PGs caused apoptotic cell death of L1210 cells which is preceded by G2/M accumulation and requires de novo protein synthesis.

Δ^{12}-prostaglandin J_2; Antiproliferative activity; DNA fragmentation; Apoptosis

1. INTRODUCTION

Cyclopentenone prostaglandins (PGs) such as PGA_2 and 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2 (Δ^{12}-PGJ_2) have potent antiproliferative activity on various cultured tumor cells [1–3]. It has been shown that these PGs block cell progression from the G1 to S phase [4]. Recently, Narumiya and coworkers reported that these PGs are actively transported into cells by a specific carrier on the cell surface and accumulated in the nuclei and that this uptake and accumulation are closely related to their growth inhibitory activity [5–7]. But the precise molecular mechanism by which these PGs exert cytostatic or cytotoxic activity remains unclarified.

Apoptosis is a controlled process of cell death which is involved in various physiological conditions including normal cell turnover, hormone-induced tissue atrophy, embryological development and effector cell mediated target cell lysis [8–10]. It is also observed in toxin-induced cell lysis and in vitro tumor cell death by cytokines such as tumor necrosis factor (TNF) and transforming growth factor-β (TGF-β) [11–13]. The cells undergoing apoptosis showed characteristic morphological features [14] and internucleosomal DNA cleavage yielding fragments in multiples of 180 bp, called ladder DNA, on agarose gel electrophoresis [15].

To investigate the mechanism for cytotoxic activity of cyclopentenone PGs, we examined the effects of these PGs on the DNA integrity, the ultrastructural morphology and cell cycle progression in L1210 cells. Our study revealed that these PGs lead to cell death via endogenous apoptotic pathway which is preceded by G2/M arrest and requires continued protein synthesis.

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Abbreviations: PG, prostaglandin; Δ^{12}-PGJ_2, 9-deoxy-$\Delta^{9,12}$-13,14-dihydro PGD_2; TNF, tumor necrosis factor; TGF-β, transforming growth factor-β.

2. MATERIALS AND METHODS

2.1. *Chemicals*

PGA_2, D_2 and E_2 were purchased from Sigma (St. Louis, MO) and Δ^{12}-PGJ_2 was kindly provided by Ohno Pharmaceutical Company (Osaka, Japan). The DNA size marker (123 ladder DNA) was obtained from BRL (Bethesda, MD) and all other reagents were of analytical grade.

2.2. *Assay of antiproliferative activity*

L1210 murine leukemia cells were obtained from American Type Culture Collection (ATCC, Rockville, MD) and cultured in RPMI 1640 medium supplemented with 10% heat-inactivated fetal calf serum and 50 μg/ml of gentamicin. Cells were plated at a density of 5×10^4/ml in 24-well culture plate and incubated with various concentrations of PGA_2 and Δ^{12}-PGJ_2. The number of viable cells was counted daily by Trypan blue exclusion test.

2.3. *Gel electrophoresis and measurement of fragmented DNA*

DNA fragmentation in L1210 cells was analyzed after the cells (2×10^6/dish) were treated with PGA_2 or Δ^{12}-PGJ_2 for indicated times. In some experiments, cells were preincubated with cycloheximide (up to 0.5 μg/ml) or actinomycin-D (2 ng/ml) for 1 h before the addition of Δ^{12}-PGJ_2. Cells were lysed in lysis buffer (500 mM Tris-HCl (pH 9.0), 2 mM EDTA, 10 mM NaCl, 1% (w/v) SDS)) and treated with proteinase K (1 mg/ml) for 24–48 h as described by Kaufmann et al. [16]. DNA was extracted and 5 μg/ml of DNA was analyzed by 1% agarose gel electrophoresis as described [17]. DNA was visualized by staining with ethidium bromide (10 μg/ml) under UV light.

DNA fragmentation was also quantitated by measuring soluble DNA fragments after cell lysis. After incubation of Δ^{12}-PGJ_2 for 48 h, cells were lysed, centrifuged at $13,000 \times g$ for 10 min and the amount

of DNA in both the pellet (intact DNA) and the supernatant (DNA fragments) was measured by using the diphenylamine reagent [18]. Calf thymus DNA was used as a standard. Fragmentation was calculated as percentage of DNA recovered in the supernatant as described previously [19].

2.4. *Examination of cell morphology*

L1210 cells treated with PGA_2 or Δ^{12}-PGJ_2 for 24 h were processed for electron microscopic examination as described by Reynold et al. [20]. The ultrathin sections were mounted on nickel grids, stained with uranyl acetate and lead citrate and photographed with a JEOL type 1200 EX (Tokyo, Japan) electron microscope at an accelerating voltage of 80 kV.

2.5. *Flow cytometric analysis*

To determine the effect of PGA_2 or Δ^{12}-PGJ_2 on the cell cycle progression of L1210 cells, cells were isolated at indicated times following treatment and analyzed using DNA reagent kit, Cycle TEST, obtained from Becton-Dickinson (San Jose, CA) and Coulter Epics profile II Flow cytometer (Hialeah, FL).

3. RESULTS

In our preliminary test, L1210 cells showed cytotoxic response to PGA_2 and Δ^{12}-PGJ_2 at a concentration of 10 μg/ml and 2.0 μg/ml, respectively. Incubation of L1210 cells with cytotoxic concentration of PGA_2 or Δ^{12}-PGJ_2 resulted in extensive degradation of DNA. As shown in Fig. 1, electrophoresis of total cellular DNA from PGA_2 or Δ^{12}-PGJ_2 treated L1210 cells revealed a typical ladder pattern of DNA fragments consisting of multiples of 180 bp. In non-treated control cells DNA fragmentation was not observed, while DNA from necrotic cells showed smear pattern of random degradation.

PGA_2 and Δ^{12}-PGJ_2 are known to be the enzymatic dehydration product of PGE_2 and PGD_2, respectively [3,12]. The cytotoxic activity of PGE_2 and PGD_2 appeared at higher concentrations. When examining the effect of PGE_2 and PGD_2 on DNA degradation, internucleosomal DNA cleavage was also caused by a cytotoxic concentration of these PGs in L1210 cells (data not shown).

Typical ladder pattern became evident 24 h after the

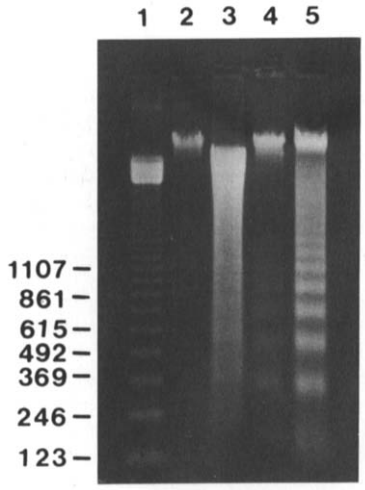

Fig. 1. Electrophoretic analysis of DNA from L1210 cells treated with PGA_2 or Δ^{12}-PGJ_2. L1210 cells were incubated without (lane 2) or with 10 μg/ml of PGA_2 (lane 4) or 2.5 μg/ml of Δ^{12}-PGJ_2 (lane 5) for 48 h. 5 μg of DNA from each sample was analyzed on 1% agarose gel electrophoresis as described in section 2. For comparison, DNA from necrotic cells incubated for 72 h after heating for 30 min at 56°C was shown in lane 3. Lane 1 is a 123 bp DNA ladder as marker.

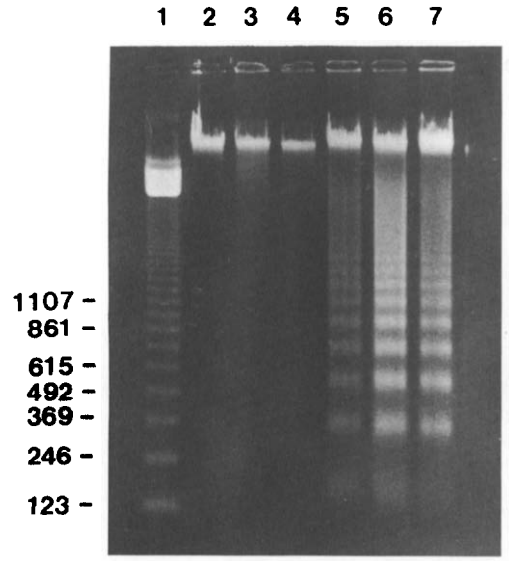

Fig. 2. Time course of DNA fragmentation in L1210 cells by Δ^{12}-PGJ_2. L1210 cells were incubated with 2.5 μg/ml of Δ^{12}-PGJ_2 for 0, 6, 12, 24, 48, or 72 h (lanes 2–8, respectively). At indicated times, DNA was isolated and analyzed as described in Fig. 1. Lane 1 represents a 123 bp DNA ladder as marker.

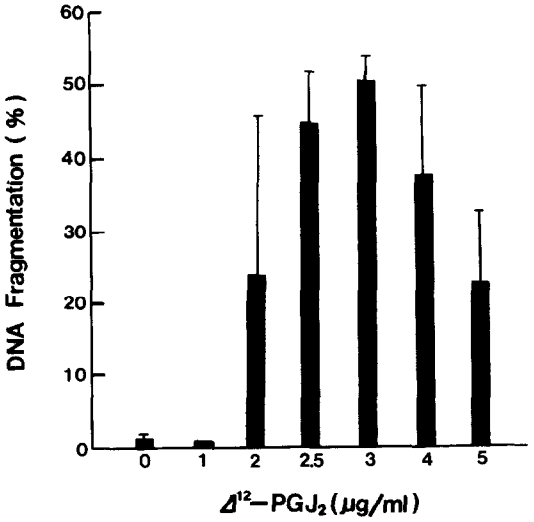

Fig. 3. DNA fragmentation in L1210 cells treated with various concentrations of Δ^{12}-PGJ_2. Cells were treated 0–5 μg/ml of Δ^{12}-PGJ_2 for 48 h and the cell lysate was centrifuged at 13,000 × *g* for 10 min. DNA fragmentation was determined by measuring DNA in the supernatant and the pellet with diphenylamine reagent. Experiments were done in triplicate.

treatment of Δ^{12}-PGJ_2 and persisted for up to 72 h (Fig. 2).

The quantitative analysis of DNA fragmentation induced by Δ^{12}-PGJ_2 is shown in Fig. 3. With 1 μg/ml of Δ^{12}-PGJ_2, which only shows a growth inhibitory effect rather than a cytotoxic effect, there was no difference in the percentage of fragmented DNA as compared with the control. The content of fragmented DNA was significantly increased in cells treated with a cytotoxic dose of Δ^{12}-PGJ_2 (≥ 2 μg/ml), reached maximum at 3 μg/ml and decreased at higher concentrations.

One of the interesting features of apoptosis is its dependency on the active synthesis of proteins under many circumstances [19,21]. So we investigated whether cycloheximide, known to affect apoptosis, can protect L1210 cells against Δ^{12}-PGJ_2-induced cytotoxic process. Fig. 4A shows that 0.5 μg/ml of cycloheximide administered 1 h before the addition of Δ^{12}-PGJ_2 completely blocked DNA fragmentation. Actinomycin-D at 2 ng/ml concentration did not inhibit DNA fragmentation. Higher concentrations of actinomycin-D could not be tested because actinomycin-D alone caused DNA fragmentation (data not shown). Using the diphenylamine assay, we also observed that the percentage of fragmented DNA gradually decreased to control levels by increasing the concentration of cycloheximide up to 0.5 μg/ml, indicating that protein synthesis is essential for the induction of DNA fragmentation by Δ^{12}-PGJ_2 (Fig. 4B).

We then examined the morphological changes induced by PGA_2 or Δ^{12}-PGJ_2 by electron microscopy to confirm the apoptotic nature as suggested by the preceding findings. The L1210 cells treated with PGA_2 or Δ^{12}-PGJ_2 for 24 h showed convolution of the nuclear membrane, fragmentation of the nucleus and formation of apoptotic body (Fig. 5A), and chromatin condensation, membrane blebbing and marked vacuolization (Fig. 5B), which are typical morphological features of apoptosis. But the cells treated with cycloheximide failed to show such findings representative of apoptosis (data not shown).

Next, we performed cell cycle analysis in order to determine whether the changes in cell cycle progression are associated with the antiproliferative activity of these PGs in L1210 cells. As shown in Fig. 6, the cells treated with a growth inhibitory dose of Δ^{12}-PGJ_2 (1 μg/ml) showed G2/M accumulation at 18 h after initiating the treatment, and then cells were released from this blockage and returned to almost normal levels at 48 h of incubation. But the treatment of L1210 cells with a cytotoxic dose of Δ^{12}-PGJ_2 (2 μg/ml) caused more profound and persistent G2/M arrest which was evident at 6 h and at a maximum at 18 h following the treatment. PGA_2 also caused L1210 cells to be blocked at the G2/M phase (data not shown). Since only the cells which have intact DNA were analyzed in our cell cycle study and DNA fragmentation was evident at 24 h after the

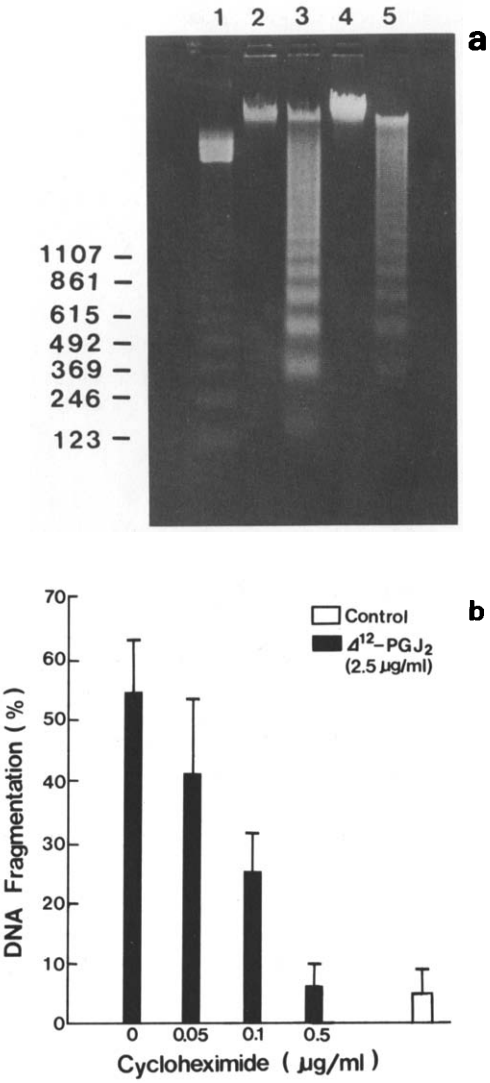

Fig. 4. Inhibition of DNA fragmentation by cycloheximide. (A) L1210 cells were preincubated for 1 h without pretreatment (lane 3), with 0.5 μg/ml of cycloheximide (lane 4), or with 2 ng/ml of actinomycin-D (lane 5). After 48 h of additional incubation with 2.5 μg/ml of Δ^{12}-PGJ_2, cells were lysed and DNA was analyzed by agarose gel electrophoresis as described in Fig. 1. Lane 1 is marker and lane 2 is DNA from control cells. (B) Cells were pretreated with various concentrations of cycloheximide for 1 h and further incubated for 48 h in the presence of 2.5 μg/ml of Δ^{12}-PGJ_2. DNA fragmentation was quantitatively determined using diphenylamine reagent as described in Fig. 3.

treatment (Fig. 2), the decrease in the cell proportion of the G2/M phase and concomitant increase of G1 phase cells observed after 24 h (Fig. 6C) might be due to the subsequent DNA degradation of G2/M arrested cells, not due to the actual progress to the G1 phase. Incubation of L1210 cells with 0.5 μg/ml of cycloheximide 1 h prior to the treatment with Δ^{12}-PGJ_2 inhibited the accumulation of cells at the G2/M phase (Fig. 6D) whereas actinomycin-D had no effect on the cell cycle arrest induced by Δ^{12}-PGJ_2 (data not shown).

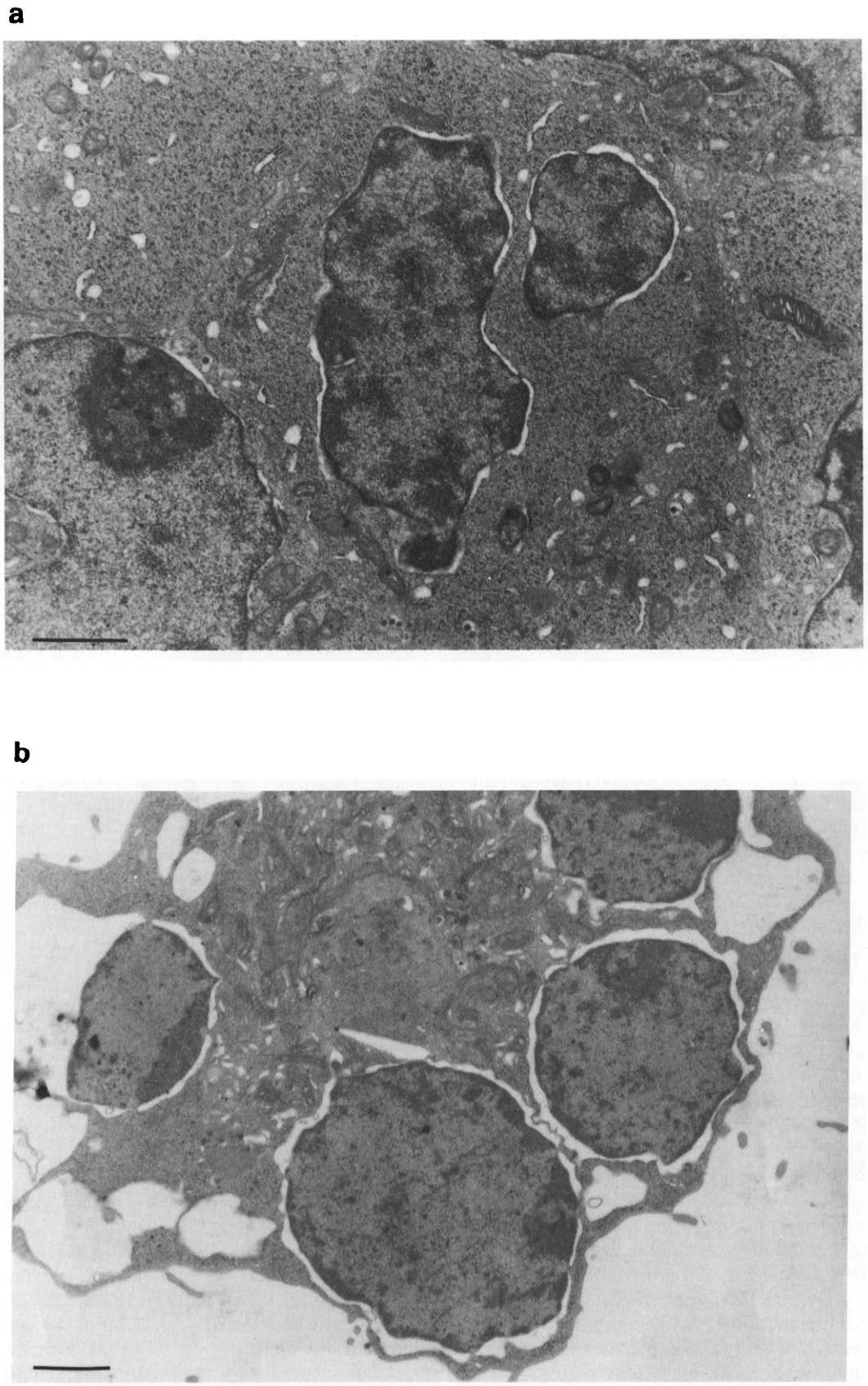

Fig. 5. Electron micrographs of PGA_2 or Δ^{12}-PGJ_2 treated L1210 cells. Cells were treated with 10 μg/ml of PGA_2 (a) or 2.5 μg/ml of Δ^{12}-PGJ_2 (b) for 24 h and prepared for electron microscopic examination as described in section 2. Bars, 1 μm.

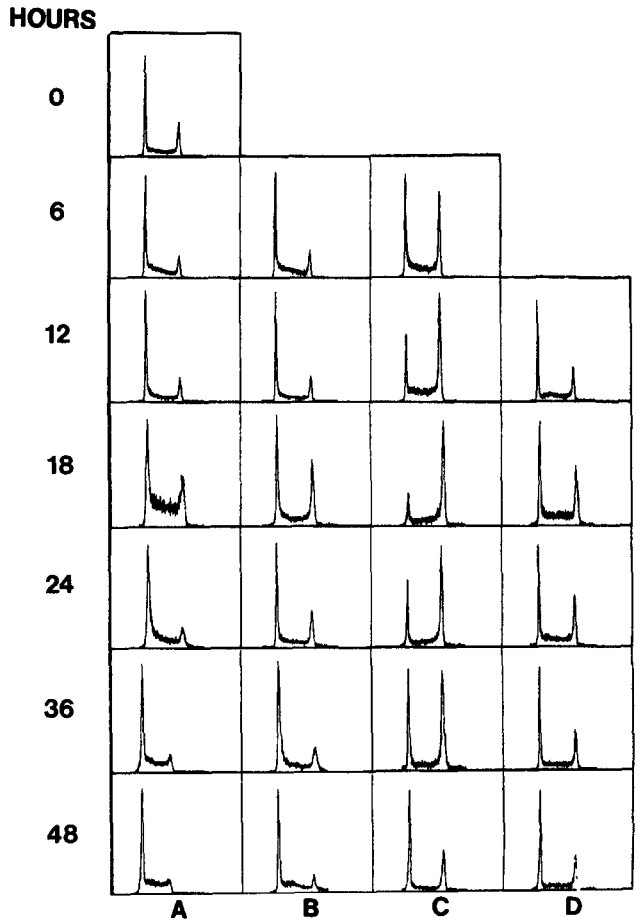

Fig. 6. Effect of Δ^{12}-PGJ_2 on the cell cycle progression of L1210 cells. The flow cytometric patterns were determined at indicated times after the treatment as described in section 2. Treatments as follows: A, control; B, 1 μg/ml of Δ^{12}-PGJ_2; C, 2 μg/ml of Δ^{12}-PGJ_2; D, 0.5 μg/ml of cycloheximide 1 h prior to the addition of 2 μg/ml of Δ^{12}-PGJ_2.

4. DISCUSSION

In the present study, we demonstrate that the L1210 cells incubated with a cytotoxic dose of PGA_2 or Δ^{12}-PGJ_2 showed many of the cardinal signs of apoptosis, indicating that cytotoxicity induced by these PGs is mediated via an endogenous apoptotic pathway. Recently, cytokines such as TNF and TGF-β, and several cancer chemotherapeutic agents were reported to induce apoptosis of cultured tumor cells in different systems [12,13,16]. So, apoptosis appears to be a significant process of cell death in tumor regression as well as in physiological condition.

Little is known about the intracellular signal pathway for apoptosis. Possible signaling mechanisms in the initiation of apoptosis include the influx of Ca^{2+} ions, a change of protein kinase C (PKC) activity and altered expression of the oncogenes c-*fos* and c-*myc* [22–24]. Also the agents that elevate cAMP levels in the cells, including PGE_2, induced apoptosis in thymocytes [25]. But the increase in cAMP levels does not seem to act as a signal for apoptosis in our experiments because PGA_2 and Δ^{12}-PGJ_2 appeared to be ineffective in activating adenylate cyclase [2,26]. In this report, we demonstrate that Δ^{12}-PGJ_2 arrested L1210 cells at the G2/M phase in the cell cycle progression before the appearance of DNA fragmentation on agarose gel electrophoresis (Fig. 2), while G1 phase arrest was reported as the major basis for antiproliferative activities of these PGs in other tumor cell lines such as HeLa and GOTO cells [4,27,28]. G2 arrest is known as a cellular response to DNA damage to prevent cells entering the mitotic phase prior to the repair of damaged DNA [29–32]. Therefore, DNA seems to be the primary target for Δ^{12}-PGJ_2-induced cytotoxicity in L1210 cells, leading to G2/M arrest in cell cycle progression. It can be postulated that a non-toxic dose of Δ^{12}-PGJ_2 causes reversible G2 arrest during which DNA damage can be repaired and cells continue to progress in their cell cycle, but toxic concentration of Δ^{12}-PGJ_2 induce lethal DNA damage resulting in persistent G2 arrest and these cells subsequently die through the process of apoptosis. This is consistent with the idea that the morphological changes observed in the cells undergoing apoptosis are related to events that normally occur at G2 to M transition such as chromatin condensation and membrane breakdown, as suggested by Sorenson et al. [33].

The association of G2 arrest and subsequent DNA degradation or cell death has already been demonstrated in several tumor cell lines, including L1210 cells, exposed to various chemotherapeutic agents [33–36]. However, the molecular mechanism initiating the sequence of cellular events which lead the G2 arrested cells to death remains unclear. Lock and Ross proposed that, in etoposide-treated CHO cells, perturbations in the activity of $p34^{cdc2}$ kinase, which is essential for passage of cells into mitosis [37], are related to transient G2 arrest and following irregular chromosome fragmentation with a concomitant progress to mitosis [38,39]. Premature chromosome condensation and nuclear membrane breakdown are also observed when S phase-arrested BHK cells are artificially driven into mitosis by caffeine [40]. In addition, it has been shown that cytotoxic effects of cell cycle phase-specific agents resulted from the dissociation of normally coupled cell cycle progression events [41]. Therefore, it will be necessary to investigate the role of cell cycle-regulatory proteins in the Δ^{12}-PGJ_2-induced G2/M arrest which is followed by apoptotic cell death.

Our results also showed that cycloheximide blocked the induction of apoptosis caused by Δ^{12}-PGJ_2. This is compatible with the results of previous studies that cycloheximide reduced Δ^{12}-PGJ_2 cytotoxicity on NCH human neuroblastoma cells and KSu osteosarcoma [36,42], indicating the requirement of continued protein synthesis for cytotoxicity of Δ^{12}-PGJ_2. In this context, decrease of DNA fragmentation observed at high (> 3

μg/ml) concentration of Δ^{12}-PGJ_2 (Fig. 3) might be the result of nonspecific inhibition of protein synthesis which occurred at these concentration of Δ^{12}-PGJ_2. Furthermore, cycloheximide also reduced G2/M accumulation induced by Δ^{12}-PGJ_2, which seems to be a prerequisite for apoptosis, suggesting that suppression of cell cycle progression by cycloheximide as well as protein synthesis inhibition contribute the protective effect of cycloheximide against Δ^{12}-PGJ_2-induced apoptosis. Several groups reported that specific proteins, including heat shock proteins, were induced in cells treated with these cyclopentenone PGs [28,43,44]. It is not certain, however, whether these PG-induced proteins are involved in the apoptotic pathway or in the regulation of cell cycle progression in L1210 cells. And the relevance of these PG-induced proteins to the gene products such as transglutaminase and TRPM-2 (testosterone-repressed prostate message-2) [45,46], known to be increased in apoptosis, is also to be determined.

Acknowledgements: We thank Emeritus Prof. Bong-Sop Shim and Associate Prof. Dae-Myung Jue for critical reading of the manuscript and discussion. This study was supported in part by a grant from the Cancer Research Center of Seoul National University (KOSEF-SRC-56-CRC-7) for 1992 and the Catholic Medical Center for 1992.

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