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Tuning Up or Down the UV-induced Apoptosis in Chinese Hamster Ovary Cells with Cell Cycle Inhibitors¶

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ABSTRACT

Exposure to UVC induces apoptosis in Chinese hamster ovary (CHO.K1) cells. While studying the underlying mechanism, we found that a variety of cell cycle inhibitors, including colcemid, hydroxyurea and mimosine, enhance the UV-induced apoptosis in these cells. Such enhancement was not dependent on the cell cycle progression nor was it related to the difference in UV sensitivity at different phases of the cell cycle. The expression of p21(waf1/cip1), a general cyclin-dependent kinase (CDK) inhibitor, was deficient in CHO.K1 cells. Ectopic overexpression of the human p21 markedly increased the survival rates of the UV-irradiated cells in the presence of colcemid. In addition, roscovitine, a small-molecule inhibitor of CDK, also inhibited the UV-induced apoptosis. These observations suggest that deregulation of CDK activity may be critical in the UV-induced apoptosis in CHO.K1 cells.

INTRODUCTION

In response to genotoxic insults, dividing cells undergo cell cycle arrest for damage repair. If the damage is beyond the cellular capacity to repair, apoptosis may occur (for reviews, see [1,2]). The cell cycle may be arrested at G1 or G2 (or both) in UV-irradiated cells (3–5). Such cell cycle arrest is believed to be the result of the checkpoint controls, which involve the ATM/ATR-p53–p21(waf1/Cip1) pathway (1,6–8). A defect in the pathway may render cells more susceptible to the killing effect of UV irradiation (9–11), although a mutation of p53 may be associated with the resistance to apoptosis and with the increase in cell transformation (reviewed in [12]).

Previously, we noted that UV irradiation (at 254 nm) caused significant apoptosis in Chinese hamster ovary (CHO.K1) cells (13) and that p21(waf1/Cip1) was not detectable despite the induction of p53 and gadd45 in the UV-irradiated cells (14). Furthermore, treatment with a cell cycle inhibitor such as colcemid did not inhibit but promoted the UV-induced cell death (13), suggesting that cell cycle arrest per se is not sufficient to prevent the apoptosis. In this report, we describe that not only the M-phase inhibitors but also the inhibitors of other cell stages have a similar proapoptotic effect. The proapoptotic effect was not related to the enrichment of a specific cell stage that is more susceptible to UV irradiation. Instead, it is caused by the deficiency of p21 expression. We confirmed that the expression of p21(waf1/cip1) was deficient in the CHO.K1 cells and found that ectopic overexpression of the human p21 markedly increased the survival rates of the UV-irradiated cells in the presence of colcemid. In addition, roscovitine, a small-molecule inhibitor of cyclin-dependent kinase (CDK), also inhibited the UV-induced apoptosis.

MATERIALS AND METHODS

Chemicals. Stock solutions of drugs were as follows: 10 μg/mL colcemid (GIBCO-BRL, Gaithersburg, MD) in phosphate-buffered saline (PBS); 40 m*M* mimosine (Sigma Chemical Co., St. Louis, MO) in PBS; 0.1 *M* hydroxyurea (Sigma) in water; 18 m*M* roscovitine (Calbiochem Co., San Diego, CA) in dimethyl sulfoxide (DMSO).

Cell cultures. The CHO.K1 cells were originally obtained from the American Type Culture Collection. The cells were maintained in 1× McCoy's 5A medium (GIBCO-BRL) supplemented with penicillin, streptomycin, 3% glutamine, 2.2% sodium bicarbonate and 10% fetal calf serum.

UV irradiation. The procedures for UV irradiation were the same as those described previously (13,14). Briefly, cells in the dish were washed with PBS, uncovered and exposed to UV light in a marked area of the tissue culture hood that had been precalibrated for the required dose using the germicidal lamp (254 nm) with the aid of a UV radiometer (UVP, San Gabriel, CA).

Survival test. Clonogenic assay was used for survival measurement (15). Cells were harvested after trypsinization and were prepared as a single-cell suspension. Aliquots with the same number of cells were dispensed into 10 cm dishes containing 10 mL normal medium. The cell density of the suspension was adjusted so that the final end-of-assay colonies were in the range 100–1000. After 6–7 days of incubation at 37°C in 5% CO_2, the numbers of colonies with >50 cells were counted.

Flow cytometric analysis. The procedures for flow cytometric analysis were the same as those used previously (13,14). In brief, cells (~2×10^6) were fixed in 75% alcohol for 12–16 h at 4°C, followed by RNase (1 mg/mL) treatment at room temperature for 30 min. Cells were then stained with propidium iodide (10 μg/mL) for 30 min before cell cycle analysis with a flow cytometer (FACScan, Becton Dickinson, Bedford, MA).

Northern blot analysis. Total RNA was extracted from cells with Trizol reagent (GIBCO-BRL Co.) according to the manufacturer's

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Abbreviations: CDK, cyclin-dependent kinase; CHO.K1, Chinese hamster ovary; DMSO, dimethyl sulfoxide; EDTA, ethylenediamine tetraacetic acid; PBS, phosphate-buffered saline.

instruction. For Northern blot analysis, total RNA (20 μg) of each sample was used and separated by electrophoresis in 1% agarose–formaldehyde gels and then capillary-transferred to Hybond-N+ membrane (Amersham Co., Arlington Heights, IL) in 20× standard saline citrate. The RNA blot was probed with ^{32}P-labeled mouse p21 cDNA (16) using the random primed labeling procedure (17). Procedures for hybridization, washing and autoradiography were according to the standard protocols (17).

Construction of tetracycline-inducible p21(waf1/cip1) expression vector, cell transfection and p21 induction. A tetracycline-inducible eukaryotic expression plasmid, pBI-EGFP (Clonetech Co., Palo Alto, CA), was used to express p21 in CHO.K1 cells. Human p21 cDNA (18) was cloned into pBI-EGFP at *Nhe*I sites. The construct, p21-BI-EGFP, was transfected into clones of CHO.K1 cells that had been stably transfected with pTet-on to express the Tet activator. Cells of 70% confluence were transfected with 5 μg of expression plasmid by the liposome-mediated method using LipofectAmine (GIBCO-BRL) according to the manufacturer's manual. After transfection, cells were incubated for 18–24 h before further treatments. For p21 induction, the transfected cells were treated with doxycycline (2 μg/mL) for 24 h before UV irradiation.

DNA fragmentation assay. DNA fragmentation assay was done as mentioned previously (13). In brief, cells (~2 × 10^6) were harvested for genomic DNA extraction in buffer containing 100 m*M* Tris–Cl, pH 7.5, 12.5 m*M* ethylenediamine tetraacetic acid (EDTA), 150 m*M* NaCl and 0.2 mg/mL proteinase K and subsequent phenol–chloroform extraction. RNase A was used to remove the RNA contaminant. DNA was further purified by ethanol precipitation. The purified DNA was dissolved in 10 m*M* Tris–HCl, pH 7.4, containing 1 m*M* EDTA and was separated by electrophoresis on a 1% agarose gel in 0.5× Tris–borate buffer.

Viability test. Cell viability was assayed with trypan blue dye exclusion (15). After treatment, cells were harvested and resuspended in 1× PBS at an appropriate cell density. An aliquot of cell suspension was mixed with the same volume of trypan blue (0.4% in 1× PBS) on a hemocytometer and examined with a microscope.

RESULTS

Enhancement of UV-induced apoptosis in CHO.K1 cells by cell cycle inhibitors

Previously, we have reported that UV irradiation induces apoptosis in CHO.K1 cells and that the cell death is exacerbated when mitotic inhibitors are included in cell cultures after irradiation (13). The purpose of the addition of M-phase inhibitors, *e.g.* colcemid, to the UV-irradiated cells was originally to study the G1 arrest. Because the mitotic inhibitors prevent the G2–M cells from reentering the cell cycle, the G1 arrest caused by irradiation could be detected unambiguously. Enhancement of cell death by the inhibitors was not seen with NIH3T3 or with the X-ray irradiated CHO.K1 cells (13). These observations were made by monitoring the sub-G1 population of cells after UV irradiation, with or without the treatment with colcemid. Colcemid was continuously present after the irradiation. The effect of colcemid on the enhancement of apoptosis was also seen when cell survival was examined in the experiments, as shown in Fig. 1A. In the experiments, after the UV treatment at various doses, cells were cultured in the presence or absence of colcemid (0.5 μg/mL) for 24 h before harvest for survival assay based on colony-forming efficiency. The presence of colcemid sensitized the cells to UV irradiation by reducing the LD50 from 28 to 8 J/m^2. By the same survival test, it was noted that the 24 h incubation period with colcemid could be reduced to about 6 h for the maximum drug effect (Fig. 1B). Thus, a relatively short period of exposure to colcemid was enough to trigger a permanent effect as seen in the cell survival test.

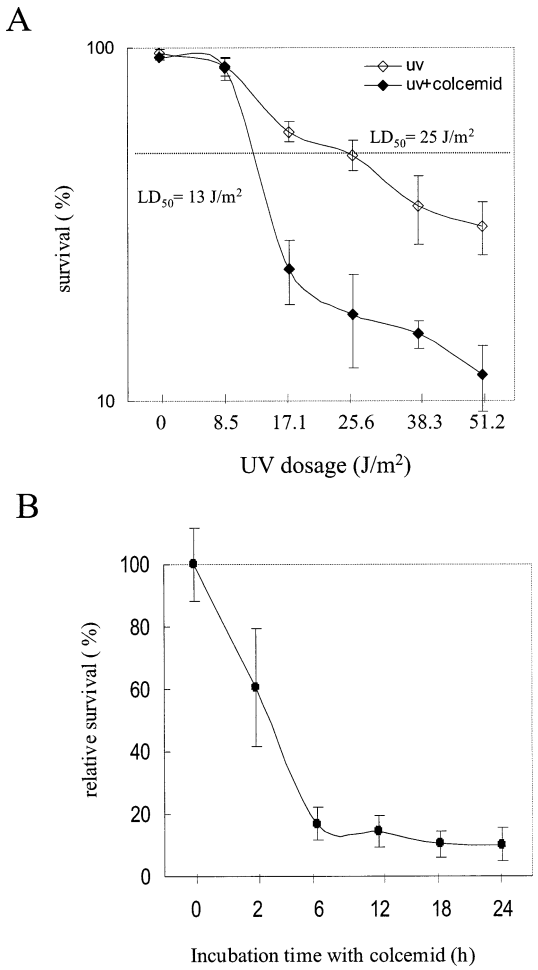

Figure 1. Effect of cell cycle inhibitors on cell survival after UV irradiation. (A) Colcemid reduced the survival of cells after UV irradiation. CHO.K1 cells of 70–80% confluence were UV irradiated at the indicated dose and then cultured in medium with or without colcemid (0.5 μg/mL) for 24 h. The cells were then rinsed, trypsinized, harvested and replated in normal medium at a suitable cell density for clonogenic formation for survival assay. (B) Determination of the least incubation period with colcemid to attain the maximum drug effect. Cells in log phase were UV irradiated at a dose of 25 J/m^2 and cultured in medium with colcemid (0.5 μg/mL) for various intervals before being subjected to the survival assay as in (A). The survivals presented herein were the relative values *versus* the control (without drug treatment).

In addition to M-phase inhibitors, other cell cycle inhibitors such as mimosine and hydroxyurea (or aphidicolin) also sensitized the cells to UV irradiation (see Fig. 2, data not shown). Mimosine is a late G1 blocker (19), whereas hydroxyurea (or aphidicolin) is an S-phase inhibitor. The cell survival decreased from about 45 to 13–14% when mimosine (or hydroxyurea) was included. The drug treatments alone gave 68% or more cell viability. Because the cell death enhancement is not specific to the M-phase inhibitors, the effect is unlikely to be associated with the mitotic cells. A similar observation was made in the flow cytometric analysis in which the sub-G1 population with or without the drug treatment was monitored (data not shown). Nevertheless, it

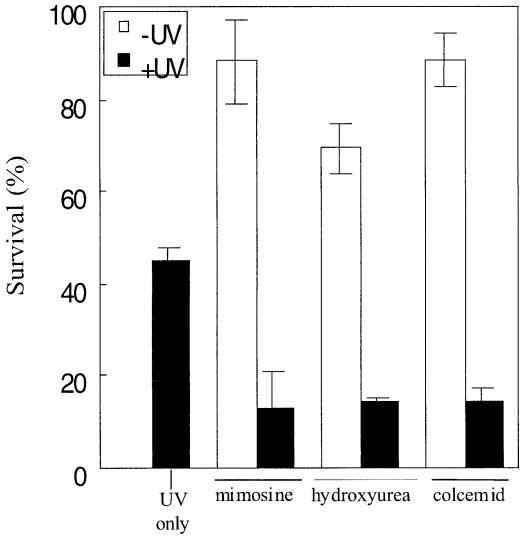

Figure 2. Survival of cells after UV irradiation was diminished by various cell cycle inhibitors. Similar to Fig. 1, cells were UV irradiated at 25 J/m^2 and cultured in medium with mimosine (0.4 m*M*), or hydroxyurea (1 n*M*) or colcemid (0.5 μg/mL) for 24 h before subsequent survival assay. Control experiments followed the same procedures, except that the treatments of UV or drugs were mock.

was noted that the kinetics of apoptosis (*i.e.* % sub-G1 *versus* time) varied with the different drugs in a decreasing order as follows: colcemid > mimosine ≫ hydroxyurea. The interpretation for this could be that the different drugs might take different routes to the common pathways that lead to apoptosis. This thought prompted us to consider if cell cycle progression is involved in the drug effects.

Enhancement of UV-induced apoptosis by cell cycle inhibitors does not require cell cycle progression

In the previous experiments, the cells *before* UV irradiation were in logarithmic phase; the cell population was asynchronous. It would be of interest to know as to what would happen if the cells were presynchronized before the UV irradiation and were kept at the same phase afterward. For instance, cells were pretreated with mimosine before irradiation and were returned to the mimosine-containing medium for 24 h after UV exposure. Under such conditions, the cell cycle progression would be prevented, and the effect of the drugs on UV-induced apoptosis could be examined in the absence of cell cycle progression. As shown in Table 1, there was no significant difference in terms of apoptosis when the experiments with or without presynchronized cells were compared. For instance, the experiment using cells presynchronized at late G1 with mimosine displayed 67.0 ± 8.6% apoptosis, whereas its counterpart experiment showed 59.6 ± 4.3% apoptosis. Similarly, 75.4 ± 9.1 and 80.1 ± 3.8% apoptosis were found in the cells presynchronized at M phase and in the asynchronous cells, respectively. Hence, cell cycle progression was not necessary for the drugs to exacerbate the UV-induced apoptosis.

Furthermore, because cells presynchronized at late G1 and M phases showed similar UV sensitivity in terms of UV-induced apoptosis (67.0 ± 8.6% *versus* 75.4 ± 9.1%, see Table 1), the UV-induced apoptosis probably did not result from the UV sensitivity of cells in a specific cell stage(s). A separate analysis of UV sensitivity of different phases was thus done to verify this suspicion (Table 2). In this study, cells synchronized at late G1, S or M phase were UV treated and left in culture medium without drugs after irradiation for 24 h before the survival assay. The survivability of cells in different phases after UV treatment all fell in the range around 40.0%; hence, UV sensitivity of the cells seems to be constant over the cell cycle.

Table 1. Effect of cell cycle inhibitors on the UV-induced apoptosis in CHO.K1 cells with or without presynchronization*

	Cell stage	Inhibitor	Apoptotic cell (%)†
Asynchronous	—	None	32.3 ± 3.5
	—	Mimosine	59.6 ± 4.3
	—	Colcemid	80.1 ± 3.8
Presynchronized	G1‡	Mimosine	67.0 ± 8.6
	G2–M‡	Colcemid	75.4 ± 9.1

*The conditions in the experiments were similar to those described in Fig. 1 unless synchronized cells were used, in which the exponential cells were pretreated with mimosine (0.4 m*M*) or colcemid (0.5 μg/mL) for 24 h before UV irradiation.
†The percentages of apoptotic cells were determined by the sub-G1 fractions of the flow cytometric profiles. Data represent the means and standard deviations of triplicate experiments.
‡Cells were primarily at G1 (78%) or G2–M (84%) after 24 h pretreatment with mimosine or colcemid, respectively.

The absence of p21 expression in UV-irradiated CHO.K1 cells, and the inhibition of UV-induced apoptosis by ectopic expression of p21

Previously, we have shown that the p21(waf1/cip1) protein, a general CDK inhibitor, was not detected in the UV-irradiated CHO.K1 cells. The absence of p21 expression was confirmed at the mRNA level by the Northern analysis (Fig. 3). Results of the analysis indicate that CHO.K1 cells, in contrast to the control cells such as NIH3T3, showed no p21 expression during the time course (0–12 h) after the UV irradiation.

Because the CHO.K1 cells appeared to be deficient in p21 expression and were susceptible to UV-induced apoptosis, a question may be asked if the p21 deficiency was linked with the UV susceptibility. To test the possibility, we performed

Table 2. Survivals of CHO.K1 cells at different cell stages after UV irradiation*

Cell stage	Survival (%)
G1	59.3 ± 9.6
S	63.2 ± 3.7
G2–M	59.2 ± 9.8

*Cells were synchronized at G1, S or G2–M by incubating exponential cells with mimosine (0.4 m*M*), hydroxyurea (1 n*M*) or colcemid (0.5 μg/mL) for 24 h, respectively. According to the flow cytometric profiles (not shown), synchronization was apparently attained. Survival was determined by clonogenic assay. Data represent the means and standard deviations of triplicate experiments.

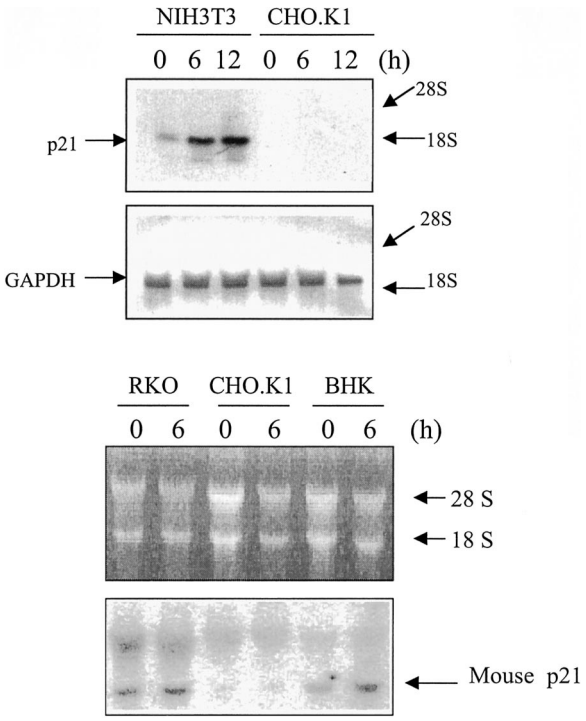

Figure 3. Northern analysis of p21. Exponentially growing cells (CHO.K1 or others) were treated with UV radiation at a dose of 25 J/m^2. After irradiation, cells were harvested for analysis of p21 expression at the indicated period of time (0–12 h). Constant amounts of total RNA were loaded in each lane. ^{32}P-labeled mouse p21 cDNA (16) was used as the probe. The levels of GAPDH mRNA (detected by GAPDH cDNA) or the RNA gel picture was used as the loading controls.

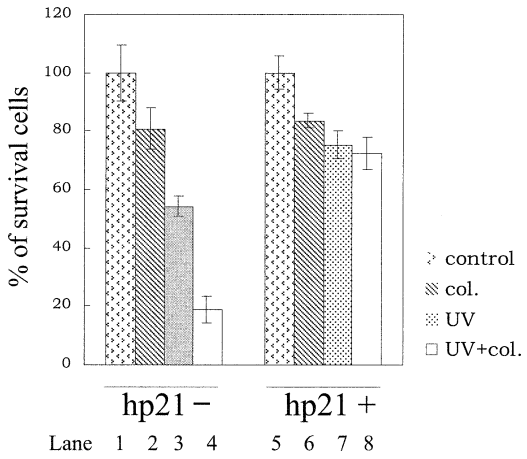

Figure 4. Ectopic overexpression of human p21 increased survival in CHO.K1 cells in response to UV radiation. Cells transiently transfected with the human p21 expression vector were treated with or without UV radiation (25 J/m^2) and recovered in the medium with or without colcemid (0.5 μg/mL, col.). Doxycycline (2 μg/mL) was present for 24 h to induce p21 before the UV irradiation. After 24 h recovery, cells were harvested and prepared as single-cell suspension for the colony-forming assay as described in Materials and Methods. The survival rate represents the ratio of the colony numbers obtained from the treated sample relative to the control (*i.e.* the exponentially growing cells without UV irradiation). Each datum was presented by the mean and standard error of three individual experiments. Symbols: hp21+ (or −), with (or without) induction of human p21.

the complementation experiments by using an inducible p21 expression vector (see Materials and Methods). The effect of ectopic p21 expression upon the cell survival after UV irradiation was studied, as shown in Fig. 4. Results of the experiments indicate that the overexpression of p21 increased the survival of the UV-irradiated cells (comparing lanes 3,4 [hp21−] and lanes 7,8 [hp21+], Fig. 4). Notably, the survival rate was increased from about 20% to more than 60% in the cells cotreated with UV and colcemid (comparing lanes 4 and 8, Fig. 4). The control experiment using the empty vector, following otherwise the same procedures, showed no enhancement of cell survival (data not shown). Thus, the ectopic expression of human p21 in CHO.K1 cells effectively inhibited the UV-induced apoptosis. This observation suggests that inhibition of CDK activity by p21 expression may ameliorate the UV-induced apoptosis. Indeed, roscovitine, a small organic molecule with selectively inhibitory activity of CDK (20,21), also inhibited the UV-induced apoptosis in CHO.K1 cells (Fig. 5). Roscovitine at a commonly used concentration (180 μ*M*) markedly inhibited the cellular DNA fragmentation caused by apoptosis (panel A, Fig. 5) and decreased the % of apoptosis of the UV-irradiated cells (panel B, Fig. 5). These findings suggest that deregulation of CDK activity may be critical in the UV-induced apoptosis in CHO.K1 cells.

DISCUSSION

Efforts have been made to explore the mechanism underlying the UV-induced apoptosis in CHO.K1 cells. It was found in this study that UV-induced apoptosis was exacerbated by different cell cycle inhibitors including colcemid, hydroxyurea and mimosine (Figs. 1 and 2). Furthermore, cell cycle progression was not necessary for the drugs to enhance the apoptosis, and such enhancement was not related to the difference of UV sensitivity at different phases of cell cycle (Tables 1 and 2). The UV-induced apoptosis and its en-

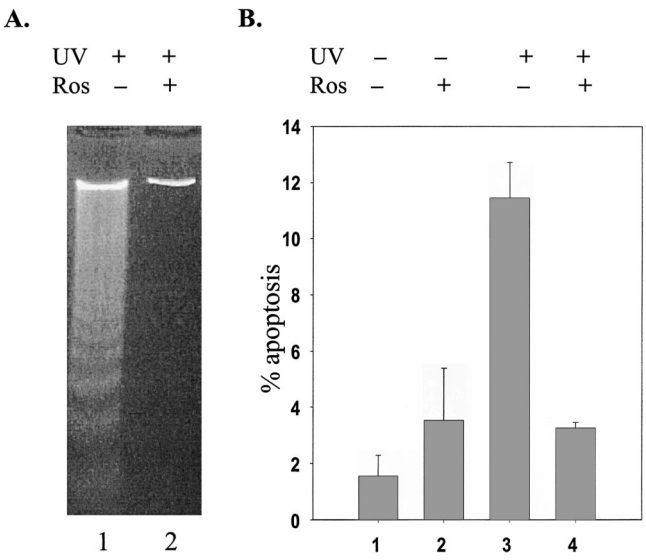

Figure 5. Roscovitine inhibited the UV-induced apoptosis. Exponentially growing cells were exposed to UV irradiation (25 J/m^2) and cotreated with roscovitine (180 μ*M*, Ros) or DMSO (the solvent for roscovitine) for 24 h before the cell harvest for (A) DNA fragmentation assay or (B) the viability assay with dye exclusion.

hancement by the drugs was associated with the lack of expression of p21(waf1/cip1) (Fig. 3), which may lead to the deregulation of CDK activity. The ectopic overexpression of the human p21 markedly increased the survival of the UV–colcemid cotreated cells (Fig. 4), and roscovitine, a small molecule that inhibits CDK, inhibited the UV-induced apoptosis (Fig. 5).

In this study, the UV-induced apoptosis was assayed by various methods including the survival of cells by colony-forming assay, the sub-G1 population in the flow cytometric analysis, the DNA fragmentation assay and the cell viability by the trypan blue exclusion. These methods, individually, may not be specific to apoptosis. However, similar conclusions were obtained by these different methods (Fig. 5, data not shown). Moreover, the UV-induced cell death was inhibited by the overexpression of p21. Thus, it is unlikely that such cell death is the result of necrosis, although more specific and more expensive assays such as annexin V staining or TUNEL assay (22) may be used.

Because mutation of p53 may make cells more resistant to the killing effect of UV (12), it might be asked whether the enhancement of UV-induced apoptosis by cell cycle inhibitors, *e.g.* colcemid, was a result of overproduction of p53. This is probably unlikely because we found that ectopic expression of wild-type human p53 in the CHO.K1 cells did not cause apoptosis ([13], data not shown).

Our study implies that deregulation of CDK activity as a result of the lack of p21 plays a role in UV-induced apoptosis in CHO.K1 cells. This is consistent with the previous report that expression of cyclin D1 after UV irradiation is essential for apoptosis (23). In our study, UV-induced apoptosis was enhanced by colcemid, and the enhanced cell death was almost completely blocked by the expression of p21 (deduced from lanes 4 and 8, Fig. 4). The data may imply that colcemid exacerbates the apoptosis by worsening the deregulation of CDK activity. This suggestion awaits further investigation. It has been reported that cells after prolonged exposure to colcemid may enter a G1-like state, and these cells undergo massive apoptosis after overexpression of c-myc (24). CDK activity was stimulated in this study (24). UV irradiation may play the same role as the overexpression of c-myc described previously.

The experiments with mimosine (Fig. 2) are worth mentioning. The drug was originally used to see if it could inhibit the UVC-induced apoptosis in CHO.K1 cells as it did in the UVB-induced apoptosis in human A431 cells (25). It has been shown that mimosine inhibits the UVB-induced apoptosis by inducing p21 (25). However, mimosine failed to inhibit the UVC-induced apoptosis in CHO.K1 cells; in fact, it aggravated the cell death. Mimosine, like p21 expression, arrested the CHO.K1 cells at G1 (data not shown). However, because p21(waf1/cip1) is a general inhibitor of CDK, CDK2 or CDK4–CDK6 activities could be inhibited by p21, whereas mimosine probably inhibits CDK2 but not CDK4–CDK6 (19). CDK2 is cyclin A- or cyclin E–dependent, whereas CDK4–CDK6 are cyclin D–dependent protein kinases. Therefore, on the basis of the previous discussion, we suspect that CDK4–CDK6 but not CDK2 play more important roles in the UV-induced apoptosis in CHO.K1 cells. We are currently examining the possibility.

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