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Effects of Cell Cycle Inhibitors on Cell Death of Human Cancer Cell Lines

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Genotoxic agents are known to cause cell death mostly by apoptosis in susceptible cells and this action plays an important role in tumor regression after chemotherapy. It has been thought that the apoptotic process may be associated with cell cycle. In the present study, the effect of aphidicolin and nocodazole, reversible cell cycle inhibitors, on the apoptosis or cell death induced by actinomycin D was studied in 3 cell lines. HeLa, SiHa and NIH/3T3 cells were treated with actinomycin D(100 nM) alone or in combination with cell cycle inhibitors for 20 hours; or presynchronized cells were treated with actinomycin D for 4 hours and further incubated for 20 hours in fresh medium. The synergistic effect of IBMX(5 or 10 μM) on the apoptosis or cell death induced by actinomycin D was also studied. The results were as follows; Majority of HeLa cells showed apoptotic changes after actinomycin D treatment. Aphidicolin or nocodazole blocked neither initiation nor progression of actinomycin D-induced apoptosis in HeLa cell. Aphidicolin pretreatment enhanced the actinomycin D-induced apoptosis of HeLa cells and cell death of SiHa cells and nocodazole pretreatment enhanced cell death of SiHa cells. IBMX moderately enhanced actinomycin D-induced cell death of NIH/3T3 and to less extent that of SiHa cells. These results show that neither initiation nor progression of actinomycin D-induced apoptosis or cell death can be blocked by cell cycle arrest, and that cAMP is partly responsible for actinomycin D-induced apoptosis in certain types of cells.

Key words: *cell death, cell cycle, aphidicolin, nocodazole, DNA damage*

Introduction

Cancer chemotherapy with cytotoxic drugs is one of the main methods of treatment for cancers and is increasingly used as an adjuvant to surgery or irradiation. Successfulness of chemotherapy and radiation therapy depends on the susceptibility of cancer cells to the cytotoxic effect of the chemotherapeutic agents or radiation.

Since many chemotherapeutic agents induce DNA damage, the tumor-specific cytotoxicity of these agents has been attributed to genotoxic effect on actively proliferating cells. However, in many cases, the cellular damage caused by active doses of these agents is not sufficient to explain the observed toxicity (Dive and Hickman, 1991). Many toxic stimuli have been shown to induce apoptosis even at doses insufficient to cause general metabolic dysfunction (Lennon *et al.*, 1991; Dive and Hickman, 1991). Apoptosis induced by anticancer drugs is p53-dependent and requires other genes which promote the apoptotic process in addition to the role of p53 (Lowe *et al.*, 1993a). Actinomycin D is known to cause DNA strand breaks by acting with type I and type II topoisomerases (Trask and Muller, 1988; Wasserman *et al.*, 1990) and is used to prompt apoptosis.

The strand breaks trigger p53 induction (Fritsche *et al.*, 1993; Nelson and Kastan, 1994) and induced p53 may be responsible for actinomycin D-induced cytotoxicity reaction. It was previously reported that p53 is responsible for cell cycle arrest induced by DNA damage (Kuerbitz *et al.*, 1992) and apoptosis by radiation damage (Lowe *et al.*, 1993b).

Correlation of the cell cycle with apoptosis is not studied well yet. Though anguidine-induced cell cycle arrest blocked the cytotoxic effect of certain anticancer drugs (Theodori *et al.*, 1981), it is postulated that apoptosis could be independent of cell cycle progression since p53 induction is not prevented by cell cycle inhibitors (Fritsche *et al.*, 1993). To test aforementioned postulation, the effects of aphidicolin, a DNA polymerase inhibitor (Lalande, 1990), and nocodazole, a mitotic inhibitor (Zieve *et al.*, 1980) on actinomycin D-induced cytotoxic effect on HeLa, SiHa, and NIH/3T3 were

studied. The effect of increased cAMP was also studied using IBMX, a cyclic nucleotide phosphodiesterase inhibitor.

Materials and Methods

Cell lines

HeLa (human cervical cancer cell line), SiHa (human cervical cancer cell line) and NIH/3T3 (mouse embryonic cell line) cell lines were maintained in DMEM supplemented with 10% FBS and subjected to drug treatment on 50-60% confluence.

Drugs

Actinomycin D, a topoisomerase inhibitor, was used to induce apoptosis. Aphidicolin and nocodazole, cell cycle inhibitors, were used to arrest cells at G1/S or mitotic phase, respectively. IBMX, a cyclic nucleotide phosphodiesterase inhibitor, was used to augment cAMP level. All agents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

Drug treatment of the cells

In the first set of experiment, 50-60% confluent cells were treated with actinomycin D (100 nM), aphidicolin (2 μg/ml), nocodazole (0.04 μg/ml), IBMX (5 or 10 μM), combination of actinomycin D and aphidicolin, combination of actinomycin D and nocodazole, and combination of actinomycin D and IBMX, and then incubated for 20 hours. In second set of experiment, cells were synchronized by 16 hours treatment with aphidicolin or nocodazole, then added with actinomycin D, incubated for 4 hours, and incubated for 20 hours in fresh medium.

Evaluation of viability and apoptosis

Cells were stained with trypan blue, and viable and nonviable cells were counted. To observe chromatin structure cells were fixed with Carnoy's fixative (glacial acetic acid: methanol, 1:3), stained with 0.1% acridine orange, washed once with 0.1 M $CaCl_2$ and observed under fluorescence microscope. Adherent and detached cells were harvested, pooled, and washed with PBS twice. Cells were lysed in a buffer containing 10 mM Tris (pH 8.0), 0.1 M NaCl, 5 mM EDTA and 1% SDS. After 3 hour-digestion with proteinase K (100 μg/ml), lysate were extracted with phenol/chloroform. High molecular weight DNA was removed by centrifugation at 13,000×g for 15 minutes at 4℃ after addition of ammonium acetate to a final concentration of 0.5 M. Low molecular weight DNA was recovered by precipitation with alcohol. DNA pellet was dissolved in TE buffer for agarose gel electrophoresis. DNA solution was treated with RNase A (100 μg/ml) for 30 minutes, analysed on 1.5% agarose gel and photographed under UV transillumination.

DNA synthesis

DNA was labelled with [^{3}H]-thymidine (5 μCi/ml) for 2 hours. Cells were fixed with 5% TCA for 15 minutes on ice and washed with ice-cold 5% TCA 4 times. TCA insoluble fraction was dissolved in 0.5 M NaOH. One tenth of the lysate was used to measure incorporated radioactivity by liquid scintillation counting.

Results

Effects of aphidicolin and nocodazole on cytotoxic effect of actinomycin D

Twenty hours treatment with actinomycin D reduced the viability of SiHa cells from 94.2% to 86.8%. Combined treatment with aphidicolin and actinomycin D showed similar viability (85.9%) to the cells treated with actinomycin D alone and combined treatment with nocodazole and actinomycin D showed 82.3% viable cells (Fig. 1). Treatment with actinomycin D greatly reduced the viability of HeLa cells from 95.0% to 9.9% (Fig. 1). Combined treatment with aphidicolin and actinomycin D left 4.9% viable cells and combined treatment with nocodazole and actinomycin D showed mildly higher viability (13.0%) than actinomycin D alone. NIH/3T3 retained relatively high viability (86.6%) even after

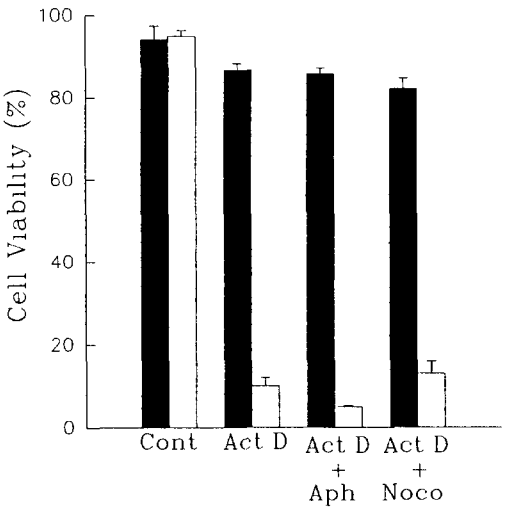

Fig. 1. Effects of actnomycin D and cell cycle inhibitors on SiHa and HeLa cells. Cells were treated with 100 nM actinomycin D or combination of actinomycin D and aphidicolin (2 μg/ml) or nocodazole (0.04 μg/ml) for 20 hours. Data are means±S.D. of 4-5 samples from 2 separate experiments. ■ SiHa cells, □ HeLa cells.

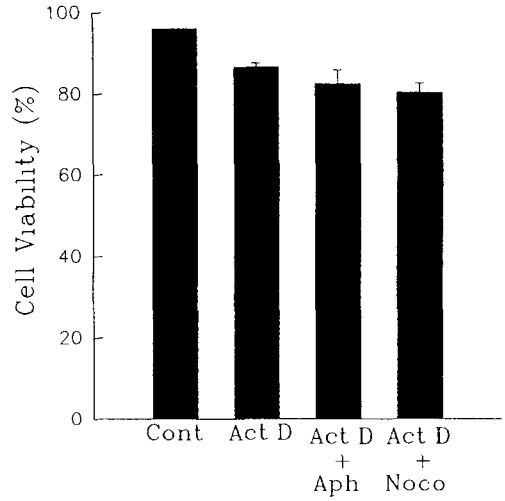

Fig. 2. Effect of actinomycin D and cell cycle inhibitors on NIH/3T3 cells. Cells were treated with 100 nM actinomycin D for 20 hours and further treated with combination of actinomycin D and aphidicolin (2 μg/ml) or nocodazole (0.04 μg/ml) for 20 hours. Data are means±S.D. of 4 samples from 2 separate experiments.

longer exposure to actinomycin D (Fig. 2).

Effect of pretreatment with aphidicolin and nocodazole on the cytotoxic action of actinomycin D

Actinomycin D alone, aphidicolin alone and nocodazole alone exerted little effect on the viability of SiHa cells (94.1%, 94.2% and 92.6%, respectively) and the viability was similar to control (Fig. 3). Actinomycin D treatment in aphidicolin-pretreated and nocodazole-pretreated cells resulted in moderate reduction in viability to 82.7% and 80.4%, respectively. In HeLa cells, 4 hour-treatment of actinomycin D reduced the viability from 96.0% to 58.8% (Fig. 3). Actinomycin D treatment in aphidicolin-pretreated and nocodazole-pretreated

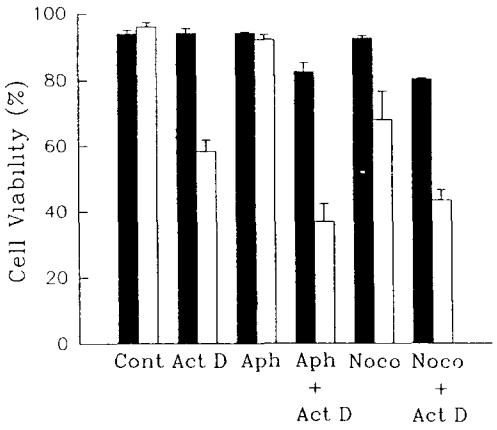

Fig. 3. Effects of actinomycin D on SiHa and HeLa cells which are synchronized with aphidicolin or nocodazole. Cells were pretreated with aphidicolin or nocodazole for 16 hours, treated with actinomycin D (100 nM) for 4 hours in the presence of cell cycle inhibitors and further incubated for 20 hours in fresh medium without any drug. Data are means±S.D. of 4 samples from 2 separate experiments. ■ SiHa cells, □ HeLa cells.

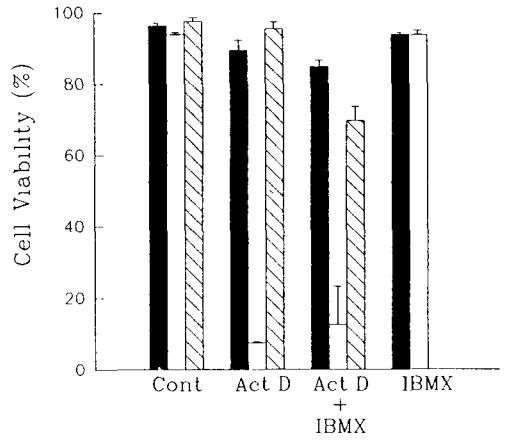

Fig. 4. Effects of actinomycin D and IBMX on SiHa, HeLa, and NIH/3T3 cells. Cells were treated with 100 nM actinomycin D or combination of actinomycin D and IBMX (5 μM for NIH/3T3 cells, 10 μM for other cells) for 20 hours. Data are means±S.D. of 3-4 samples from 2 separate experiments. ■ SiHa cells, □ HeLa cells, ▨ NIH/3T3 cells.

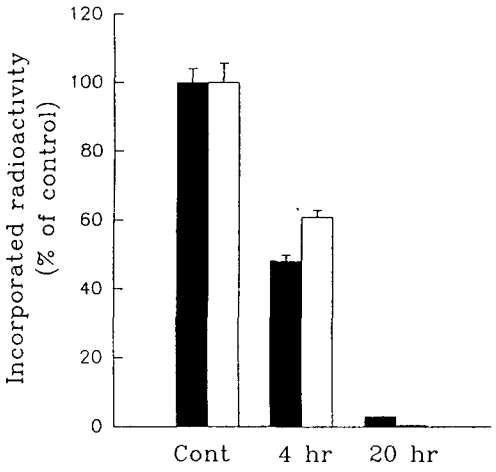

Fig. 5. Effects of actinomycin D on thymidine incorporation of SiHa and HeLa cells. Cells were treated with 100 nM actinomycin D for 4 or 20 hours. Data are means±S. D. of 3 samples. ■ SiHa cells, □ HeLa cells.

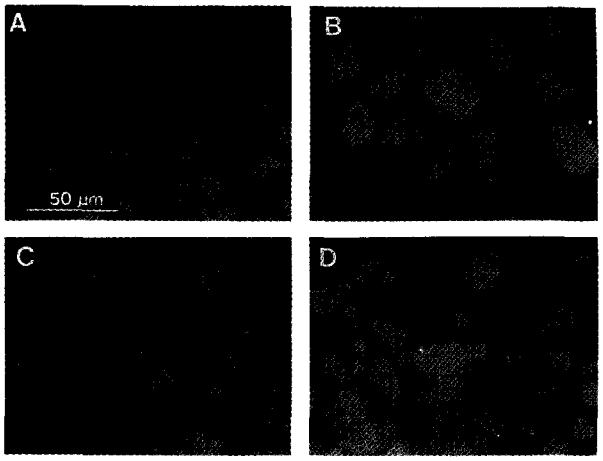

Fig. 6. Fluorescence photomicrograph of acridine orange stained control and actinomycin D-treated cells. A. SiHa, Control, B. SiHa, 20 hours actinomycin D-treated (100 nM), C. HeLa, Control, D. HeLa, 20 hours actinomycin D-treated (100 nM). Excitation at 530 nm.

cells showed lower viability (36.9% and 43.4%) than in the cells treated with actinomycin D alone.

Effect of IBMX on the cytotoxic action of aphidicolin

NIH/3T3 and SiHa cells were relatively resistant to the cytotoxic action of actinomycin D and resultant viability were 95.3% and 89.6%. Combined treatment of NIH/3T3 with IBMX and actinomycin D resulted in additional reduction in viability to 69.5% (Fig. 4) and combined treatment of SiHa cells resulted in mild reduction to 84.8%

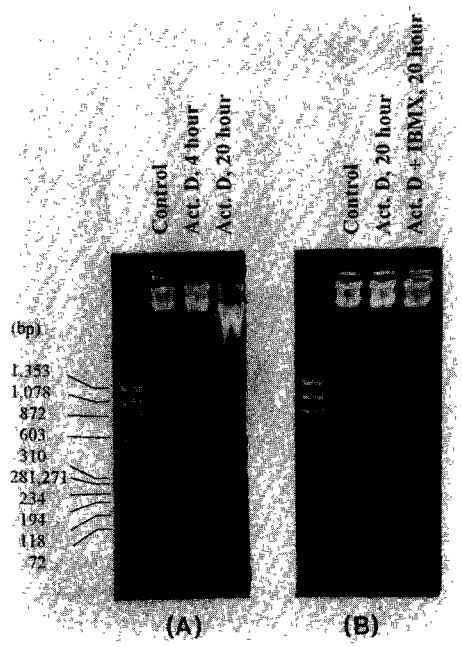

Fig. 7. Photograph of ethidium bromide stained DNA after electrophoresis on 1.5% agarose. A. DNA extracted from HeLa cells after 4 or 20 hours treatment with actinomycin D (100 nM). B. DNA extracted from SiHa cells after 20 hours treatment with actinomycin D (100 nM) or combination of actinomycin D and IBMX (10 μM). ϕ X174/Hae III fragments were used as a DNA size standard. Act.D: actinomycin D

(Fig. 4). No further reduction in viability was observed in HeLa cells (Fig. 4).

Effect of actinomycin D on DNA synthesis

The DNA synthesis of SiHa and HeLa cells reduced to 48.2% and 60.9% of control after 4 hour treatment with actinomycin D. Almost complete suppression of DNA synthesis was observed after 20 hours treatment with actinomycin D (Fig. 5).

Fragmentation of nucleus

SiHa cells generally showed normal intact nuclear appearance (Fig. 6) and minimal degradation of DNA as shown by agarose gel electrophoresis of DNA even after 20 hours of actinomycin D treatment (Fig. 7). On the other hand, most of HeLa cells treated with actinomycin D for 20 hours showed apoptotic bodies and/or condensed chromatin (Fig. 6) and the representative DNA ladder pattern was not observed though DNA degradation was obviously observed (Fig. 7).

Discussion

Morphologically distinct type of cell death was reported previously, which is characterized by nuclear chromatin and cytoplasmic condensation and following nuclear fragmentation and cellular budding to produce membrane bounded structures with well preserved organelles (Kerr *et al.*, 1972). This type of cell death, apoptosis, was also noted in actively proliferating normal and neoplastic cells after administration of cancer chemotherapeutic agents including actinomycin D (Searl *et al.*, 1974). It is reported that most cell deaths by cytotoxic drugs used in cancer chemotherapy are attributed to apoptosis (Dive and Hickman, 1991; Lowe *et al.*, 1993a). More recently the apoptotic process has been studied extensively and reviewed well by Wyllie (1993) and the apoptotic mechanism attracts a great concern until now. In addition to aforementioned morphological changes, cells undergoing apoptosis show activation of calcium-magnesium sensitive endonuclease (Wyllie, 1980), which reduces the DNA of apoptotic cells to a series of fragments. Recently it is reported that fragmentation of DNA into 300 Kbp and/or 50 Kbp is essential in apoptosis (Oberhammer *et al.*, 1993). Apoptosis requires p53 protein and also needs other gene products to efficiently perform apoptosis in susceptible cells (Lowe *et al.*, 1993a; Wyllie, 1993). p53 plays a key role in apoptosis and can be induced by DNA damage regardless of cell cycle progression (Fritsche *et al.*, 1993). In the present study, HeLa cell was most sensitive to the cytotoxic action of actinomycin D and the cell death was not blocked by aphidicolin or nocodazole. This result coincides with cell cyclin-dependent induction of p53 by DNA-damage (Fritsche *et al.*, 1993). Teodori *et al.* (1981) reported that cell cycle inhibition reduces cytotoxicity of several anticancer drugs. However, it seems that the reduced cytotoxicity of certain anticancer drugs by anguidine, which was used in their experiment, is attributed to protein synthesis inhibition not to cell cycle inhibition since protein synthesis inhibition may block synthesis of several gene products necessary for apoptosis. Since aphidicolin-pretreatment and nocodazole-pretreatment did not block cell death after short-term treatment with actinomycin D, it seems that initiation of apoptosis by DNA damage is independent of cell cycle progression. Moreover, the independence from cell cycle of the progression of actinomycin D-induced apoptosis can be postulated from the fact that combined treatment of aphidicolin and actinomycin D, or nocodazole and actinomycin D did not yield higher viability of the cells than the viability of the cells treated with actinomycin D alone. In addition cells arrested at G_1/S boundary seems to be a little more susceptible to cytotoxic effect of DNA-damaging agent.

Among the cells used in the present study, SiHa and NIH/3T3 cells were more resistant than HeLa to the cytotoxic action of actinomycin D. One of the possible mechanism is the difference in the effectiveness of cell cycle arrest at G_1 or G_2 phase and DNA repair in actinomycin D-resistant cells. Phosphodiesterase inhibitors are known to release G_1 and G_2 arrested cells and prevent p53 induction after DNA-damage (Kastan *et al.*, 1991). In NIH/3T3 cells, this mechanism seems to play a role, but in SiHa cells it was not the case. Other mechanism which makes the difference in susceptibility to actinomycin D is not clear at present. The difference in several oncogenes such as *c-myc* and *bcl-2* are the probable candidates (Wyllie, 1993).

In the present study the independence of DNA damage-induced apoptosis from cell cycle and the participation of cAMP, though in part, in the cytotoxicity of actinomycin D were shown.

Conclusion

To study the correlation of apoptotic process with cell cycle, the effect of aphidicolin and nocodazole, reversible cell cycle inhibitors, on the cell death induced by actinomycin D was studied. HeLa, SiHa, and NIH/3T3 cells were treated with actinomycin D alone or in combination with cell cycle inhibitors for 20 hours; or presynchronized cells were treated with actinomycin D for 4 hours and further incubated for 20 hours in fresh medium. The synergistic effect of IBMX on the cell death induced by actinomycin D was also studied.

Majority of Hela cells showed apoptotic changes

after actinomycin D treatment. Aphidicolin or nocodazole blocked neither initiation nor progression of actinomycin D-induced apoptosis in HeLa cell. Aphidicolin pretreatment enhanced the actinomycin D-induced apoptosis of HeLa and cell death of SiHa cells and nocodazole pretreatment enhanced cell death of SiHa cells. IBMX moderately enhanced actinomycin D-induced cell death of NIH/3T3 and to less extent that of SiHa cells. These results show that neither initiation nor progression of actinomycin D-induced apoptosis or cell death can be blocked by cell cycle arrest, and that cAMP is partly responsible for actinomycin D-induced apoptosis in certain types of cells.

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