STIMULATION AND INHIBITION OF DNA SYNTHESIS AND CELL GROWTH BY PARAQUAT IN CULTURED CELLS

Reiko TANAKA and Yasuji AMANO*

Department of Pharmacology and Central Laboratory*
Akita University School of Medicine, Akita 010, Japan

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Abstract.....Paraquat, a popular herbicide, is known to be very toxic to man and animals. Using cultured mammalian cells (human embryo lung cell, R-66), the effect of paraquat has been studied. With autoradiography, stimulation of DNA synthesis was observed when the cells were exposed to 0.08 or 0.4 μM of paraquat; however, DNA synthesis was inhibited with 40 μM of paraquat. To determine cell growth rate, accumulation of mitotic cells was determined after addition of colcemid (0.2 $\mu\text{g/ml}$) into the culture medium. The ratios of mitotic cells observed were proportional to the level of DNA synthesis. And the stimulation and inhibition of cell growth were observed even after washing off paraquat from media.

Key words : Paraquat, R-66, DNA synthesis, cell growth.

INTRODUCTION

Paraquat (PQ, 1, 1'-dimethyl-4, 4'-bipyridylium dichloride), is known to be very toxic to man and animals (Haley et al., 1979). Many reports have indicated that the herbicidal and toxic effects of PQ, which include lipid peroxidation (Hassan and Fridovich, 1979; Mirsa and Gorsky, 1981; Trush et al., 1981), depletion of cellular NADPH (Witshe et al., 1977; Forman et al., 1980) and other biological reactions in cells (Freeman and Crapo, 1982), are mediated by the generation of superoxide anions in electron transfer reactions (Bus et al., 1974).

However, toxic effects of PQ, which are not affected by superoxide dismutase, have been reported (Patterson and Rhodes, 1982). These effects also include inhibition of DNA synthesis and DNA single-strand breaks in mouse lymphoblasts (Ross et al., 1979).

In the present paper, the effects of PQ on DNA synthesis and cell growth using cultured human embryo lung cells have studied to elucidate the effects of PQ in cells, especially in lower concentrations than those previously reported.

MATERIALS AND METHODS

R-66 cells (human embryo lung cells) were grown in Eagle's minimum essential medium (Nissui) supplemented with 5% calf serum and 6 $\mu\text{g}/\text{ml}$ of kanamycin, at 37°C.

DNA synthesis was determined by the incorporation of ^3H -thymidine (Radiochemical Ctr., Amersham, 20 mCi mg) into the nuclei of cells. R-66 cells (1×10^5 in 1 ml) were seeded into small rectangular bottles in which cover glass strips were placed. After 20 hr of incubation at 37°C, the cells were incubated with various concentrations of PQ (Wako Pure Chemical Industries, Ltd) for various periods, then radioactive thymidine was added to the medium. After 2 hr of incubation with 0.5 μCi ^3H -thymidine, the cells were fixed in a mixture of ethanol and acetic acid (3 : 1), then air dried. The dried cells on cover glass strips, pasted onto micro-slide glass, were coated with Sakura NR-M₂ emulsion by dipping and then exposed for 2 weeks at -80°C. After development, the cells were stained with Giemsa's solution (MERCK) and silver grains on the nuclei of cells were counted using a microscope.

To estimate cell growth rates, the cells (1×10^5 in 1 ml of culture medium) were seeded into test tubes (15 $\times$ 100 mm), and after 20 hr of incubation at 37°C, various concentration of PQ were added to the culture media. Every 24 hr after the addition of PQ, cells were harvested from three tubes randomly selected and counted by a hemocytometer, then cell growth rates were calculated.

In some instances, the numbers of mitotic cells subsequent to PQ treatment were determined for estimating cell growth rate, instead of the total cell counting method. Colcemid (0.2 $\mu\text{g}/\text{ml}$) was added to each culture and incubated for the last 20 hr of each incubation time, then the ratio of mitotic cells to total cell number was calculated, and expressed as a mitotic index.

To determine the long-term effect of PQ on R-66 cells the following methods were used. First, R-66 cells were cultivated in media containing different concentrations of PQ and subcultures were made in the PQ-contained media, at intervals of four days, for several generations. At the end of each generation, mitotic indices were calculated. Second, the cells were cultivated in PQ containing media for 2 days or 6 days and subcultures were continued without PQ.

RESULTS

The results of DNA synthesis determined by autoradiography are summarized in Table 1 and Fig. 1.

The effect of PQ on DNA synthesis in R-66 cells is biphasic. The highest value of silver grain count per nucleus was obtained when there was 0.4 μM of PQ in the culture medium. However, at 40 μM of PQ, the silver grain count was extremely low. At 2.0 and 10.0 μM , no differences were found in comparison with the control.

The effect of PQ on the growth of R-66 cells is shown in Fig. 2. At 0.4 and 0.08 μM of PQ, cell growth was significantly enhanced. On day 1 of incubation, cell numbers were $1.6 \times 10^5/\text{tube}$ without PQ and $1.8 \times 10^5/\text{tube}$ with 0.4 μM PQ. On day 4 of incuba-

Effect of paraquat on cultured cells.

Fig. 1 Relation between paraquat concentration and silver grain counts on nuclei. Incubation of cells with paraquat and incorporation of $[6\text{-}^3\text{H}]$ -thymidine were performed by the same technique as that which yielded the data shown in Table 1.

Table 1 Incorporation of ^3H -thymidine into nuclei of R-66 cells exposed to paraquat.

Paraquat Conc. (μM)	Grains/nucleus
0	23.0 ± 0.6
0.08	$32.7 \pm 0.8^{**}$
0.4	$37.5 \pm 0.8^{**}$
2.0	26.2 ± 0.7
10.0	24.1 ± 0.9
40.0	$11.0 \pm 0.6^{**}$

$n=90$, $^{**}p<0.01$

Cells were incubated with paraquat for 24 hr, and labeled with $0.5 \mu\text{Ci}$ $[6\text{-}^3\text{H}]$ -thymidine for 2 hr.

Fig. 2 Growth curves of cells incubated with paraquat.

tion, cell numbers had increased to 2.6×10^5 and 4.4×10^5 , respectively.

Accumulations of mitotic cells (mitotic indices) after incubation with colcemid are summarized in Table 2. With 0.08 and 0.4 μ M of PQ, mitosis of R-66 cells was significantly enhanced.

To determine the prolonged effect of PQ of on cell growth, R-66 cells were washed after incubation with PQ for 2 days or 6 days, and cultivated without PQ for several generations at intervals of four days. At each generations, mitotic indices were calculated and the results are shown on Table 3. The cells cultivated in 0.4 μ M of PQ show a high mitotic index even after the third subculture.

Table 2 Accumulation of mitotic cells incubated with paraquat and colcemid for the last 20 hr.

Paraquat Conc. (μ M)	Mitotic cells (%) period of incubation with paraquat (days)		
	1	2	5
0	32.0 ± 2.2	28.2 ± 0.6	20.5 ± 1.3
0.08	29.4 ± 1.4	35.2 ± 3.2	$34.7 \pm 0.6^{**}$
0.4	35.1 ± 2.9	$32.7 \pm 1.6^*$	$32.6 \pm 2.7^*$
40.0	29.0 ± 3.1	$10.9 \pm 4.0^{**}$	$6.1 \pm 2.7^{**}$

n=2-4, * p<0.05, ** p<0.01

Cells were subcultured at 4 days, and incubation with paraquat was continued.

Table 3 Accumulation of mitotic cells treated with paraquat for 2 days or 6 days and repeatedly subcultured every 4 days without paraquat.

Paraquat Time# Conc. (days) (μ M)	Mitotic cells (%) Subculture (days after washing off paraquat)				
	I(2)	II(6)	III(12)	VI(21)	VII(26)
0	32.1 ± 1.7	34.3 ± 1.3	31.3 ± 1.3	49.9 ± 4.0	25.1 ± 1.7
2	$47.4 \pm 3.9^*$	$45.2 \pm 0.4^{**}$	$44.9 \pm 1.5^{**}$	46.9 ± 3.0	21.5 ± 0.7
40.0	$7.7 \pm 2.4^{**}$	$14.3 \pm 2.1^{**}$	34.4 ± 2.2	48.6 ± 5.0	34.4 ± 6.6
0	23.6 ± 1.3	37.7 ± 1.8	17.3 ± 2.3	47.7 ± 2.4	29.2 ± 2.4
6	$38.5 \pm 0.9^{**}$	$51.2 \pm 2.0^{**}$	$33.3 \pm 3.0^{**}$	43.1 ± 2.1	32.7 ± 2.2
40.0	$0.1 \pm 0.1^{**}$	$0.1 \pm 0.1^{**}$	$2.5 \pm 0.1^{**}$	$24.7 \pm 2.0^{**}$	29.0 ± 3.8

: Treatment with paraquat. n=2-4, * p<0.05, ** p<0.01

Cells were treated with colcemid for the last 20 hr of each incubation period.

DISCUSSION

Paraquat has been studied by many investigators because of the toxicity to man and of its chemical nature as a superoxide generator.

However, few investigators have indicated a direct interaction between PQ and DNA, which is not affected by the superoxide dismutase or catalase. Ross et al. (1979) found DNA strand breaks in L1210 mouse leukemia cells treated with PQ. Also, using "macrophage-like" P388D₁ cells, Carmines and his co-workers (1981) observed inhibition of DNA synthesis by PQ.

On the other hand, Ahmed and his co-workers (1977) reported unscheduled DNA synthesis induced by diquat (1, 1'-ethyne-2, 2'-bipyridylum dibromide), which is a member of the same chemical family as PQ.

In the present paper, it was clarified that PQ has a biphasic effect on R-66 cells: a cytotoxic effect at higher concentration (40 μ M) and enhancement of cell division at lower (0.4-0.08 μ M) concentration (Fig. 2).

A similar result was also obtained in the ³H-thymidine incorporation experiment (Fig. 1). A higher concentration of PQ suppressed DNA synthesis in the cells but a lower concentration of PQ enhanced thymidine incorporation.

A long-term effect of PQ on the growth of R-66 cells (Table 3) showed that cells treated with low concentration of PQ (0.4-0.08 μ M) had increased mitotic indices, even after the third (12 days) subculture without PQ.

The two results, namely increased cell growth rate and enhanced DNA synthesis, are possibly due to a common effect of PQ, but the nature of this action is not yet clear.

And only from those results described in the present paper, it is not able to estimate the mechanism of effect of PQ, especially to conclude that the superoxide production by PQ have contributed to those stimulatory and inhibitory effects on DNA synthesis, or that PQ reacts directly with DNA in nucleus. Furthermore, it is possibly supposed that the different rule is present between low concentrations and high concentrations of PQ.

In our previous experiment, it was found that the melting point (T_m) of freshly prepared salmon sperm DNA was markedly increased by the addition of PQ, strongly suggesting a direct interaction between double strand DNA and PQ (unpublished data). The experimental results with transport of PQ into cultured mammalian cells in relation to the effects on DNA and production of superoxide would be described in next paper, while reduced paraquat could diffuse across the cell envelope and react with dioxygen in *Escherichia coli* (Hassan, 1979).

PQ or other chemical agents have been investigated using concentrations at which inhibitory toxicity is expressed on a cell. However it is suggested that PQ is capable of influencing cell growth at far lower concentrations than inhibitory concentration and that it may play a important role in genetical phenomena.

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