Free Rad. Res. Comms., Vol. 18, No. 3, pp. 139-145
Reprints available directly from the publisher
Photocopying permitted by license only

THE EFFECT OF PARAQUAT ON THE RADIOSENSITIVITY OF MELANOMA CELLS: THE ROLE OF SUPEROXIDE DISMUTASE & CATALASE

ALICJA JAWORSKA*, NADA STOJCEVIC-LEMIC# and ANTHONY H.W. NIAS#

**Department of Radiation Biology and Health Protection, Institute of Nuclear Chemistry and Technology, 03-195 Warsaw, Poland and #Richard Dimbleby Department of Cancer Research, St. Thomas's Hospital, London SE1 7EH, United Kingdom*

(Received: September 9, 1992; in final form, December 1, 1992)

The activities of reactive oxygen species scavenging enzymes, superoxide dismutases (SODs) and catalase (in cells of two melanomas (mouse B16 and human SK23) and in Chinese hamster ovary (CHO) cells were examined. Melanoma cells are relatively depleted in activities of superoxide dismutases and catalase as compared to CHO cells. Short equitoxic (500 μM for CHO and B16 cells and 5 μM for SK23 cells) paraquat treatment (15 min before the X-irradiation, 45 min in postirradiation period - the total time of treatment was 1 h) caused an increase in radiation resistance, measured as colony forming ability, in two of the three lines examined.

It is proposed that PQ may exert its radioprotective effect by induction of antioxidant enzymes.

KEY WORDS: Paraquat, radiation resistance, melanoma cells, superoxide dismutase, catalase.

INTRODUCTION

Paraquat (PQ) is a widely used herbicide. Its high cytotoxicity for mammals is exerted mainly by the deleterious effect on lung tissue. It is well established that the ability of this compound to generate O_2^- in the cell through redox cycling reactions (1) is responsible for the toxic action of PQ. It was found in several studies that paraquat induce superoxide dismutases in both bacterial (2) and mammalian systems (3). Induction occurs rapidly and is by suppressed inhibitors of both translation and transcription (4,5).

In this study we used two melanoma cell lines (human SK23 and murine B16) and as a reference line Chinese hamster ovary (CHO) cells. Our aim was to establish whether there is any correlation between PQ toxicity and activities of antioxidant enzymes, catalase and superoxide dismutases; and secondly whether a short PQ pretreatment would modify the radiation resistance of above mentioned cell lines as we found previously for mouse lymphoma L5178Y cells (6).

Address for correspondence: Alicja Jaworska Department of Radiation Biology and Health Protection, Institute of Nuclear Chemistry and Technology, Dorodna 16, 03-195 Warsaw, Poland telephone: (0-4822) 11-07-36 fax: 11-15-32

MATERIALS AND METHODS

Cells, Culture Conditions and Paraquat Treatment

Monolayer cultures of human melanoma SK23, mouse melanoma B16 and CHO cells were grown in Falcon flasks in minimal essential medium (MEM) supplemented with L-glutamine and 15% fetal calf serum and, in the case of CHO cells, with non-essential amino-acids. Cultures were trypsinized and passaged twice weekly to maintain the logarithmic growth. Asynchronous log-phase cells were taken for experiments. They were seeded in 25 ml Falcon flasks two days before X-irradiation or PQ treatment.

PQ (Sigma) was added 15 min before X-irradiation and removed after 1 hour. Thus, it was present for approximately 45 min after irradiation. PQ was added in equitoxic doses i.e. doses which reduce the clonogenic survival to 30% (500 μm for CHO and B16 cells and 5 μM for SK23 cells). For enzymes activities estimation $10^7 - 10^8$ cells in log phase of growth were taken. Cultures were washed with phosphate-buffered saline (PBS) and trypsinized. The cell suspensions were centrifuged two times at 50 × g for 10 min. The cell pellets were stored frozen in liquid nitrogen.

Estimation of Enzyme Activities

1. Cell homogenate preparation

For superoxide dismutases (SOD) estimation, cell pellets were suspended in 5×10^{-2} mol dm^{-3} HEPES (Sigma), pH 7.5, and sonicated by three consecutive 10 s bursts at 20 kilocycles/s while on ice. Crude homogenates were used in SOD assay.

For catalase estimation cell pellets were suspended in 5×10^{-3} mol dm^{-3} phosphate buffer, pH 7.0, containing 0.1% Triton X-100, homogenized in a glass homogenizer, frozen and thawed three times. This procedure gives, in our hands, better results than sonication. Homogenates were centrifuged at 18,000 × g for 30 min. Catalase activity was determined in the supernatant.

2. SOD assay

The activity of SOD was determined by the "xanthine-xanthine oxidase" assay (7) using nitroblue tetrazolium (NBT) as a detector of O_2^-; HEPES (N-[hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) (Merck) was used instead of phosphate buffer. No NBT reductase activity was detected at the protein concentration used in the assay. The unit of SOD activity was defined as the amount of the enzyme which will reduce the reaction rate by 50 per cent under the assay conditions. The reaction mixture contained an aliquot of sonicate, 10^{-3} mol dm^{-3} DTPA (diethylenetriamine-pentaacetic acid) (Merck), 5×10^{-2} mol dm^{-3} HEPES pH 7.5, 5.6×10^{-5} mol dm^{-3} NBT (Sigma), 1 IU of catalase (Sigma), 10^{-4} mol dm^{-3} xanthine (Merck) and xanthine oxidase (Sigma). The amount of xanthine oxidase was adjusted, so that the absorbance change was 0.020/min without SOD. Manganese-containing SOD was assayed in the presence of 2 × 10 mol dm^{-3} KCN (Merck) to inhibit copper-zinc SOD activity. Samples were preincubated with cyanide for 30 min. The amount of Cu,Zn-SOD was calculated by subtraction of Mn-SOD activity from the total SOD activity.

3. Catalase assay

Catalase activity was determined by monitoring decomposition of H_2O_2 at 240 nm during 1 min from the start of the reaction (8). One unit of catalase activity was defined as the amount of enzyme which is required to catalyze decomposition of 1 μmole of H_2O_2 during 1 min.

4. Protein estimation

Protein was determined by a modification of Bradford's method (9).

Irradiation Procedure

Attached cells in 25 ml Falcon flasks were irradiated at room temperature with a Maximar X-ray machine (250 kV) at a dose rate of 1. 2 Gray per min (Gy min^{-1}), 0.83 mm Cu HVT.

Clonogenic Assay

To determine survival in terms of colony-forming ability, cells were plated in 25 ml Falcon flasks. In control flasks 250 cells (CHO and B16) or 500 cells (SK23) were seeded. Colonies were stained and counted after 8 days (CHO and B16) or 14 days (SK23). Plating efficiencies of the untreated cultures were 95 per cent for CHO, 57 per cent for B16 and 19.5 per cent for SK23 cells.

Determination of Paraquat Toxicity

Cell cultures were incubated with PQ at concentrations between 10^{-2} and 10^{-8} mol dm^{-3} for 1 h and then PQ was removed by medium change. Toxicity was determined by clonogenic assay.

RESULTS AND DISCUSSION

Activities of Cu,Zn-SOD, MN-SOD and catalase are presented in table 1. We found that human melanoma SK23 is deficient in activity of SODs, as compared to mouse melanoma B16 and CHO cells. Activity of catalase is lower in both mice and human melanomas than in CHO cells as well as in HeLa cells (637.29 IU/mg protein). We estimated catalase activity in HeLa to have a reference of human origin. The results indicate that human melanoma SK23 is deficient in both catalase and SODs activities.

TABLE 1
Activities of superoxide dismutases and catalase in SK23, B16 and CHO cells

Cell line	SODs [U/mg protein] $x \pm SD$ Cu,Zn-SOD	Mn-SOD	Catalase [IU/mg protein] $x \pm SD$
SK23	2.8 ± 1.6	2.2 ± 1.2	284 ± 46
B16	6.4 ± 2.7	3.9 ± 1.3	306 ± 37
CHO	10.0 ± 4.5	3.1 ± 1.2	676 ± 109

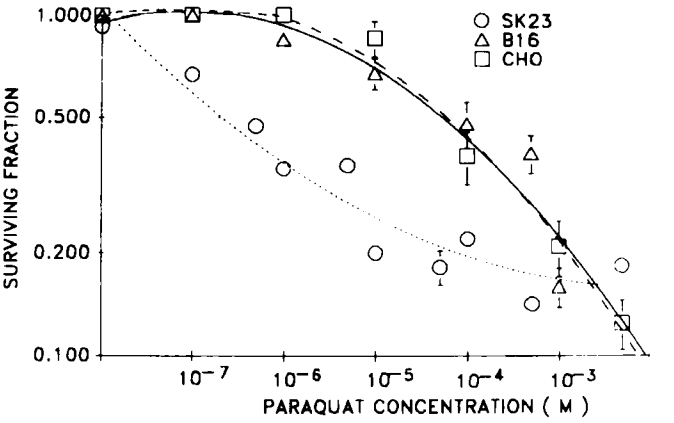

FIGURE 1 Toxicity of PQ in SK23, B16 and CHO cells. Cells were incubated with paraquat for 1 h. PQ was removed by medium change. Survival was estimated, as described in Materials and Methods, in 3 separate experiments. Bars indicate standard errors.

We then estimated paraquat toxicity for SK23, B16 and CHO cells. SK23 cells are much more susceptible to the toxic action of PQ, than B16 and CHO cells (Figure 1). The last finding, together with relative deficiency in catalase and SOD activities, may be attributed to the fact that SK23 cells were derived from a relatively early passage (6th) as compared to the established CHO and B16 cells. It is well known that established cell lines passaged for many generations in atmospheres containing oxygen at much higher partial pressures than *in vivo* may have adapted to the increased oxidative stress (10). It is worth noting that the toxicity of paraquat may be inversely related to activities of SODs in these cell lines.

Further, it was found that human melanoma SK23 cells are more radiation-sensitive than B16 and CHO. Survival curves are presented as dashed curves in Figure 2.

Short PQ pretreatment and the presence of the compound during irradiation and for about half an hour in the postirradiation period caused an increase in radiation resistance in two of the three cell lines examined (SK23 and CHO). We did not find any significant difference in radiation sensitivity in the case of B16 cells. The respective survival curves (normalized to survival of unirradiated cells treated with PQ) are presented in Figure 2.

Parameters of the curves have been estimated according to the linear-quadratic model, reported to be most suitable for melanoma cells (11); they are presented in Table 2. In the last column the α/β ratio as a radiation response parameter is shown. It is worth noting that this ratio decreases for all three cell lines examined. It decreases also in the case of B16 cells although the difference between PQ treated and untreated cells in terms of colony forming ability is very small. This case shows that α/β ratio is not always a good measure of radiation sensitivity. It can be misleading, if both the slope and the shoulder of the curve are changing as a result of combined treatment.

Results of this work indicate that a connection exists in the cell lines examined between activities of antioxidant enzymes removing $O_2^{\cdot-}$ and H_2O_2, PQ toxicity and sensitivity to ionizing radiation. Firstly, in SK23 cells, which are more sensitive to X-rays than the other two cell lines (B16 and CHO) we found a relatively low

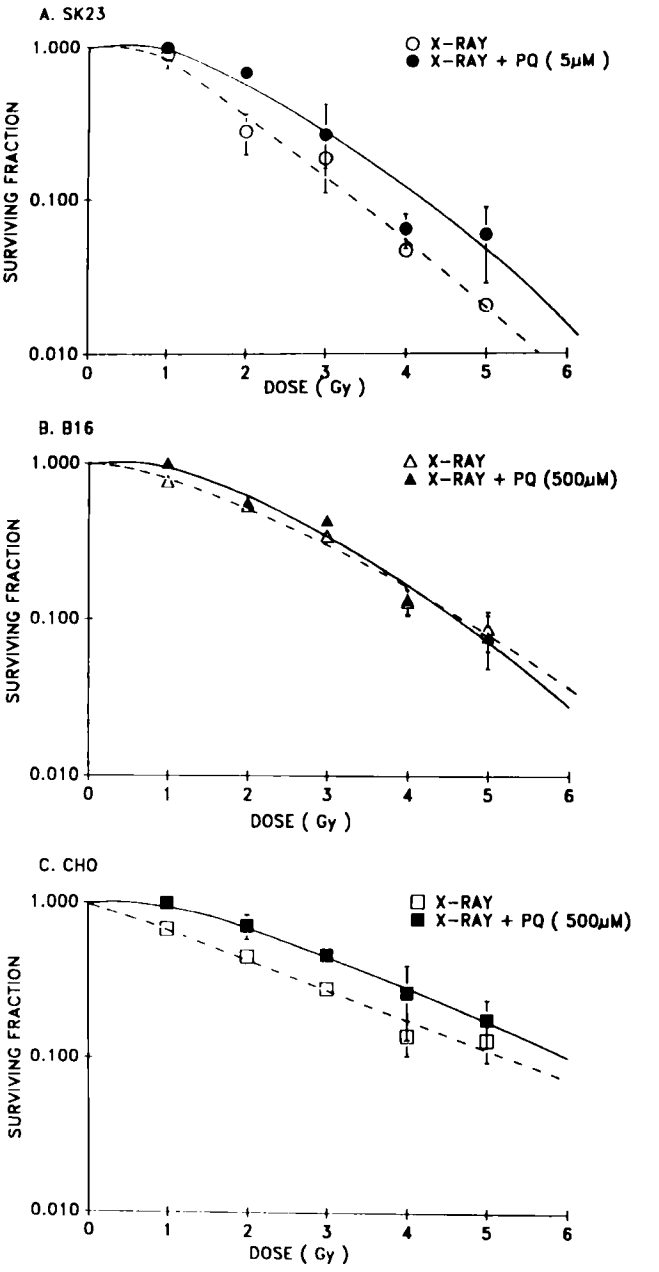

FIGURE 2 X-ray survival curves for PQ treated (solid lines) and untreated (dotted lines) cells: A. SK23, B. B16, C. CHO cells. Mean results from 4 experiments, with standard errors indicated.

TABLE 2
Parameters of X-ray survival curves, estimated with linear-quadratic equation $\ln S = -\alpha D - \beta D^2$ for cells treated (+) or untreated with PQ (−). Conditions of PQ treatment as described in Materials and methods

Cell line	PQ treatment	$\alpha \pm$ SD [Gy^{-1}]	$\beta \pm$ SD [Gy^{-2}]	α/β [Gy]
SK23	−	0.549 ± 0.117	0.055 ± 0.029	10.0 ± 4.5
	+	0.222 ± 0.055	0.220 ± 0.024	1.0 ± 1.4
B16	−	0.209 ± 0.027	0.051 ± 0.009	4.1 ± 0.2
	+	0.097 ● 0.049	0.082 ± 0.017	1.2 ± 0.4
CHO	−	0.382 ± 0.086	0.015 ± 0.025	25.5 ± 9.4
	+	0.102 ± 0.024	0.114 ± 0.014	0.9 ± 0.1

activity of SODs and catalase. Secondly, activity of SOD correlated inversely in these lines with the toxic action of PQ. This confirms that the toxic action of PQ is due $O_2^{-\cdot}$ generation in SK23, B16 and CHO cells. We did not find such connections in the case of catalase. However, we found lower catalase activity in melanoma cells both human and mouse than in CHO and HeLa cells.

The exact mechanism of PQ action is still under discussion, although the generation of $O_2^{-\cdot}$ seems to be proven (12,13). It was proposed that after superoxide production the subsequent events may be production of hydroxyl radical, singlet oxygen, tetravalent iron radical, alteration in nucleic acid metabolism, inhibition of DNA synthesis, intracellular lipid peroxidation, thiol oxidation, disulfide production and decreases in decreased nucleotide concentration such as NADPH (14 and references therein).

PQ may exert its toxic action by its influence on the scavenging potential in the cell. It was recently found that, apart from induction of SODs, PQ can affect H_2O_2 metabolism by induction of selenoenzyme glutathione peroxidase (15); at the cellular level it is difficult to discern this effect from the induction of SOD. This finding may explain why the higher activity of SODs itself, as in the cells overexpressing SOD (16), is not a sufficient condition for removing of reactive oxygen species, if not followed by scavenging of H_2O_2. Hence, the discrepancy can be explained between the results confirming (17,18) and discrediting antioxidant enzymes and, in particular, SODs as modulators of radiation resistance (16,19).

The same arguments can be applied to the discrepancy in the results of PQ influence on radiation resistance. In some reports PQ was found to be a weak radiosensitizer (4,20,21). However, it should be pointed out that high doses of the compound or prolonged treatment were used. In both cases $O_2^{-\cdot}$ accumulates in the cell and elevated enzymatic defence may not be sufficient to counteract its deleterious effects.

Acknowledgements

We are grateful to Professor Irena Szumiel for her critical remarks during preparation of this paper. This work was supported by the grant from the international Atomic Energy Agency.

References

1. J. Krall, A.C. Bagley, G.T. Mullenbach, R.A. Hallewell and R.E. Lynch (1988) Superoxide mediates the toxicity of paraquat for cultured mammalian cells. *Journal of Biological Chemistry*, **263**, 1910–1914.
2. H.M. Hassan, and I. Fridovich (1977) Regulation of the synthesis of superoxide dismutase in Escherichia Coli. Induction by methyl viologen. *Journal of Biological Chemistry*, **252**, 7567–7672.
3. A.C. Bagley, J. Krall and R.E. Lynch (1986) Superoxide mediates toxicity of paraquat for Chinese hamster ovary cells. *Proceedings of the National Academy of Sciences. USA*, **83**, 3189–3193.
4. Ch.R. Geard, C.M. Shea and M.A. Georgsson (1984) Paraquat and radiation effects on mouse $C_3H1/2$ cells. *International Journal of Radiation Oncology, Biology, Physics*, **10**, 1407–1410.
5. J.B. Stevens and A. Autor (1977) Induction of superoxide dismutase by oxygen in neonatal rat lung. *Journal of Biological Chemistry*, **252**, 3509–3514.
6. A. Jaworska and O. Rosiek (1991) Paraquat increases superoxide dismutase activity and radiation resistance in two mouse lymphoma L5178Y cell strains of different radiosensitivities. *International Journal of Radiation Biology*, **60**, 899–906.
7. I.B. Bize, L.V. Oberly, and M.P. Norris (1980) Superoxide dismutase and superoxide radical in the Morris hepatomas. *Cancer Research*, **40**, 3686–3693.
8. R.F. Berrs and I.W. Sizer (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195**, 133–140.
9. T. Spector (1978) Refinement of the Coomasie Blue method of protein quantitation. *Analitical Biochemistry*, **86**, 142–146.
10. R. Ham, W.L. McKeehan (1979) Media and Growth Requirements. In *Methods in Enzymology* **53**, *Cell culture* (ed. W.B. Jacoby & I. Pastan), Academic Press, Inc. London. p. 74.
11. K.H. Yohem, D.J. Slymen, M.D. Bregman, F.L. Meyskens, Jr. (1990) Radiosensitivity of murine and human melanoma cells: A comparative study with different models. *Pigment Cell Research*, **3**, 157–161.
12. R.C. Miller, T. Fujikura, T. Hiraoka and H. Tenon (1986) Paraquat induced the radiosensitization of mammalian cells. *Journal of Radiation Research*, **27**, 163–170.
13. C.S. Moody and H.M. Hassan (1982) Mutagenicity of oxygen free radicals. *Proceedings of the National Academy of Sciences. USA*, **89**, 2855–2859.
14. M.J. Kelner and R. Bagnell (1989) Paraquat resistance associated with reduced NADPH reductase in an energy dependent paraquat-accumulating cell line. *Archives of Biochemistry and Biophysics*, **274**, 366–374.
15. J.M. Kelner and R. Bagnell (1990) Glutathione-dependent enzymes alone can produce paraquat resistance. *Free Radicals in Biology and Medicine*, **9**, 149–153.
16. M.D. Scott (1989) Superoxide dismutase amplifies organismal sensitivity to ionizing radiation. *Journal of Biological Chemistry*, **264**, 2498–2501.
17. T.M. Nicotera, A.W. Block, Z. Gibas and A.A. Sandberg (1985) Induction of superoxide dismutase, chromosomal aberration and sister chromatid exchanges by paraquat in Chinese hamster fibroblasts. *Mutation Research*, **151**, 263–268.
18. A. Petkau (1987) Role of superoxide dismutase in modification of radiation injury. *British Journal of Cancer*, **55(suppl. 8)**, 87–95.
19. S.L. Marklund, N.G. Westman, G. Roos and J. Carlsson (1984) Radiation resistance and the Cu,Zn-superoxide dismutase, Mn-superoxide dismutase, catalase and glutathione peroxidase activities of seven human cell lines. *Radiation Research*, **100**, 115–123.
20. T.X. Peng, A. Moya, and F.J. Ayala (1986) Irradiation resistance conferred by superoxide dismutase. Possible adaptative role of radiation effects on mouse $C_3H/10T1/2$ cells. *International Journal of Radiation Oncology, Biology, Physics*, **10**, 1407–1410.
21. R.C. Miller, T. Fujikura, T. Hiraoka and H. Tenon (1986) Paraquat induced radiosensitization of mammalian cells. *Journal of Radiation Research*, **27**, 163–170.

Accepted by Prof. H. Sies