Free Rad. Res. Comms., Vol. 11, Nos. 1-3, pp. 137-144
Reprints available directly from the publisher
Photocopying permitted by license only

DEVELOPMENT OF MECHANISMS OF PROTECTION AGAINST OXIDATIVE STRESS IN DOXORUBICIN-RESISTANT RAT TUMORAL CELLS IN CULTURE

M.N. BENCHEKROUN, P. CATROUX, D. MONTAUDON and J. ROBERT

Fondation Bergonié and Université de Bordeaux II, 180, rue de Saint-Genès — 33076 Bordeaux Cédex, France

(Received December 15, 1989; in final form April 9, 1990)

We have compared some mechanisms involved in the defense against doxorubicin-induced free radical damage in rat hepatoma and glioblastoma cell lines and their doxorubicin-resistant variants presenting an overexpression of the multidrug resistance gene.

Immediate *in vivo* production of malondialdehyde was minor and was not different in sensitive and resistant cells. Alpha-tocopherol was undetectable in all cell lines. Glutathione levels were not different in sensitive and resistant cells and these levels did not vary upon doxorubicin treatment. Resistant cells exhibited either a 50% decrease (hepatoma) or a 25% increase (glioblastoma) of glutathione-S-transferase activity. Glutathione reductase presented no important change upon acquisition of resistance. In contrast, selenium-dependent glutathione peroxidase activity was consistently 2–6-fold increased in the resistant cells, which suggests a magnification of protection mechanisms against hydroxyle radical formation from H_2O_2 in resistant cells. Depletion of glutathione levels by buthionine sulfoximine sensitized hepatoma resistant cells to doxorubicin, but had no effect on doxorubicin cytotoxicity to glioblastoma cells.

KEY WORDS: Multidrug-resistance, glutathione, glutathione peroxidase.

INTRODUCTION

The development of doxorubicin resistance during infratoxic exposure of cultured cells generally leads to a multidrug resistance phenotype characterized by the overproduction of a membrane glycoprotein responsible for drug extrusion (see for review[1]). The overexpression of the gene encoding this glycoprotein, called *mdr1*, has been recognized in several multidrug-resistant cell lines selected with a variety of anticancer drugs, and has been presented as the unique mechanism for resistance, especially since it was shown that gene transfer induces in sensitive cells the multidrug-resistant phenotype.[2]

Several features are not explained, however, by this conception of P-glycoprotein as the unique mechanism for doxorubicin resistance. Alteration of topoisomerase II, a target of several anticancer drugs, also leads to doxorubicin resistance.[3] Other cell lines display a reduction of net drug incorporation without any evidence of P-glycoprotein overexpression.[4,5] Clinical studies have failed to demonstrate the overexpression of the *mdr1* gene in solid tumors such as breast cancer, even in the cases when *in vitro* and *in vivo* resistance were documented.[6] It has been shown for a long time that doxorubicin cytotoxicity could be mediated by the free radical damage to macromolecules which occur through the formation of doxorubicin semiquinone radical.[7,8]

Therefore, the involvement of detoxication mechanisms against free radical formation or damage from anthracyclines can be postulated as a possible mechanism for drug resistance in tumoral cells. Alterations of the glutathione redox cycle have been observed in various multidrug-resistant cells much as MCF7 human breast cancer cells[9,10] or P388 mouse leukemia cells.[9,11] Increase of glutathione-S-transferase activity,[9,12] of glutathione peroxidase activity,[9,10] tolerance to superoxide ion,[13] sensitization to anthracycline action by glutathione depletion[14] have been observed by various authors working on different models of multidrug-resistant cells.

We had observed in our doxorubicin-resistant cell lines that they were able to incorporate much more drug than sensitive cells when exposed to similarly cytotoxic concentrations.[15] We postulated, therefore, that drug extrusion through the P-glycoprotein pump could not explain by itself the doxorubicin resistance of these cells. Moreover, cells of different origins could present the same resistance factor, and drastically differ in the level of *mdr1* gene overexpression.[16] This prompted us to evaluate in our cell lines the detoxication mechanisms against free radical formation or damage as a possible mechanism of drug resistance, added to the classical *mdr* mechanism.

MATERIAL AND METHODS

Cell Culture

The C6 clone originated from a rat glioblastoma induced by N-nitrosomethylurea[17] and the HTC clone from a spontaneous rat hepatoma.[18] Resistant cells were obtained by 10-week successive exposures to increasing amounts of doxorubicin (0.001, 0.003, 0.01, 0.03, 0.1, 0.3, 0.5 or 1.5 μg/ml) and cultivted as monolayers as already described.[16] Experiments were done with the C6 and HTC cells cultivated for at least 1 year in the presence of 0.5 and 1.5 μg/ml doxorubicin respectively.

Doxorubicin sensitivity was evaluated by incubating the cells 72 h after plating, for 2 h at various doxorubicin concentrations; cells were counted after growth in normal medium for two cell generations. Cell survival was then plotted against doxorubicin concentration and IC50 (concentration of doxorubicin yielding a 50% inhibition of cell number) was determined.

Doxorubicin-Induced Lipid Peroxidation

Lipid peroxidation produced by doxorubicin was evaluated either in cell homogenates or in intact cells. Cell homogenates were obtained in 50 mM Tris-HCl buffer, 150 mM KCl, pH 7.4, and were then incubated with 2.5 mM NADPH and 0 or 100 μM doxorubicin for 1 h at 37° under a 100% oxygen atmosphere in the dark, as described by Mimnaugh *et al.*[19] Addition of trichloroacetic acid terminated the reaction and provided an acid-soluble supernatant for malondialdehyde (MDA) assay.

Intact living cells were incubated for 1 h with doses of doxorubicin providing similar intracellular concentrations in sensitive and resistant cells; the doses were 10 μg/ml and 100 μg/ml in sensitive and resistant cells respectively. Cells were then harvested in 0.15 M NaCl immediately after drug exposure, and homogenized with a Potter for MDA assay. Cell media were also analyzed with the same technique. In all cases, MDA was quantified by the thiobarbituric reaction as described by Ohkawa

et al.[20] for cell homogenates, and by an HPLC procedure with fluorometric detection for intact cells and culture media.[21]

Tocopherol Assay

Alpha-tocopherol was assayed by the technique of Buttriss and Diplock,[22] on an HPLC column of C18-bonded silica with fluorimetric detection. This technique gave a lower limit of sensitivity of 10^{-11} moles.

Glutathione and Glutathione-Related Enzymes

Total glutathione was assayed in cells by the spectrophotometric technique of Brehe and Burch[23] using glutathione reductase (Sigma). Inhibition of reduced form by N-ethyl maleimide showed that no detectable oxidized form was present in the cells.

Glutathione transferase activity was assayed by the technique of Habig *et al.*[24] with 1-chloro-2,4-dinitrobenzene as a substrate.

Glutathione reductase activity was assayed by the technique of Cohen and Duvel.[25]

Glutathione peroxidase activity was assayed by the technique of Lawrence and Burk[26] either with 0.25 mM H_2O_2 as a substrate (selenium-dependent enzyme) or with 1.5 mM cumene peroxide as a substrate (total enzyme).

Modulation of Glutathione Levels by Buthionine Sulfoximine (BSO)

BSO treatment was realized in the cells in order to deplete glutathione levels. In all sensitive or resistant lines, a 90–95% depletion was obtained in 24 h treatment at 0.2 mM. We have verified that BSO itself had no cytotoxic activity on the cells. After a 24 h exposure to BSO, cell survival was always higher than 90% for BSO concentrations ranging from 0.05 mM to 1 mM. The effect of BSO on doxorubicin cytotoxicity was evaluated by the ratio of the IC50 obtained without and with BSO incubation; this parameter was referred to as degree of potentiation.

RESULTS AND DISCUSSION

Doxorubicin-Induced Lipid Peroxidation

When evaluated in cell homogenates, with NADPH as an electron donor, and with a 100% oxygen atmosphere, malondialdehyde basal production was slightly higher in

TABLE I
Lipid peroxidation in homogenates of C6 and HTC cells

	C6 glioblastoma		HTC hepatoma	
	sensitive	resistant	sensitive	resistant
MDA nmol/mg prot				
− dox	1.53 ± 0.23	3.03 ± 0.21	1.22 ± 0.21	1.75 ± 0.35
+ dox	2.31 ± 0.21	9.51 ± 1.60	2.34 ± 0.13	5.19 ± 0.53

Lipid peroxidation was evaluated in cell homogenates in Tris-KCl buffer, in the presence of 2.5 mM NADPH, under an atmosphere of 100% oxygen, for 1 h at 37°. Malondialdehyde production was estimated with the thiobarbituric reaction; the MDA-TBA complex was estimated by spectrophotometry (532 nm). Doxorubicin was at the concentration of 100 μM (58 μg/ml).

TABLE II
Lipid peroxidation in intact C6 and HTC cells

	C6 glioblastoma cells		HTC hepatoma cells	
	sensitive	resistant	sensitive	resistant
MDA nmol/mg prot				
− dox	0.308 ± 0.023	0.294 ± 0.026	0.135 ± 0.033	0.129 ± 0.024
+ dox	0.332 ± 0.007	0.301 ± 0.063	0.127 ± 0.005	0.122 ± 0.029

Lipid peroxidation was evaluated in cell extracts after incubation with doses of doxorubicin providing similar intracellular concentrations in the cells (10 μg/ml in sensitive cells, 100 μg/ml in resistant cells). Malondialdehyde production was estimated with the thiobarbituric reaction; the MDA-TBA complex was estimated by HPLC (column: microbondapak-C18; solvent: phosphate buffer/methanol 60/40; detection: fluorometry with excitation at 536 nm and emission at 557 nm).

doxorubicin-resistant cells than in sensitive cells. In the presence of doxorubicin (100 μM), malondialdehyde production increased by 50% in sensitive cells and was 3-fold increased in resistant cells (Table I). these levels of lipid peroxidation remained however low when compared to levels obtained in animal organs; this is generally the case in tumoral cells.[27]

In order to know if there was a significant production of lipid peroxides in the conditions that induce cytotoxicity, MDA production was estimated in intact cells incubated with cytotoxic concentrations of doxorubicin (Table II). When evaluated immediately after drug exposure, MDA production remained very low, both in sensitive and resistant cells, and only an HPLC technique could quantify the MDA-TBA adduct. No release of MDA in the culture medium of the cells treated with doxorubicin was evidenced.

Alpha Tocopherol Levels

Alpha-tocopherol remained under the limit of detection of our HPLC technique in all the cell lines tested. No alpha-tocopherol was either detected in the fetal bovine serum

TABLE III
Levels of glutathione and glutathione-related enzymes in C6 and HTC cells

	C6 glioblastoma cells		HTC hepatoma cells	
	sensitive	resistant	sensitive	resistant
Glutathione nmol/mg prot				
− dox	70.9 ± 8.1	85.5 ± 14.2	124 ± 8	95.8 ± 8.5
+ dox	76.2 ± 4.9	90.1 ± 3.6	126 ± 13	107 ± 14
Glutathione S-transferase (nmol/mg prot/min)	74.6 ± 3.1	93.3 ± 10.6	696 ± 19	257 ± 14
Glutathione reductase (nmol/mg prot/min)	36.9 ± 3.4	37.1 ± 5.9	103 ± 7	84.2 ± 4.6
Glutathione peroxidase (nmol/mg prot/min)				
selenium dependent	23.5 ± 3.6	153 ± 26	32.9 ± 6.5	62.0 ± 1.5
non-selenium dependent	57.9 ± 7.8	319 ± 61	534 ± 98	349 ± 30

Glutathione and glutathione-related enzymes were evaluated by spectrophotometric methods as described in Materials and Methods.

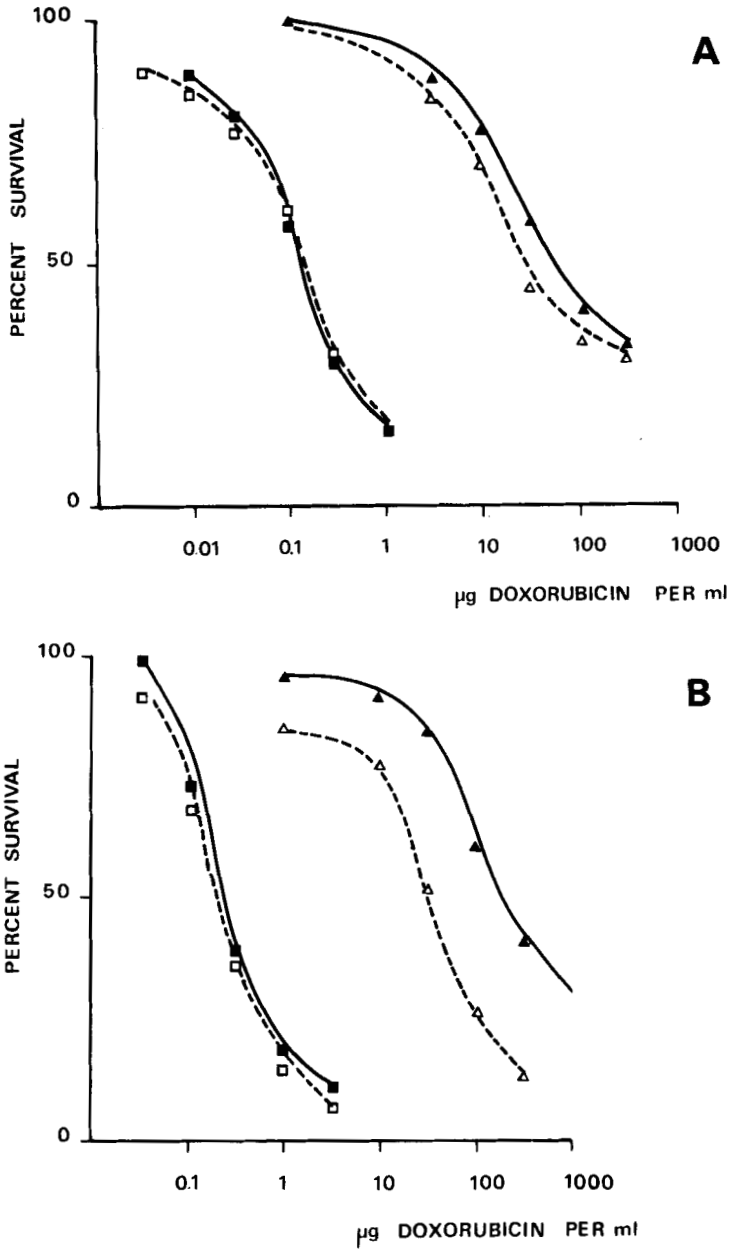

FIGURE 1 Effect of buthionine sulfoximine (BSO) on doxorubicin cytotoxicity in C6 (A) and HTC cells (B). BSO was added at the concentration of 0.2 mM and maintained for 24 h in the cell culture medium. These conditions provided a 90–95% depletion of glutathione and was not cytotoxic to the cells. Cytotoxicity of doxorubicin was evaluated by 2 h incubations with the drug at different concentrations and further growth for 2 cell generations. Cells were suspended and counted in a Coulter ZM counter. Square symbols: sensitive cells; triangle symbols: resistant cells; solid lines: control culture; dotted line: BSO-incubated cultures.

used in the cell culture media; we concluded that this compound could play no role in free radical scavenging in our cell lines.

Glutathione and Glutathione-Relted Enzymes

Glutathione levels were measured in the various cell lines (Table III) either in usual growth conditions or in the presence of cytotoxic concentrations of doxorubicin for 2 hours (IC50 dose). No significant difference was seen in the doxorubicin-resistant variants; no influence of doxorubicin upon glutathione levels could be detected.

Glutathione S-transferase activity was much higher in the hepatoma cell line than in the glioblastoma line, probably due to the origin of the tissue. A slight but significant increase of this activity was seen in the glioblastoma resistant cells, and a 50% decrease was seen in the HTC resistant cells. These results contrast with those observed on other cell lines, such as human breast MCF-7 cells, which exhibit a 40-fold increase of this activity.[9]

No significant change in glutathione reductase was exhibited in the resistant cells as compared to the sensitive ones.

Glutathione peroxidase was studied either with H_2O_2 as a substrate (selenium-dependant enzyme) or with cumene peroxide (total enzyme). We observed in the glioblastoma line a 6-fold increase of both activities upon resistance acquisition; a 2-fold increase of the selenium dependent enzyme was observed in the hepatoma resistant cells, but the non-selenium-dependent enzyme, which had a very high basal activity in sensitive cells, was decreased in the resistant cells. We think that the non-selenium-dependent enzyme may in fact represent a peroxidase activity of glutathione S-transferase, as shown by others.[12]

Modulation of Glutathione Levels by Buthionine-Sulfoximine

We observed in C6 cells a very slight effect of BSO on doxorubicin cytotoxicity (Figure 1); the degree of potentiation was about 1.4 in resistant cells. In HTC cells however, glutathione depletion was more efficient on doxorubicin cytotoxicity, with a degree of potentiation of 5.2 in the resistant line. It seems therefore that glutathione availability is a factor which participates to the expression of resistance, without playing a major role. Due to their very high seleno-dependent glutathione peroxidase activity, C6 resistant cells may not be as sensitive as HTC resistant cells to glutathione depletion.

CONCLUSIONS

We show in this preliminary paper that mechanisms other than drug extrusion may be involved in doxorubicin resistance in our cell lines. Doxorubicin is present in resistant cells at concentrations which are lethal to sensitive cells; this does not result in an immediate increase of lipid peroxidation, but in the enhancement of some detoxication mechanisms dependent on the glutathione redox cycle: the increase of selenium-dependent glutathione peroxidase activity might be responsible for the detoxication of H_2O_2 coming from superoxide ions. We are now exploring the mechanisms for production and detoxication of H_2O_2 species, superoxide dismutase and catalase.

References

1. G. Bradley, P.F. Juranka and V. Ling (1988) Mechanism of multidrug resistance. *Biochimica et Biophysica Acta*, **948**, 87–128.
2. P. Gros, Y. Ben-Neriah, J. Croop and D. Housman (1986) Isolation and expression of a cDNA (mdr) that confers multidrug resistance. *Nature*, **323**, 728–731.
3. W.T. Beck, M.C. Cirtain, M.K. Danks, R.C. Felsted, A.R. Safa, J.S. Wolverton, D.P. Suttle and J.M. Trent (1987) Pharmacological, molecular and cytogenetic analysis of "atypical" multidrug-resistant human leukemic cells. *Cancer Research*, **47**, 5455–5460.
4. T. McGrath and M.S. Center (1987) Adriamycin resistance in HL60 cells in the absence of detectable P-glycoprotein. *Biochemical and Biophysical Research Communication*, **145**, 1171–1176.
5. Pharmacological and biological evidence for differing mechanisms of doxorubicin resistance in two human tumor cell lines *Cancer Research*, **48**, 2793–2797.
6. D.E. Merkel, S.A.W. Fuqua, A.K. Tandon, S.M. Hill, A.V. Buzdar and W.L. McGuire (1989) Electrophoretic analysis of 248 clinical brest cancer specimens for P-glycoprotein overexpression or gene amplification. *Journal of Clinical Oncology*, **7**, 1129–1136.
7. C.E. Myers, W.P. McGuire, R.H. Liss, I. Ifrim, K. Grotzinger and R.C. Young (1977) Adriamycin: the role of lipid peroxidation in cardiac toxicity and tumor response. *Science*, **197**, 165–167.
8. J.H. Doroshow (1983) Anthracycline-stimulated superoxide, hydrogen peroxide, and hydroxyle radical production by NADH dehydrogenase. *Cancer Research*, **43**, 4543–4551.
9. R.A. Kramer, J. Zakher and G. Kinm (1988) Role of the glutathione redox cycle in acquired and de novo multidrug resistance. *Science*, **241** 694–697.
10. B.K. Sinha, E.G. Mimnaugh, S. Rajazopalan and C.E. Myers (1989) Adriamycin activation and oxygen free radical formation in human breast tumor cells: prospective role of glutathione peroxidase in adriamycin resistance. *Cancer Research*, **49**, 3844–3848.
11. S.V. Singh, S. Nair, H. Ahmad, Y.C. Awasthi and A. Krishan (1989) Glutathione-S-transferases and glutathione peroxidases in doxorubicin-resistant murine leukemic P388 cells. *Biochemical Pharmacology*, **38**, 3505–3510.
12. A. Batist, A. Tulpule, B.K. Sinha, A.G. Katki, C.E. Myers and K.H. Cowan (1986) Overexpression of a novel anionic glutathione transferase in multidrug resistant human breast cancer cells. *Journal of Biological Chemistry*, **261**, 15544–15549.
13. E.G. Mimnaugh, L. Dusre, J. Atwell and C.E. Myers (1989) Differential oxygen radical susceptibility of adriamycin-sensitive and -resistant MCF-7 human breast tumor cells. *Cancer Research*, **49**, 8–15.
14. J. Lutzky, M.B. Astor, R.N. Taub, M.A. Baker, K. Bhalla, J.E. Gervasoni, M. Rosado, V. Stewart, S. Krishna and A.A. Hindenburg (1989) Role of glutathione and dependent enzymes in anthracycline-resistant HL60/AR cells. *cancer Research*, **49**, 4120–4125.
15. B. Schott and J. Robert (1989) Comparative cytotoxicity, DNA synthesis inhibition, and drug incorporation of eight anthracyclines in a model of doxorubicin-sensitive and -resistant rat glioblastoma cells. *Biochemical Pharmacology*, **38**, 167–172.
16. B. Schoot, S. Huet, M.N. Benchekroun, D. Londos-Gagliardi, P. Vrignaud, D. Montaudon and J. Robert (1989) Pharmacological and molecular characterization of three rodent cell lines in culture selected for resistance to doxorubicin. In: Anticancer drugs (Tapiero, H., Robert, J. and Lampidis, T.J. eds), pp 245–252, Coloque INSERM/John Libbey Eurotext, Paris, vol. 191.
17. P. Benda, J. Lightbody, G. Sato, L. Levine and W. Sweet (1968) Differentiated rat glial cell strain in tissue culture. *Science*, **161**, 370–371.
18. E.B. Thompson, G.M. Tomkin and J.F. Curran (1966) Induction of tyrosin α-ketoglutarate transaminase by steroid hormones in a newly established tissue culture cell line. *Proceedings of the National Academy of Sciences, USA*, **56**, 296–304.
19. E.G. Mimnaugh, T.A. Trush and T.E. Gram (1986) A possible role for membrane lipid peroxidation in anthracycline nephrotoxicity. *Biochemical Pharmacology*, **35**, 4327–4336.
20. H. Ohkawa, N. Ohishi and K. Yagi (1979) Assay for lipid peroxide in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, **95**, 351–358.
21. S.H.Y. Wong, J.A. Knight, S.M. Hopfer, O. Zaharia, C.N. Leach and F.W. Sunderman (1987) Lipoperoxides in plasma as measured by liquid-chromatographic separation of malondialdehyde-thiobarbituric acid adduct. *Clinical Chemistry*, **33**,214–220.
22. J.L. Buttriss and A.T. Diplock (1984) High-performance liquid chromatography methods for vitamin E in tissues. *Methods in Enzymology*, **105**, 131–138.
23. J.E. Brehe and H.B. Burch (1976) Enzymatic assay for glutathione. *Analatical Biochemistry*, **74**, 189–197.
24. W.H. Habig, M.J. Pabst and W.B. Jakoby (1974) Glutathione S-transferase, the first enzymatic step

in mercapturic acid formation. *Journal of Biological Chemistry*, **249**, 7130–7139.
25. M.B. Cohen and D.L. Duvel (1988) Characterization of the inhibition of glutathione reductase and the recovery of enzyme activity in exponentially growing murine leukemia (L1210) cells treated with 1,3-bis (2-chloroethyl)-1-nitrosourea. *Biochemical Pharmacology*, **37**, 3317–3320.
26. R.A. Lawrence and R.F. Burk (1976) Glutathione peroxidase activity in selenium deficient rat liver. *Biochemical and Biophysical Research Communication*, **71**, 952–958.
27. K.V. Cheeseman, S. Emery, S.P. Maddix, T.F. Slater, G.W. Burton and k.U. Ingold (1988) Studies on lipid peroxidation in normal and tumour tissues. The Yoshida rat liver tumour. *Biochemistry Journal*, **250**, 247–252.

Accepted by Prof. H. Sies