Doxorubicin-induced oxygen free radical formation in sensitive and doxorubicin-resistant variants of rat glioblastoma cell lines

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We have studied the formation of hydroxyl radical (OH•) induced by doxorubicin in a series of doxorubicin- or vincristine-selected variants of C6 rat glioblastoma cells in culture by electron-spin resonance spectroscopy using 5,5'-dimethyl-1-pyrroline-1-oxide as a spin trap. Wild-type cells, sensitive to doxorubicin, exhibited in the presence of this drug a concentration-dependent OH• formation which could be inhibited by preincubation with superoxide dismutase, catalase or an antibody against cytochrome P450-reductase. In highly doxorubicin-resistant cells, OH• formation was reduced to about 20% of the level obtained in sensitive cells. In cells presenting a very low level of resistance to doxorubicin or in cells selected with vincristine, both presenting a pure multidrug-resistant phenotype, OH• formation was identical to that obtained in sensitive cells. In cells of intermediate resistance or in revertant cells, intermediate levels of OH• formation were obtained. Protection against OH• formation and action can be identified at the levels of superoxide dismutase and glutathione peroxidase activities, which are both enhanced in the resistant cells.

Anthracycline; Electron spin resonance spectroscopy; Free radical; Anticancer drug; Redox activation; Lipid peroxidation

1. INTRODUCTION

The exact role of doxorubicin-induced free radical formation in the cytotoxicity of this anticancer drug has been the matter of important controversy [1]. It is now generally accepted that the nuclear enzyme DNA-topoisomerase II is one of the primary targets of doxorubicin, which induces the formation of cleavable complexes and transforms this enzyme into an endogenous poison. This does not exclude, however, other possible toxic roles of doxorubicin at the cellular level. As several quinone compounds, doxorubicin can induce free radical formation through one-electron activation by NADPH-cytochrome P450 reductase [2]. The resulting oxygen free radicals, $O_2^{-•}$ and OH•, which are successively formed, can damage cell components at the level where they occur. This toxicity is probably not responsible for doxorubicin-induced tumor cell kill in the in vitro models used for cytotoxicity evaluation ; it can be hypothesized, however, that in cells lacking topoisomerase II activity as a primary hypersensitive target, the damages caused by oxygen free radicals can explain cell death. This could be for instance the case of cells resistant to doxorubicin [3] especially since they have been shown to develop no DNA breaks at cytotoxic exposures [4]. We have recently shown that it was possible

to evaluate the degree of doxorubicin-induced lipid peroxidation in tumor cells [5], and that it was related to the glutathione peroxidase activity of the cells [6]. We wanted then to know if this was related to the formation of oxygen free radicals; we have therefore evaluated the formation of hydroxyl radical upon doxorubicin exposure in cells sensitive and resistant to this drug, especially in cells having developed mechanisms of resistance other than P-glycoprotein overexpression, and displaying an intracellular tolerance to the drug [7]. We show in this paper that doxorubicin was able to induce hydroxyl radical formation in C6 rat glioblastoma cells, and that this production was decreased especially in the cells displaying a non-MDR resistance to this drug as an additional mechanism to classical MDR. We also show that there is a correlation between the decrease of doxorubicin-induced free radical formation and the superoxide dismutase and glutathione peroxidase activities of the cell lines.

2. MATERIALS AND METHODS

2.1. Cell culture
C6 rat glioblastoma cells [8] were grown with stepwise increasing amounts of doxorubicin so as to obtain a series of lines of increasing resistance. These lines have been already characterized in previous papers [7,9]. Briefly, C6 0.001 currently grow with 0.001 mg doxorubicin per ml medium and are 5-fold resistant to this drug through a pure MDR mechanism; C6 0.1 cells grow with 0.1 mg/ml doxorubicin, are 50-fold resistant to this drug, and part of this resistance cannot be explained only by *P*-glycoprotein overexpression; C6 0.5 cells grow

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with 0.5 mg/ml doxorubicin, are 400-fold resistant, but overexpress P-glycoprotein to the same level as C6 0.1 cells, indicating that most of the resistance acquired by this line as compared to the C6 0.1 one is unrelated to P-glycoprotein expression. A revertant line, C6 Rev, was obtained from C6 0.5 cells by one year culture in drug-free medium, and presents a degree of resistance of 2-fold as compared to C6 sensitive cells. To this panel of doxorubicin-selected lines was added a vincristine-selected line, C6 1V, growing with 1 μg/ml vincristine, and presenting both a high degree of resistance to vincristine (400-fold) and to doxorubicin (100-fold).

An important feature, unrelated to classical multidrug resistance, is the possible intracellular accumulation of doxorubicin in the most resistant cells selected with doxorubicin, without concomitant cytotoxicity. This 'intracellular tolerance' can be quantified as 'intracellular IC_{50}', i.e. the accumulation of doxorubicin accompanying 50% growth inhibition of the cell line. This intracellular IC_{50} is identical in C6 sensitive, C6 0.001, C6 Rev and C6 1V cells; it is in contrast 4-fold higher in C6 0.1 cells and 17-fold higher in C6 0.5 cells than in sensitive cells (see [7]).

Cells were routinely cultivated in DMEM supplemented with 10% fetal calf serum, in a 5% CO_2 atmosphere at 37°C. They were replicated each week and the culture medium was changed 2 times between passages.

2.2. Estimation of free radical formation

We have used electron spin resonance (ESR) spectroscopy with 5,5'-dimethyl-1-pyrroline-1-oxide (DMPO) as a spin trap, according to Kalyanaraman [10], as already described [3]. The formation of the complex DMPO-OH• is detected by this technique and reveals the presence of hydroxyl radicals. The cells were harvested after trypsinization and washed twice with ice-cold phosphate-buffered saline without calcium or magnesium. They were used at a density of 5×10^6/ml and the reaction medium (total volume: 0.4 ml) contained DMPO (50 mM), NADPH (1 mM), and doxorubicin (50–300 mM) in phosphate-buffered saline (50 mM, pH 7.4). A Varian E-109 spectrometer operating at 9.5 GHz with a field modulation of 100 kHz was used for these studies. The DMPO-OH• spectrum consists of a quartet (1 : 2 : 2 : 1) with hyperfine splitting constants of 14.9 G [11]. Experiments were performed either on intact cells or on cells sonicated on ice for 3×15 s at 50 W. Reactions were also done in the presence of 40 μl of 10% ethanol, which is a known scavenger of OH•, and forms DMPO-carbon centered radicals. Preincubations (30 min on ice) with 100 μg/ml superoxide dismutase (Sigma), 100 μg/ml catalase (Sigma), or with an antibody against rat cytochrome P450-reductase (a generous gift from Dr. Krishna, National Institutes of Health, Bethesda) were also performed. Similar preincubations in the absence of enzymes or antibodies had no effect on the ESR spectra recorded.

2.3. Evaluation of glutathione and of detoxifying enzymes

Reduced glutathione was assayed in the cells by the spectrophotometric technique of Brehe and Burch [12] using glutathione reductase (Sigma). Glutathione peroxidase was estimated by the technique of
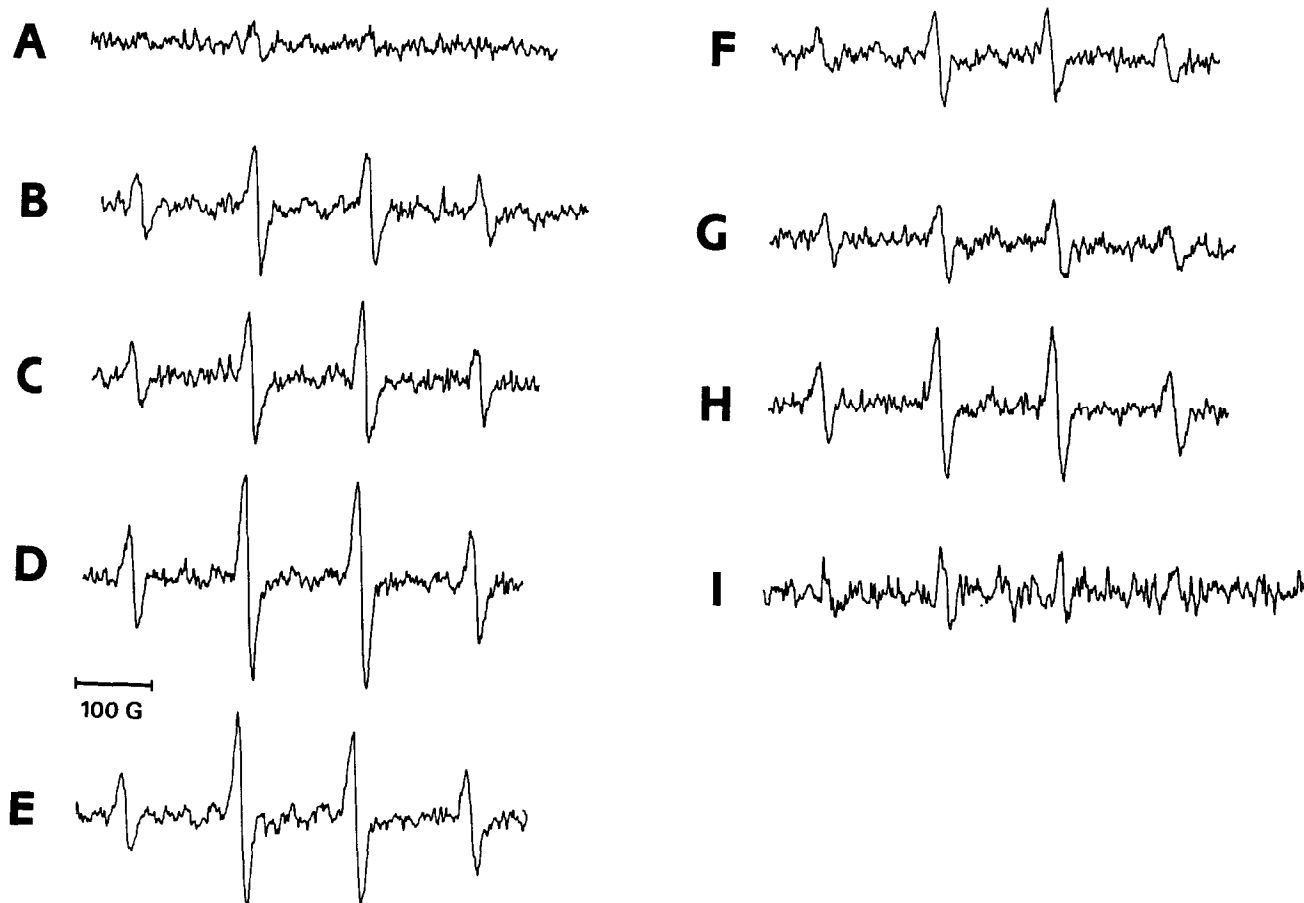

Fig. 1. ESR spectra obtained from C6 sensitive cells (5×10^6 cells/ml) in the presence of DMPO (50 mM) and NADPH (1 mM) in phosphate-buffered saline (50 mM, pH 7.4) (final volume: 0.4 ml). The hyperfine coupling constants for DMPO-OH were: $a^N = a^H = 14.9$ G. The ESR settings were: center field = 3364 G; microwave power = 20.0 G; modulation amplitude = 2.0 G; receiver gain = 8×10^4. (A) Control without doxorubicin; (B) doxorubicin: 50 mM; (C) doxorubicin: 100 mM; (D) doxorubicin: 200 mM; (E) doxorubicin: 300 mM; (F) doxorubicin: 200 μM; the reaction was done in the presence of 40 μl of 10% ethanol; (G) doxorubicin: 200 mM ; the cells were preincubated with superoxide dismutase (100 μg/ml for 30 min on ice; (H) doxorubicin: 200 mM; the cells were preincubated with catalase (100 μg/ml) for 30 min on ice; (I) doxorubicin: 200 mM; antibody to cytochrome P450-reductase was added to the lysed cell preparation.

Lawrence and Burk [13] using the supernatant at $25,000 \times g$ of a cell sonicate. The assay was performed in the presence of reduced glutathione (1 mM) and a glutathione regenerating system. The peroxide source was either cumene hydroperoxide (1.5 mM) for total glutathione peroxidase activity, or hydrogen peroxide (0.25 mM) for selenium-dependent glutathione peroxidase activity. Bovine erythrocyte glutathione peroxidase was used as a standard for calibration of the assay.

Superoxide dismutase was evaluated by the technique of Marklund and Marklund [14] on the same cell preparation as the one used for glutathione peroxidase evaluation. Superoxide dismutase activity is expressed in arbitrary International Units (IU), 1 IU being able to inhibit by 50% the autooxidation of pyrogallol.

Catalase was evaluated according to Beers and Sizer [15] on the same cell preparation.

3. RESULTS

Fig. 1 presents a series of ESR spectra obtained from C6 sensitive cells. No signal was present in the absence of either doxorubicin or the cell preparation. There was a concentration-dependent increase of the DMPO adduct formation up to 200 mM doxorubicin. Incubation with ethanol strongly decreased the signal, indicating that the OH° formed from doxorubicin were free hydroxyl radicals trapped by DMPO. The addition of superoxide dismutase or of catalase provided respectively a 70% and a 30% inhibition of adduct formation, indicating that $O_2^{-\bullet}$ and H_2O_2 were important intermediates in the production of OH°. Incubation of lyzed cells with an antibody against cytochrome P450-reductase completely abolished the ESR signal. Fig. 2 presents ESR spectra obtained from the C6 doxorubicin resistant variants incubated with 200 mM doxorubicin. C6 0.001 and C6 1V cells were able to form the same amount of DMPO-OH adduct as C6 sensitive cells, whereas C6 Rev, C6 0.1 and C6 0.5 cells respectively formed 1.5, 3.4 and 4.5 less adducts than C6 sensitive cells. This reduction was only partly due to the reduced doxorubicin accumulation occurring in multidrug resistant cells, since after sonication, lyzed C6 0.5 cells still presented a 3-fold reduction of DMPO adduct formation as compared to C6 sensitive cells. As in sensitive cells, preincubation of the C6 1V or C6 Rev cells with superoxide dismutase or catalase strongly decreased the ESR signal. Table I presents the levels of reduced glutathione as well as the activities of glutathione peroxidase, catalase and superoxide dismutase measured in the various cell lines. It appears that C6 0.1 and C6 0.5 cells have 3–4-fold higher levels of both selenium-dependent and non-selenium-dependent enzyme activities as compared to sensitive and C6 0.001 cells. It also appeared in the same cell lines an increase of superoxide dismutase activity, whereas basic glutathione levels as well as catalase activity remained unchanged in the cell lines studied.

4. DISCUSSION

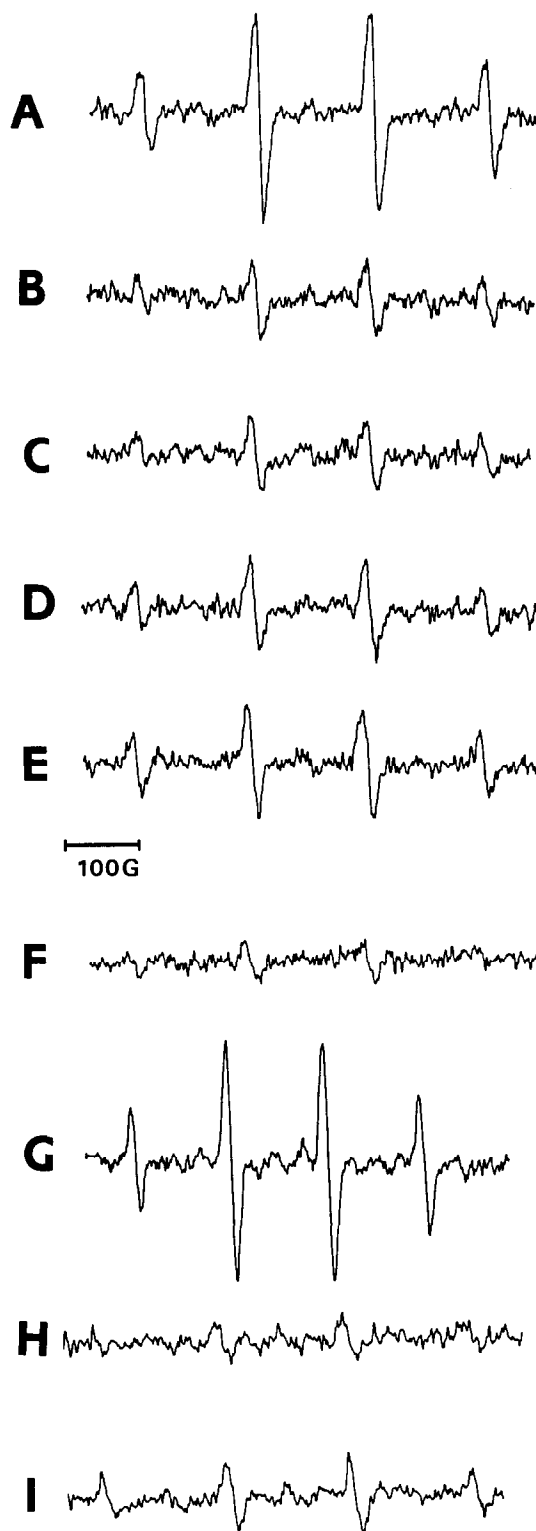Sinha et al. [3] were the first to show the differential

Fig. 2. ESR spectra obtained from C6 cell resistant variants (5×10^6 cells/ml) in the presence of DMPO (50 mM), NADPH (1 mM) and doxorubicin (200 mM). (A) C6 0.001 cells; (B) C6 0.1 cells; (C) C6 0.5 cells; (D) lyzed C6 0.5 cells; (E) C6 Rev cells; (F) C6 Rev cells preincubated with superoxide dismutase (100 μg/ml) for 30 min on ice; (G) C6 1V cells; (H) C6 1V cells preincubated with superoxide dismutase (100 μg/ml) for 30 min on ice; (I) C6 1V cells preincubated with catalase (100 μg/ml) for 30 min on ice.

Table I

Glutathione levels and free-radical scavenging enzyme activities in the cell lines studied

	Glutathione	Glutathione peroxidase nmol/mg protein/min		Catalase μmol/mg protein/min	Superoxide dismutase UI/mg protein/min
		Se-dependent	non Se-dependent		
C6	70.9 ± 8.1	23.5 ± 3.6	57.9 ± 7.8	9.30 ± 2.56	4.26 ± 0.61
C6 0.001	68.0 ± 9.0	30.7 ± 4.9	65.2 ± 6.5	8.41 ± 1.75	3.80 ± 0.45
C6 0.1	76.2 ± 9.6	91.2 ± 12.0	205 ± 43	8.00 ± 2.00	7.10 ± 1.00
C6 0.5	85.5 ± 14.2	153 ± 26	319 ± 61	7.87 ± 2.10	9.72 ± 1.83
C6 Rev	52.0 ± 2.9	50.3 ± 14.4	133 ± 24	nd	6.24 ± 0.77

formation of hydroxyl radicals by doxorubicin in sensitive and resistant tumor MCF-7 cells in culture. It was not clear at that time if this reduction was or was not part of the multidrug resistance phenotype. Other authors have then strongly doubted the role of hydroxyl radicals in the cytotoxic effect of doxorubicin [1]. It appears from our work that the early development of doxorubicin resistance is not accompanied by a reduction of free radical production; the C6 0.001 and the C6 1V lines appear as pure MDR lines, their resistance to doxorubicin being only due to the overexpression of P-glycoprotein. In contrast, the C6 0.5 line, which can tolerate high intracellular amounts of drug, presents several features not directly associated with classical MDR: it is able to strongly decrease free radical formation, as shown in this paper; it is also able to avoid the doxorubicin-induced lipid peroxidation as shown earlier [6]. The C6 0.1 and C6 Rev lines behave somewhat like the C6 0.5 line, but the inhibition of DMPO adduct formation occurs to a much lesser extent.

It can be hypothesized that in cell lines selected with doxorubicin to a high level of resistance, like MCF-7 doxR cells studied by Sinha et al. [3] or C6 0.5 cells studied here, mechanisms other than P-glycoprotein-mediated drug efflux could occur and participate to the phenotype of resistance; these mechanisms might protect the various intracellular targets of doxorubicin. At the level of the endoplasmic reticulum, where high levels of doxorubicin could cause lipid peroxidation via free radical formation, detoxification procedures would be necessary, explaining then the tolerance that these highly resistant cells develop as a mechanism additional to MDR. In contrast, in the line selected with vincristine, an anticancer drug recognized by P-glycoprotein but not able to form free radicals upon activation, as well as in the first steps of selection of resistant cells by doxorubicin, these systems of detoxification would not develop, since no decrease in doxorubicin-induced DMPO adduct formation occured.

Two systems at least can be involved in our cell lines for the protection against free radical production and effects: the increase of superoxide dismutase activity, which can detoxify $O_2^{\bullet -}$, and the increase of glutathione peroxidase, which can detoxify hydrogen and organic peroxides formed from oxygen free radicals. We have already shown that there is a significant inverse relationship between glutathione peroxidase activity and doxorubicin-induced lipid peroxidation in cell lines of different origin [6]. We show here that the decrease in doxorubicin-induced lipid peroxidation observed in the resistant cells can also be attributed to a diminished formation of hydroxyl radical, which might be explained at least in part by an overactivity of superoxide dismutase in the resistant cells. This may contribute to the non-MDR mechanism of resistance of the cells studied.

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