THE ROLE OF OXYGEN-DERIVED FREE RADICALS IN THE CYTOTOXICITY OF DOXORUBICIN IN MULTIDRUG RESISTANT AND SENSITIVE HUMAN OVARIAN CANCER CELLS

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SUMMARY

The role of oxygen-derived free radicals in the cytotoxicity of doxorubicin (Dox) was studied in a Dox sensitive human ovarian cancer cell line (A2780) and its multidrug resistant counterpart (2780AD) using reactive oxygen scavengers. In both cell lines, a significant inhibition of Dox toxicity was found after treatment with the hydroxyl radical scavengers, N-acetylcysteine, sodium benzoate and dimethyl sulfoxide, but not with mannitol. The protection was similar in sensitive and resistant cells: 13–39% less growth inhibition was found at Dox concentrations of 0.2 and 0.5 μM for A2780 as well as at 20 and 50 μM for 2780AD. This protection was not due to effects of the scavengers on Dox accumulation, as shown by uptake experiments with radio-labelled Dox. The superoxide anion free radical scavenger ascorbic acid or the enzyme superoxide dismutase as well as the hydrogen peroxide scavenger catalase did not protect cells against Dox-induced cell growth inhibition. Preloading the cells with the enzymes, a treatment which resulted in a two to nine-fold increase in their cellular contents, was not effective either. It is concluded that hydroxyl radicals, but not superoxide anion or hydrogen peroxide likely play a role in the antitumor activity of Dox in sensitive and resistant human ovarian cancer cells.

Key words: Doxorubicin; Multidrug resistance; Free radicals.

INTRODUCTION

Dox is an active agent in the treatment of human cancer. Its wide spectrum of activity includes acute leukemias, breast, ovarian, small cell lung cancer and

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soft tissue sarcomas [19]. Despite the fact that different mechanisms of action have been claimed, up to now it is not known which one is responsible for antitumor activity. Among the several possibilities, Dox-derived free radicals have been implicated. Dox can be converted into its semiquinone free radical by several NADPH or NADH-dependent enzymes and in the presence of oxygen, superoxide anions, hydrogen peroxide and hydroxyl radicals can be formed [1,4]. These oxygen-derived free radicals are most probably involved in Dox-induced cardiotoxicity [3], but their role in its antiproliferative activity has not been clearly established.

Dox has been shown to produce DNA-protein cross-links and DNA single strand breaks [12]. Such breaks might be responsible for antitumor activity [14]. It is thought that non-protein-associated breaks are caused by direct action of Dox-derived free radicals [12], while the protein-associated breaks may be the result of the formation of Dox-topoisomerase complexes [18]. Dox-induced non-protein-associated DNA breaks have been observed at low [7] or only at high Dox concentrations [12]. This type of DNA damage, which probably occurs only in euoxic cells, could be reduced by scavengers of oxygen-derived free radicals [13].

Thus, since the relative contribution of free radicals to DNA damage and probably also to cytotoxicity, seems to depend on the Dox concentration used, it is of interest to compare cell lines which can stand highly different concentrations of Dox. For this purpose Dox resistant and sensitive cell lines seem to be appropriate.

In this paper the Dox sensitive human ovarian cancer cell line A2780 and its multidrug resistant counterpart 2780AD, which is 120 times less sensitive to Dox, were used. In these cell lines the role of oxygen-derived free radicals in Dox-induced cytotoxicity was studied using scavengers of oxygen-derived free radicals.

MATERIALS AND METHODS

Chemicals

Doxorubicin (adriablastin) was from Laboratoire Roger Bellon (Neuilly-sur-Seine, France). [14-14C]Doxorubicin (53.3 Ci/mol) was purchased from Amersham Radiochemical Center (Amersham, U.K.). N-Acetylcysteine (NAC), sodium benzoate (SB), mannitol (MAN), superoxide dismutase (SOD) and catalase (CAT) were obtained from Sigma Chemical Corp. (St Louis, MO, USA). Ascorbic acid (AA) was from Gibco (New York, USA) and dimethyl sulfoxide (DMSO) was from J. Baker Chemicals B.V. (Deventer, The Netherlands). All stock solutions were prepared in 0.9% NaCl.

Cells and cell culture

The Dox sensitive human ovarian carcinoma cell line A2780 and its resistant counterpart 2780AD were kindly supplied by Dr. Ozols (N.C.I., Bethesda, MA, U.S.A.). Cells were maintained in plastic flasks (C.A. Greiner, Nurtingen, F.R.G.) in a humidified atmosphere at 5% CO₂ and cultured in Dulbecco's
modified minimal essential medium with glutamine (Gibco, Europe Ltd, Paisley, Scotland), containing 20 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES) and supplemented with 10% fetal bovine serum (Flow Laboratories, Irvine, U.K.). 2780AD cells were cultured in the continuous presence of 2 μM Dox. The cells were grown in drug-free medium for a period of 1—2 weeks before cytotoxicity or accumulation experiments. Doubling times were 17 and 24 h, respectively for A2780 and 2780AD. Cells were detached in Ca2+- and Mg2+-free Hanks balanced salt solution (pH 7.4) containing trypsin (0.0025%, w/v) and EDTA (0.25%, w/v) for 2 min at 37°C.

Cell growth inhibition assays

Cells in log-phase were harvested from culture flasks and plated in 6-well tissue culture clusters (Costar, Cambridge, MA, U.S.A.) at a density of 1—5 × 10⁴ viable cells per well, depending on the cell type used. SOD, AA and DMSO were added to cells from concentrated stock solutions. NAC, SB and MAN were prepared in culture medium at their final concentrations. After a 30-min pre-incubation with the free radical scavengers, Dox was added from a 100-fold concentrated stock solution and cells were incubated for another 2 h at 37°C. Then the medium was removed and fresh drug-free medium was added. All controls were treated as described above, but instead of Dox, the same volume of 0.9% NaCl was added. Cells were then cultured for 3 days and after that time, they were detached and counted with a Coulter Counter (Sigmex microcell counter CC-110). Cell numbers were corrected for the number of cells present at the time of the addition of the drug, thus indicating cell growth. Cell growth was expressed as percentages of control values.

Scrape loading experiments

The scrape-loading method [11] was used to introduce SOD or catalase into cells. The cells were scraped at 0°C in cell culture medium without serum with a plastic device with a hinge between the handle and the scraping surface. After scraping and washing with phosphate buffered saline (PBS), the cells were resuspended in culture medium and plated as described under cell growth inhibition assays. Cells were then cultured for 3—24 h. After that one part of the cells was trypsinized, washed and collected for enzyme measurements, while the other part was used for Dox cytotoxicity experiments as described under cell growth inhibition assays. Cell viability (routinely > 95%) was determined by the trypan blue exclusion test. SOD activity was assayed by the method of Joenje et al. [9], while catalase activity was determined according to Del Río et al. [2].

Dox accumulation experiments

Log-phase cells were harvested, washed and resuspended to a concentration of 2 × 10⁶ cells/ml in growth medium without NaHCO₃, but with 20 mM HEPES (pH 7.4). Two hundred fifty microliters of the cell suspension and 250 μl of a twofold concentrated NAC or SB solution in medium were mixed at 0°C in 2 ml Eppendorf vials. For DMSO, 25 μl of a 22-fold concentrated solution was mixed
with 500 µl of a cell suspension (10⁶ cells/ml). Thereafter, 25 µl of Dox solution was added, giving a final concentration of 2 µM. Control suspensions contained 0.9% NaCl instead of drug. Cells were incubated during 90 min at 37°C. Cell samples were quickly cooled with ice-cold PBS and centrifuged for 30 s at full speed in an Eppendorf 3200 centrifuge. After an additional washing step, cells were transferred in 450 µl PBS to the liquid scintillation fluid Optifluor (Packard, Groningen, The Netherlands) and counted. Intracellular Dox contents are expressed as pmol/10⁶ cells, after correction for immediate binding at 0°C.

RESULTS

In Fig. 1 it is shown that the hydroxyl radical scavenger DMSO was able to protect the sensitive human ovarian cancer cell line A2780 against Dox-induced cell growth inhibition. In order to investigate whether at higher Dox concentrations free radicals are involved in Dox cytotoxicity to a larger extent, the resistant cell line 2780AD was also treated with DMSO. The protective effect by DMSO was similar in both cell lines. Also, the protective effect seen for each cell line was not dependent on the external Dox concentration (Fig. 2). The effects of other non-structurally related hydroxyl radical scavengers NAC, SB and MAN are shown in Table 1 and 2. NAC and SB showed a clear inhibition of the cytotoxic effect of Dox in sensitive and in resistant cells to a similar extent but MAN had no effect. Since scavengers may influence membrane properties

![Graph showing the effect of DMSO on Dox-induced cell growth inhibition in A2780 and 2780AD. The concentrations of DMSO depicted are those that showed the maximal protection. ○, A2780 without DMSO; ●, A2780 plus DMSO; ○, 2780AD without DMSO; ●, 2780AD plus DMSO. Data represent cell growth and are means ± S.E. from 4 or 5 experiments. *, significantly different from control cells (P < 0.05. Student’s t-test).]
TABLE 1
EFFECT OF HYDROXYL RADICAL SCAVENGERS ON Dox-INDUCED CELL GROWTH-INHIBITION IN A2780*

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Dox (μM)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
<td>0.2</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>None</td>
<td>65 ± 4</td>
<td>31 ± 0</td>
<td>10 ± 1</td>
<td>2 ± 2</td>
</tr>
<tr>
<td>NAC (100 mM)</td>
<td>79 ± 6</td>
<td>51 ± 9</td>
<td>23 ± 2*</td>
<td>7 ± 2*</td>
</tr>
<tr>
<td>SB (100 mM)</td>
<td>89 ± 7</td>
<td>70 ± 5*</td>
<td>32 ± 4*</td>
<td>13 ± 2*</td>
</tr>
<tr>
<td>MAN (200 mM)</td>
<td>61 ± 7</td>
<td>28 ± 9</td>
<td>9 ± 6</td>
<td>2 ± 2</td>
</tr>
</tbody>
</table>

*Data indicate cell growth (% of controls) and are means ± S.E. of 3 or 4 experiments.
*Significantly different from controls. *P < 0.01 (Student’s *t*-test).
*Significantly different from controls. *P < 0.05 (Student’s *t*-test).

[10], it was checked whether the protective effects found could be explained by scavenger-induced reduction of Dox accumulation. In Fig. 2, it is shown that the intracellular accumulation of Dox at 2 μM of extracellular Dox in both cell lines was not significantly inhibited by those reactive oxygen scavengers. Qualitatively similar results were found when Dox concentrations close to the IC₅₀ (concentration of Dox that inhibits cell growth for 50%) were used (0.3 μM for A2780 and 50 μM for 2780²⁴) (data not shown).

Neither the superoxide anion scavengers SOD and AA, nor the hydrogen peroxide scavenger CAT showed any protection against Dox cytotoxicity (Tables 3, 4 and 5). Even when A2780 cells were postincubated for 3 days with SOD, no effect was found (data not shown). In order to increase cellular SOD contents, the enzyme was introduced into the cells using the scrape-loading

TABLE 2
EFFECT OF HYDROXYL RADICAL SCAVENGERS ON Dox-INDUCED CELL GROWTH-INHIBITION IN 2780²⁴*

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Dox (μM)</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>20</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>None</td>
<td>91 ± 5</td>
<td>70 ± 11</td>
<td>46 ± 9</td>
<td>28 ± 4</td>
</tr>
<tr>
<td>NAC (100 mM)</td>
<td>89 ± 6</td>
<td>86 ± 10</td>
<td>80 ± 12*</td>
<td>77 ± 15*</td>
</tr>
<tr>
<td>SB (100 mM)</td>
<td>93 ± 4</td>
<td>85 ± 9</td>
<td>74 ± 3*</td>
<td>55 ± 9*</td>
</tr>
<tr>
<td>MAN (200 mM)</td>
<td>89 ± 3</td>
<td>67 ± 9</td>
<td>47 ± 3</td>
<td>26 ± 2</td>
</tr>
</tbody>
</table>

*Data indicate cell growth (% of controls) and are means ± S.E. of 3 experiments.
*Significantly different from controls. *P < 0.05 (Student’s *t*-test).
Fig. 2. Effect of hydroxyl radical scavengers on Dox accumulation in 2870 and in 2870AD. Data indicate relative Dox accumulation (% of controls) and are mean ± S.E. of 4 to 6 experiments. * significantly higher than controls (P < 0.05, Student's t-test).

method [11]. Scraped-loaded cells showed a 4.3 (S.D. 0.5, N = 4)-fold increase in SOD activity compared to controls, 12 h after scraping and a 6—9-fold increase if SOD activity was determined 4 h after scraping. However, loading with SOD did not result in protection against Dox-induced cytotoxicity: IC50 values (µM) were 0.11 ± 0.02 and 25 ± 2 for SOD-loaded A2870 and 2870AD cells, respectively, while the values for control cells were 0.11 ± 0.02 and 24 ± 3 (mean ± S.E. of 2 or 3 experiments). Also scrape-loading of A2870 cells in the presence of catalase resulted in a 3—5-fold increase in cellular contents as measured 2—4 h after scraping, without affecting the sensitivity of the cells to Dox (IC50 was 0.14 ± 0.01 without catalase and 0.14 ± 0.02 plus catalase). In addition no effects of preloading with catalase were found for 2870AD cells, despite a 2-fold increase in enzyme content.

### TABLE 3

**EFFECT OF SUPEROXIDE ANION RADICAL SCAVENGERS ON Dox-INDUCED CELL GROWTH-INHIBITION IN A2870**

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Dox (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.1</td>
</tr>
<tr>
<td>None</td>
<td>81 ± 7</td>
</tr>
<tr>
<td>SOD (150 µg/ml)</td>
<td>81 ± 7*</td>
</tr>
<tr>
<td>AA (0.2 µM)</td>
<td>82 ± 9</td>
</tr>
</tbody>
</table>

*Data indicate cell growth (% of controls) and are means ± S.E. of 3 or 4 experiments.
*None of the treatments is significantly different from controls. P > 0.05 (Student's t-test).
TABLE 4
EFFECT OF SUPEROXIDE ANION RADICAL SCAVENGERS ON Dox-INDUCED CELL GROWTH-INHIBITION IN 2780AD

<table>
<thead>
<tr>
<th>Scavenger</th>
<th>Dox (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
</tr>
<tr>
<td>None</td>
<td>91 ± 5</td>
</tr>
<tr>
<td>SOD (150 µg/ml)</td>
<td>92 ± 5b</td>
</tr>
<tr>
<td>AA (0.2 µM)</td>
<td>90 ± 4</td>
</tr>
</tbody>
</table>

*Data indicate cell growth (% of controls) and are means ± S.E. of 2 or 3 experiments.

None of the treatments is significantly different from controls. P > 0.05 (Student’s t-test).

DISCUSSION

From the results presented in this study we conclude that hydroxyl radicals play a role in the Dox-induced cell growth inhibition of sensitive as well as resistant human ovarian cancer cells. DMSO, NAC and SB, three non-structurally related hydroxyl radical scavengers all protected against Dox-induced cell growth inhibition, without reducing the intracellular accumulation of Dox. It is of considerable interest that hydroxyl radicals play a role already at low, clinically achievable, concentrations (0.1 – 0.5 µM). These results are in agreement with those reported recently in other tumor cells [5,6]. Overall, those studies indicate a role for free radicals in Dox-cytotoxicity at much lower concentrations than suggested by Potmesil et al., who measured free radical-induced DNA damage at Dox concentrations > 2.8 µM, which is above the concentrations affecting cell growth [12].

TABLE 5
EFFECT OF CAT (3000 UNITS/ML) ON Dox-INDUCED CELL GROWTH-INHIBITION IN A2780 AND 2780AD

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Dox (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.05b/5c</td>
</tr>
<tr>
<td></td>
<td>CAT</td>
</tr>
<tr>
<td>A2780</td>
<td>89</td>
</tr>
<tr>
<td>2780AD</td>
<td>82</td>
</tr>
</tbody>
</table>

*Data indicate cell growth (% of controls) and are means of 2 experiments. S.D.s are < 10%. Treated cells were not significantly different from controls. P > 0.05 (Student’s t-test).

bDox concentrations used in A2780.

cDox concentrations used in 2780AD.
In our experimental model only membrane penetrating agents were able to protect against the Dox-induced cytotoxicity. MAN, a hydroxyl radical scavenger that does not penetrate into cells, lacks this protective effect. Also non-penetrating proteins like SOD and CAT were not able to protect A2780 and 2780AD against Dox-induced cytotoxicity. These results suggest that there is no deleterious effect of free radicals produced near the cell surface. The opposite conclusion was drawn by other authors for Ehrlich tumor and human breast cancer cells [5,6,16]. Perhaps these discrepancies are due to differences between cell lines or to differences in experimental protocols. The lack of increasing cellular SOD or catalase contents even suggests strongly that superoxide anions or hydrogen peroxide are not involved at all in Dox cytotoxicity. At present we do not know why we were able to show an involvement of hydroxyl radicals but not of superoxide anions or hydrogen peroxide, which are well known precursors of hydroxyl radicals in the Haber Weiss and Fenton reactions [8]. One possibility might be that the internalized enzymes are located too far from the sites where the potentially damaging species are formed.

It is remarkable that the protection offered by hydroxyl radical scavengers is similar in A2780 and 2780AD, despite the much higher external concentration of Dox used for resistant cells. Also the intracellular Dox content in 2780AD exceeds by far that in A2780 at equitoxic concentrations [17]. One possible explanation could be that Dox-induced free radical formation in certain cellular compartments, at Dox concentrations which are affecting cell growth, is similar for both cell lines. In this respect it is of particular interest that Dox is located mainly in the cytoplasm of 2780AD cells and in the nucleus of A2780 cells [17]. Also it could be shown that resensitization of resistant cells with calcium channel blocking agents was accompanied by a redistribution of Dox from the cytoplasm to the nucleus [17]. It thus seems that the bulk of Dox molecules in 2780AD had no effect on cell growth. The results of that study suggested that nuclear concentrations of Dox are the same in 2780AD and A2780 when these cells were incubated with equitoxic Dox concentrations. It is tempting to speculate that this might be the reason why free radical scavengers had the same protective effect in A2780 and 2780AD at equitoxic concentrations, despite the fact that intracellular drug contents were largely different.

In conclusion, our results suggest that free radicals and in particular hydroxyl radicals, likely play a role in Dox antitumor activity at clinically achievable concentrations. In the search for new Dox analogs it has to be considered that the limitation of free radical production might result not only in a decreased cardiotoxicity but also in less therapeutic potential.

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REFERENCES


