Effects of Anthracyclines on Oxygenated and Hypoxic Tumor Cells

Katherine A. Kennedy, Jill M. Siegfried, Alan C. Sartorelli, and Thomas R. Tritton

Department of Pharmacology and Developmental Therapeutics Program, Comprehensive Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06510

ABSTRACT

The cytotoxic effects of anthracyclines and other chemotherapeutic agents were examined in normally aerated and hypoxic Sarcoma 180 and EMT6 tumor cells in vitro. Adriamycin, daunomycin, and mitomycin C were selectively toxic to hypoxic Sarcoma 180 cells. The augmented sensitivity was not the result of an increase in susceptibility of oxygen-deprived cells toward antitumor agents in general. 1,3-Bis(2-chloroethyl)-1-nitrosourea, for example, exhibited equal cytotoxicity toward normally aerated and hypoxic cells, while streptonigrin was selectively toxic toward normally aerated cells. The cellular levels of [3H]daunomycin in both Sarcoma 180 and EMT6 cells were not different under the two conditions of oxygenation, and no greater production of either the alcohol or aglycone metabolites of daunomycin occurred in hypoxic cells, compared with their normally aerated counterparts. In addition, analysis of cellular pellets for residual drug remaining after exhaustive extraction showed no significant difference between normally aerated and hypoxic cells. The effects of reoxygenation of hypoxic cells on their sensitivity to mitomycin C and to Adriamycin were studied in both Sarcoma 180 and EMT6 cells. The enhanced efficacy of mitomycin C as a cytotoxic agent observed under hypoxia was reversed after a 2-hr reoxygenation. In contrast, the augmented toxicity of Adriamycin toward hypoxic cells was not reversible in either cell line after 2 or 4 hr of reoxygenation. The results suggest that neither the formation of a reactive oxygen species nor direct involvement of an alkylating agent generated by drug metabolism is an obligatory step in the cytotoxic action of these anthracyclines.

INTRODUCTION

The anthracycline antibiotics ADR and DNR are prototypes of a class of potent antineoplastic agents which are widely used clinically (5, 9, 45). Studies in laboratory animals and in cell culture have led to many hypotheses for the mechanism of action of these agents, including inhibition of DNA and RNA polymerases (7, 26, 44, 47), induction of DNA breaks (9, 33, 34), generation of free radicals (4, 12, 15, 31), and production of alkylating metabolites from the anthracycline molecule (13, 27, 38, 39). Moore (27) has proposed that ADR and DNR undergo metabolic bioreductive activation leading to the formation of quinone-methide metabolites capable of alkylating cellular macromolecules in a manner analogous to that suggested for quinones such as MC (22). In support of this hypothesis, anthracycline ring structures bound to DNA were found in vitro both after chemical reduction of the drugs and after incubation with liver microsomes under anaerobic atmospheres in the presence of DNA (38, 39). Additional evidence for the generation of reactive species was provided by Ghezzi et al. (13), who found radiolabeled residues bound to protein after incubation of microsomes with [14C]ADR.

Previous reports from this laboratory have shown that Sarcoma 180 and EMT6 tumor cells are able to activate the bioreductive alkylating agent MC (18) and that hypoxic EMT6 cells in vitro are more susceptible to both MC (18, 42) and ADR (42) than are normally aerated cells. We have extended our investigations to include a measurement of the comparative sensitivities of oxygenated and hypoxic Sarcoma 180 cells to MC, ADR, DNR, and other chemotherapeutic agents and the sensitivity of hypoxic EMT6 and Sarcoma 180 cells to ADR and MC following reoxygenation. We have also examined the metabolism and irreversible binding of [3H]DNR under different states of oxygenation in sensitive and ADR-resistant Sarcoma 180 cells. The results obtained suggest that mechanisms other than bioreductive activation are responsible for the enhanced cytotoxicity of anthracyclines toward hypoxic EMT6 and Sarcoma 180 cells.

MATERIALS AND METHODS

Drugs. ADR, DNR, streptonigrin, and BCNU were the gifts of Dr. John D. Douros, Division of Cancer Treatment, National Cancer Institute. DNOL was donated by Dr. M. Israel, Sidney Farber Cancer Institute (Boston, Mass.). MC was generously supplied by Dr. Maxwell Gordon, Bristol-Myers Co. (Syracuse, N. Y.). The radiochemical purity of generally labeled [3H]DNR (1.3 Ci/mmol; New England Nuclear, Boston, Mass.) was determined to be 92% by HPLC analysis, as described below. The aglycone DNONE accounted for 5% of the total amount of radiolabeled material, and 3% was due to [3H]exchange. No DNOL was found in the radiolabeled sample. Drugs were dissolved in 70% ethanol prior to addition to cell culture systems.

Tumor Cells and Cytotoxicity Studies. Experiments were performed using Sarcoma 180 or its ADR-resistant variants, S180A3 and S180A5 or EMT6 cells in vitro. The techniques used for propagation of cells and measurement of their survival by colony formation have been described in detail (18, 30, 32, 33). Colony-forming efficiencies for Sarcoma 180 cells were 40 to 80% for normally aerated cells and 20 to 60% for hypoxic cells (see below). The cloning efficiency for EMT6 cells was between 60 and 70% for both normally aerated and hypoxic cells.
hypoxic cells. Results for all cytotoxicity experiments were expressed as a percentage of the appropriate vehicle-treated cells included in each experiment.

**Hypoxic and Reoxygenation Conditions.** Cells were made hypoxic or normally aerated by sealing 25-cm culture flasks (EMT6 cells), or 100-ml sterile bottles (Sarcoma 180 cells) with thick rubber sleeves. The flasks were fitted with 20-gauge needles for inflow and outflow of gases and exposed to a continuously flowing humidified mixture of 95% N₂:5% CO₂ or 95% air:5% CO₂ (Matheson Gas Products, East Rutherford, N. J.). With this technique, drug could be added directly to cultures without interfering with the gas flow by injection of the agent through the sleeve. Normally aerated cultures were incubated in a humidified atmosphere of 95% air:5% CO₂ for 4 hr, while hypoxic cells were preincubated for the same period of time in a humidified atmosphere of 95% N₂:5% CO₂ prior to addition of drug. Exposure to chemotherapeutic agents was for 1 or 2 hr. After the treatment period, cells were prepared for measurement of colony-forming ability as mentioned above. For experiments designed to examine the effects of reoxygenation on the susceptibility of cells to either ADR or MC, cells were pretreated for 4 hr with 95% N₂:5% CO₂. After the preincubation period under hypoxic conditions, the cells were exposed to continuously flowing 95% air:5% CO₂ for 0 to 4 hr. Two hr prior to the completion of each reoxygenation treatment, drug or vehicle was administered. Concurrent controls with vehicle-treated and drug-treated normally aerated cells were also used. Colony-forming ability was then measured after treatment.

**DNR Metabolism by Normally Aerated and Hypoxic Cells.** Metabolism of [³H]DNR was studied in ADR-sensitive and -resistant Sarcoma 180 cells under conditions of normal aeration and hypoxia. Cells were treated as described, except that the 10-fold resistant cell line, S180A3, was exposed to twice the concentration of [³H]DNR than were the parent Sarcoma 180 cells, in order to achieve equal intracellular levels of drug. Radioactivity in fractions of medium, washes, and whole cells was determined. Cells (2 x 10⁸) were collected by centrifugation, and the resulting pellets were washed and then lysed in distilled H₂O. Prior to extraction with ethyl acetate and n-propyl alcohol (9:1, v/v) in the presence of 2% Na₂CO₃ (pH 9.0), unlabeled DNR, DNOL, and DNONE were added as carriers. Four extraction volumes (5 ml each) were combined and dried under N₂, and the residue was reconstituted prior to HPLC analysis. Extraction efficiency was 98% for DNR and DNOL and 95% for DNONE, and no detectable conversion of DNR to DNONE or DNOL occurred during the extraction procedure. The HPLC column effluent was collected in 0.5-ml fractions, and the radioactivity in each fraction was measured. The amount of [³H] which chromatographed with the unlabeled carrier was used to quantitate the amount of metabolite present in the sample.

The amount of drug irreversibly bound to macromolecules of sensitive and resistant cells after exposure to [³H]DNR for 2 hr under conditions of normal aeration or hypoxia was assessed after treatment of cells as described above. At the end of the drug exposure time, 2 x 10⁸ cells were collected by centrifugation, washed with ice-cold phosphate-buffered saline, and lysed in distilled H₂O. The cell pellets were extracted with ethyl acetate:n-propyl alcohol (9:1, v/v) in the presence of Tris-HCl buffer (pH 8.3) containing 3% (w/v) SDS. Addition of SDS to the pellet releases drug intercalated into DNA. Extraction was continued until background levels of radioactivity were found in the organic phase. Approximately 20 extraction volumes were required. The pellets were solubilized with 1 N NaOH, neutralized with 1 N HCl, and assayed for radioactivity. Drug levels in both normally aerated and hypoxic tumor cells were estimated as described previously. Extraction of cell pellets by an alternate method using AgNO₃ (35) yielded similar results, but for simplicity only the data obtained with SDS are shown.

**HPLC Analyses of DNR and Its Metabolites.** Measurement of DNR and its metabolites, DNOL, and DNONE, was performed by HPLC using a µBondapak phenyl reverse-phase column (Waters Associates), as described by Israel et al. (17). The column effluent was monitored at 254 nm, using a UV detector and a Perkin-Elmer fluorescence detector with excitation and emission wavelengths of 485 and 595 nm, respectively.

**Determination of Radioactivity.** Radioactivity was measured by liquid scintillation counting in a Beckman Model 7500 scintillation spectrometer (Beckman Instruments). The scintillation cocktail was Aquasol (New England Nuclear), and counting efficiencies were determined by using an internal standard.

**Statistics.** The data obtained for normally aerated and hypoxic cells were analyzed using a paired t test (41).

**RESULTS**

Charts 1 and 2 show the results of cytotoxicity studies in normally aerated and hypoxic cells obtained by treatment with ADR, DNR, MC, streptonigrin, and BCNU. Of these compounds, ADR, DNR, and MC were more cytotoxic to hypoxic
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Chart 2. Survival of normally aerated and hypoxic Sarcoma 180 cells after exposure to MC, streptonigrin, and BCNU. Exponentially growing cells were exposed to varying concentrations of drug for 2 hr under conditions of normal aeration or hypoxia, and cytotoxicity was estimated by the ability of cells to form colonies in soft agar, as described in "Materials and Methods." Data are expressed as a percentage of control survival; bars, S.E. Asterisks, significant differences in survival at a given dose between normally aerated and hypoxic cells at \( p \leq 0.075 \) (*) and \( p \leq 0.05 \) (**).

The effects of reoxygenation of hypoxic cells on sensitivity to MC and ADR were measured to determine if the enhanced toxicity of ADR and MC toward hypoxic cells was reversible upon cellular reoxygenation. Chart 3 shows that both MC and ADR were selectively toxic to hypoxic Sarcoma 180 and EMT6 cells (0-hr reoxygenation). Upon reoxygenation of both cell types, MC and ADR demonstrated major differences in their effects. Reoxygenated cells showed no enhanced sensitivity to MC when compared with their normally aerated counterparts. In contrast, reoxygenated cells were as sensitive to ADR as were hypoxic cells. Hypoxic Sarcoma 180 cells required at least 18 hr of reoxygenation before the sensitivity of these cells toward ADR was similar to that of normally aerated cells (data not shown).

Drug levels in normally aerated and hypoxic Sarcoma 180 and EMT6 cells were measured under both conditions using

Differential cytotoxicity by Adriamycin toward normally aerated, hypoxic, and reoxygenated Sarcoma 180 or EMT6 cells exposed to ADR or MC. Sarcoma 180 cells (S180) were exposed to 0.1 \( \mu \text{M} \) ADR (○, oxygenated; •, hypoxic) or 0.5 \( \mu \text{M} \) MC (○, oxygenated; ■, hypoxic) for 2 hr with 0-, 2-, or 4-hr reoxygenation, and EMT6 cells were exposed to 0.01 \( \mu \text{M} \) ADR (○, oxygenated; •, hypoxic) or 0.1 \( \mu \text{M} \) MC (○, oxygenated; ■, hypoxic) for 1 hr with 0-, 2-, or 4-hr reoxygenation. Survival of cells was assessed by colony formation. Differential cytotoxicity is the difference in the survivals of reoxygenated cells compared to normally aerated cells exposed to the same drug concentration as defined by:

\[
\text{Differential cytotoxicity} = \frac{100}{\text{cloning efficiency hypoxic cells (drug treated)}} - \frac{100}{\text{cloning efficiency hypoxic cells (control)}} - \frac{100}{\text{cloning efficiency normally aerated cells (drug treated)}} - \frac{100}{\text{cloning efficiency normally aerated cells (control)}}
\]
enhancement of DNR cytotoxicity toward hypoxic cells (Chart 0.1 IM [3H]DNR. At this concentration, there was significant these cells than in their well-oxygenated counterparts. The cytotoxicity of DNR to hypoxic cells, less drug was present in aerated and hypoxic cells. In this case, despite the greater resistant Sarcoma 180 cells was studied under conditions of (approximately 40-fold resistant) cells were incubated with 0.1 IM of DNR to achieve comparable drug levels (see Ref. 5). Cells were collected by centrifugation, extracted with ethyl acetate/isopropyl alcohol, and the organic extracts were analyzed by HPLC, as described in "Materials and Methods." Of the DNR present, 1% represented 8500 dpm, and column recovery was greater than 90% of the amount injected. Mean 3H exchange was 4.2 ± 0.7% of DNR present.

Table 1

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Oxygenated cells</th>
<th>Hypoxic cells</th>
<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180</td>
<td>25.4 ± 1.0*</td>
<td>18.4 ± 1.3</td>
<td></td>
</tr>
<tr>
<td>EMT6</td>
<td>11.4 ± 3.0</td>
<td>11.6 ± 2.5</td>
<td></td>
</tr>
</tbody>
</table>

Metabolism of [3H]DNR in normally aerated and hypoxic Sarcoma 180 and S180A3 cells

Cells were incubated with 0.25 μM (Sarcoma 180) and 0.5 μM (S180A3) [3H] DNR to achieve comparable drug levels (see Ref. 5). Cells were collected and the pellets were exhaustively extracted until no additional radiolabel could be removed. As can be seen in Table 3, a small but measurable amount of bound material was observed in all cell lines under both normal aeration and hypoxia. However, the same or less irreversibly bound material was found in cells incubated under hypoxic conditions than in their normally aerated counterparts. Similarly, comparisons between the cell lines with regard to drug sensitivity revealed that the most highly resistant cell line (S180A5) had the largest percentage of covalently bound material and that under hypoxia this level of binding decreased. It should be noted that we have not determined the molecular form of the nonextractable material. This does not affect our conclusions, however, that very little of the originally added material cannot be extracted and that the small amount remaining is not greater in hypoxic cells.

DISCUSSION

Anthraclycline-induced cytotoxicity has been postulated by several authors to involve either oxygen-based radicals or an anthracycline-based free radical, with consequent damage to DNA or other cellular macromolecules (4, 6, 10, 12, 13, 15, 31, 38, 39). The results presented in this report demonstrate that the first of these mechanisms does not play a critical role in the susceptibility of hypoxic Sarcoma 180 or EMT6 cells to the anthracyclines, because O2 is not required for anthracycline-induced cytotoxicity. Hypoxic cells of both the Sarcoma 180 or EMT6 tumor cell lines were more sensitive to both ADR and DNR than were their normally aerated counterparts. The enhanced cytotoxicity of the anthracyclines to the hypoxic cells used in these experiments does not appear to be the result of increased sensitivity of the hypoxic cells toward all cytotoxic agents. BCU, for example, was equitoxic toward normally aerated and hypoxic Sarcoma 180 (Chart 2) and EMT6 tumor cells (43). Such a result would be predicted for a direct-acting alkylating agent such as BCU. Furthermore, streptonigrin, another quinone antibiotic, was selectively toxic to normally aerated Sarcoma 180 cells (Chart 2) and EMT6 cells (43). Streptonigrin toxicity is thought to involve degradation of DNA through an oxygen-based toxic species (11). This hypothesis would predict that an oxygen-containing atmosphere is essential for the cytotoxic properties of this quinone-containing compound.

The prototype bioreductive alkylating agent, MC, as well as the anthracyclines, have been proposed to undergo similar metabolic activation to form quinone methides or a semiquinone-free radical (4, 13, 27, 31, 38, 39). If this hypothesis is correct, because the hypoxic state promotes the stability of the

Table 3

Residual radioactivity in anthracycline-sensitive and -resistant Sarcoma 180 cells exposed to 0.1 μM [3H]DNR

<table>
<thead>
<tr>
<th>Cell line</th>
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<th>Mean ± S.E.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sarcoma 180</td>
<td>0.36*</td>
<td>0.38</td>
<td></td>
</tr>
<tr>
<td>S180A3</td>
<td>0.23</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>S180A5</td>
<td>1.45</td>
<td>0.28</td>
<td></td>
</tr>
</tbody>
</table>

Mean of duplicate determinations.
reduced species, it should favor the formation of these reactive species with resultant alkylation of cellular macromolecules (18, 20). Indeed, we have shown previously that MC is reductively activated to a metabolite(s) capable of alkylation in cell-free preparations from Sarcoma 180 and EMT6 cells (18) and in liver microsomes and nuclei (19). The system responsible for this activation requires a NADPH-generating system and is inhibited by the presence of O2 in the incubation atmosphere. The results presented here and elsewhere (43) demonstrate clearly that MC is differentially toxic to hypoxic cells in vitro, presumably by virtue of selective metabolic activation.

The results obtained upon reoxygenation, however, suggest that MC and ADR are acting by different mechanisms to exert their enhanced activity toward hypoxic cells. Consistent with the concept that MC is a bioreductive alkylating agent (22), this antibiotic showed no enhanced cytotoxicity to hypoxic cells that were reoxygenated. This finding suggests that sufficient O2 is present in the normal aeration atmosphere of reoxygenated cells to inhibit the generation of the reactive species. In contrast, ADR retained its selective cytotoxicity toward hypoxic Sarcoma 180 and EMT6 tumor cells that were reoxygenated (Chart 3). The sensitivity of reoxygenated Sarcoma 180 cells to ADR did not return to that of normally aerated cells until at least 18 hr after hypoxic stress was reversed (data to be presented elsewhere). These findings led to the conclusion that the enhanced lethal effects of ADR toward hypoxic or reoxygenated cells may be a secondary result of a cellular response resulting from the imposition of hypoxia, and not because of bioreductive activation.

The effect of oxygen deficiency on the cytotoxicity of ADR has been variable, with reports of oxygenated cells having greater (24, 40, 42), equal (16), and lesser (14, 43) sensitivity. The reasons for the differences between these results and those presented in this report are not clear, but it is possible that cell lines differ in their response to hypoxia. It is notable, however, that Smith et al. (40) could not reverse the protection from ADR afforded by hypoxia for up to 9 hr after reoxygenation. Twenty-four hr of reoxygenation were required before drug sensitivity was restored (40).

Additional evidence which supports the hypothesis that mechanisms other than bioreductive activation may be of importance to anthracycline-induced cytotoxicity were obtained by measurement of the metabolism of [3H]DNOR in Sarcoma 180 and S180A3 cells under the 2 conditions of aeration. No differences in the production of DNONE or DNOL were determined between hypoxic and aerated cells (18, 20). Indeed, we have shown previously that MC is reductively activated to a metabolite(s) capable of alkylation in cell-free preparations from Sarcoma 180 (18) and in liver microsomes and nuclei (19). The system responsible for this activation requires a NADPH-generating system and is inhibited by the presence of O2 in the incubation atmosphere. The results presented here and elsewhere (43) demonstrate clearly that MC is differentially toxic to hypoxic cells in vitro, presumably by virtue of selective metabolic activation.

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Additional evidence which supports the hypothesis that mechanisms other than bioreductive activation may be of importance to anthracycline-induced cytotoxicity were obtained by measurement of the metabolism of [3H]DNOR in Sarcoma 180 and S180A3 cells under the 2 conditions of aeration. No differences in the production of DNONE or DNOL were detected between either sensitive or resistant cells exposed to an oxygenated atmosphere or to a hypoxic atmosphere. Indeed, only minor production of these 2 metabolites occurred. Both of these metabolites are produced by reductive processes in cells and tissues such as liver and kidney (1, 2, 46). DNONE is formed by a ketoreductase, and DNONE is the product of reductive or hydrolytic cleavage of the sugar, daunosamine. The formation of DNONE from DNR is known to be sensitive to O2 (23), and aglycone metabolites can be produced under anaerobic conditions in liver microsomes (3) and by xanthine oxidase (31). A semiquinone free radical intermediate has been postulated for these reactions, which in the presence of molecular oxygen would be dissipated. Cleavage would result only if no electron acceptor were present. A NADPH:cytochrome P-450 reductase has also been reported (a) to produce an ADR-dependent free radical paramagnetic resonance signal under anaerobic conditions and (b) to stimulate oxygen consumption (4). However, Handa and Sato (15) found that stimulation of oxygen consumption by ADR did not occur in intact Ehrlich ascites cells, as it did in the presence of MC. It is possible that transfer of an electron to the quinone ring is not favored in whole cells. Because of the short lifetime of the semiquinone free radical in the presence of oxygen, these reactive species probably produce only local damage. The free radical may be more stable under low oxygen tension, and thus toxic reactions are more likely to be enhanced under hypoxic conditions. It is unlikely, however, that drug intercalated into DNA could be activated, since the polargraphic half-wave potential of ADR disappears upon binding to DNA (8), and the generation of the paramagnetic free radical signal has been shown to be totally inhibited by the addition of DNA. (36). Furthermore, the anthracycline free radical is not apparently involved in producing damage to DNA, inasmuch as DNA strand scission requires the presence of O2 (6).

The data reported in this paper also suggest that hypoxic cells do not exhibit greater irreversible binding of [3H]DNOR to macromolecular material when compared to their normally aerated counterparts. Thus, we conclude that other factors are important for anthracycline-induced cytotoxicity, although it is conceivable that different macromolecules are irreversibly alkylated in hypoxic and oxygenated cells. Cellular membranes may be a relevant target for anthracyclines, for example, because ADR has been shown to enhance the agglutination rate of Sarcoma 180 cells by concanavalin A (28), and to cause morphological changes in human erythrocyte ghosts (25). In addition, the fluidity of cell membranes, as measured by the behavior of the fatty acid spin-label 5-doxylstearic acid, increases in a dose-dependent manner after exposure to ADR (29). Enhanced membrane glycoprotein synthesis has also been reported by Kessel (21) to occur after treatment of neoplastic cells with anthracyclines. Further experiments to compare the role of anthracycline-mediated lesions to both membranes and DNA in normally aerated and hypoxic tumor cells are currently under way in our laboratories.

REFERENCES


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