Oxygen and Exposure Kinetics as Factors Influencing the Cytotoxicity of Porfiromycin, a Mitomycin C Analogue, in Chinese Hamster Ovary Cells

Raymond S. Marshall and A. Michael Rauth

Department of Medical Biophysics, University of Toronto, and Ontario Cancer Institute, Toronto, Ontario, Canada M4X 1K9

ABSTRACT

Some factors affecting the cytotoxicity of porfiromycin (PM), an analogue of mitomycin C (MMC), were investigated in suspension cultures of wild-type (AA8-4) and repair-deficient (UV-20) Chinese hamster ovary cells. Oxygen was an important modulator of PM toxicity in AA8-4 cells. The aerobic toxicity was significantly less, and toxicity under extremely hypoxic conditions was significantly greater for PM than MMC. Porfiromycin cytotoxicity at intermediate O2 levels was similar to that observed previously for MMC. While the aerobic/hypoxic ratio was greater for PM than MMC, survival at intermediate oxygen concentrations could limit the therapeutic utility of these drugs as adjuncts to radiotherapy. Ascorbic acid was found to increase the aerobic, but not hypoxic, cytotoxicity of PM in AA8-4 cells, as was observed previously for MMC. Investigation of various exposure times and drug concentrations revealed that drug toxicity for both aerobic and hypoxic cells was dependent on the product of drug concentration and time, and that the aerobic/hypoxic differential observed in AA8-4 cells was constant over a broad range of exposure conditions. The sensitivity of UV-20 cells was also a linear function of concentration and time, but no aerobic/hypoxic differential was observed in these cells. It is suggested that the sensitivity of UV-20 to PM and MMC, and its lack of an hypoxic/aerobic differential could result from lethality being due to a different lesion than in wild-type cells.

INTRODUCTION

Hypoxic populations of cells in solid neoplasms may limit tumor curability as a result of their intrinsic radioresistance, resistance to some drugs, and relative inaccessibility to some chemotherapeutic agents (1). Combining agents which are preferentially toxic to such hypoxic populations with localized radiation therapy, which effectively reduces the aerobic tumor population, could, therefore, be one rational approach to increase local tumor control. The systemic introduction of such a chemotherapeutic drug requires that the agent have minimal toxicity toward normal, presumably well-oxygenated tissues. MMC\(^2\) has been identified as a clinically utilized chemotherapeutic agent which demonstrates preferential toxicity toward cells under hypoxic as compared to aerobic conditions \textit{in vitro} (2). Compounds which require metabolic reductive activation prior to production of cytotoxic species which alkylate cellular macromolecules have been termed bioreductive alkylating agents (3).

While preferential hypoxic cytotoxicity is a required feature of a compound that can usefully be combined with radiation, the cytotoxicity at intermediate oxygen levels is also crucial. Ideally, the agent should have no toxicity at oxygen concentrations above that at which radiation sensitivity is maximal (1.0% O\(_2\)). Below this concentration the agent should have maximal toxicity. The oxygen dependency of MMC cytotoxicity has been determined previously and found to approximate this ideal situation (4). However, while it demonstrated preferential hypoxic cytotoxicity, significant aerobic toxicity was also observed. As well, its toxicity only increased below 0.1% oxygen, and this increase was relatively gradual. Therefore, it would be useful if MMC analogues could be identified which demonstrate less aerobic and/or more hypoxic cytotoxicity.

It has been demonstrated previously that PM, a MMC analogue with a methyl group attached to the aziridine ring, has similar hypoxic and decreased aerobic toxicity compared to MMC (5). This could make it a better agent than MMC for the purposes of combined radiochemotherapy. However, the oxygen dependency of its toxicity at intermediate oxygen levels is not known. If it is more sensitive to oxygen inhibition than MMC, cells at oxygen levels sufficiently low for radiobiological hypoxia may be spared, hence masking this potential increase in therapeutic ratio. To ascertain whether this in fact occurs, the oxygen dependency of PM has been investigated and determined using CHO cells in suspension culture. ASC, an agent known to selectively increase the aerobic cytotoxicity of MMC (4), has been investigated for its ability to modulate aerobic PM cytotoxicity. The ability of ASC to inhibit catalase activity (6) may reduce the ability of the cell to cope with the oxidative stress induced through futile cycling of aerobically activated mitomycin compounds.

The degree of preferential hypoxic cytotoxicity demonstrated by bioreductive alkylating agents depends on the exposure conditions utilized (4, 7, 8). For example, the aerobic/hypoxic ratio of cytotoxicity may vary as function of drug concentration (c) and time of exposure (t) which may, in turn, affect drug effectiveness. The lack of an aerobic/hypoxic differential demonstrated by a MMC-sensitive, DNA repair-deficient CHO mutant (4) might, therefore, be attributable to the extremely low levels of drug utilized for such investigations, rather than a difference in repair capability of the cell. Such a characteristic would have significant implications for the clinical use of this class of agents in that appropriate levels of drug would have to be achieved and maintained before any selective toxicity could be obtained. To investigate this factor, the aerobic and hypoxic toxicity of PM and MMC was investigated for a series of drug concentrations and a wide range of exposure times in both the wild-type and drug-sensitive CHO cells.

MATERIALS AND METHODS

Cells. A cloned subline of CHO cells, AA8-4, and a repair-deficient mutant of this subline, UV-20 (9), were utilized in these experiments. Both cell lines were obtained from Dr. L. H. Thompson (Lawrence Livermore Laboratory, Livermore, CA). Asynchronous cell populations were maintained in exponential growth in suspension culture in alpha-minimal essential medium with 10% FBS (Bocknek, Canada), with typical doubling times of 12 to 14 h. Both cell lines were tested for Mycoplasma and found to be negative.

Chemicals. Porfiromycin (a gift from Upjohn Pharmaceutical Co., Kalamazoo, MI) and mitomycin C (Boehringer Mannheim, West Ger-
cells (5). This suggests that an increased therapeutic ratio could be obtained clinically with PM, provided its oxygen sensitivity is similar to MMC. Fig. 1 demonstrates that PM toxicity toward CHO cells in suspension culture increased as the amount of oxygen in solution during exposure decreased below 0.1%. The survival curves did not vary at oxygen tensions of 0.1% and greater. Although PM was less toxic than MMC under aerobic conditions, survival was reduced to 10% under such exposure conditions.

To determine the oxygen dependency of PM cytotoxicity and compare it with that previously determined for MMC (4), survival at 5 h after addition of 1 µg/ml of PM was determined for a series of oxygen tensions. Survival was then plotted as a function of the percentage of oxygen in solution (Fig. 2). Results previously obtained for MMC using identical incubation and counting techniques and the same concentration and exposure time (4) are presented as a line without data points. PM demonstrated less aerobic and greater hypoxic cell toxicity than MMC. For both PM and MMC, toxicity did not increase significantly until the percentage of oxygen in solution was below 0.1%. Below this level PM caused a very rapid increase in toxicity, whereas MMC toxicity increased gradually. Due to the precipitous drop in survival, data for 0.001% O2 were obtained by extrapolating curves in Fig. 1 out to 5 h. PM toxicity was compared with that previously obtained for MMC using identical incubation and counting techniques. Different symbols represent determinations from separate experiments.

RESULTS

Porfiromycin has been shown previously to exhibit greater selective cytotoxicity toward hypoxic versus aerobic EMT6 cells than MMC, primarily as a result of decreased toxicity to aerobic cells (5). This suggests that an increased therapeutic ratio could be obtained clinically with PM, provided its oxygen sensitivity is similar to MMC. Fig. 1 demonstrates that PM toxicity toward CHO cells in suspension culture increased as the amount of oxygen in solution during exposure decreased below 0.1%. The survival curves did not vary at oxygen tensions of 0.1% and greater. Although PM was less toxic than MMC under aerobic conditions, survival was reduced to 10% under such exposure conditions.
demonstrated much greater toxicity under extremely hypoxic conditions than MMC. While the aerobic/hypoxic survival ratio was greater for PM, the oxygen dependency for this transition remained similar to that of MMC, suggesting an enhanced therapeutic kill of hypoxic cells relative to aerobic cells could be obtained with PM.

Ascorbic acid increases the aerobic but not the hypoxic cytotoxicity of MMC (4). It is possible that the decreased aerobic toxicity of PM might result in ascorbic acid not having an effect on PM cytotoxicity. However, exposures of CHO cells to 1.0 μg/ml of PM in the presence of 2.84 mM ASC (10 times that normally found in grown medium) increased the aerobic, but not the hypoxic, cytotoxicity of PM (Fig. 3). The modification of aerobic toxicity was similar for PM and MMC, although its absolute magnitude was less due to the reduced aerobic toxicity of PM.

The selective toxicity of PM towards hypoxic cells has been demonstrated at one concentration of drug (Fig. 1). It has been observed recently in chemical systems that the spectrum of lesions produced in DNA after MMC exposures is a function of the mode by which it is activated, as well as its rate of activation (11). This might suggest that exposures conducted at different drug concentrations but normalized with respect to exposure time may lead to different relative survivals, and possibly a different degree of selective toxicity. To investigate this phenomenon in the present cellular system, the CHO cell line, AA8-4, was exposed to 0.5 to 6 μg/ml of PM for 1 h, 0.05 to 0.5 μg/ml for 16 h, or 1 μg/ml for 0 to 5 h. When the percentage of survival for all conditions was plotted versus the product of c x t, the results shown in Fig. 4 were obtained. For all exposure conditions it appears that the absolute degrees of aerobic and hypoxic toxicities were similar for the different conditions. For example, whether an exposure was conducted for 1 h at 5 μg/ml, 5 h at 1 μg/ml, or 16 h at 0.3 μg/ml, the survival under aerobic conditions was identical, as was the toxicity under hypoxic conditions. Therefore, the magnitude of the aerobic/hypoxic differential was not dependent upon either the length of the exposure period or concentration of PM, provided the exposure was expressed as c x t. Similar results were obtained for MMC (data not shown).

UV-20 is a mutant CHO cell line which is extremely sensitive to the cytotoxic effects of bifunctional alkylating agents, resulting from its deficient repair of damage (9, 12). Previously, we were unable to demonstrate any selective hypoxic cell toxicity in this cell line with exposures of 0.01 μg/ml of MMC for up to 5 h (4). This lack of aerobic/hypoxic differential might have been a consequence of the extremely low concentrations of drug utilized in those experiments. To explore this further we have exposed UV-20 to 0.15 to 1 μg/ml of PM for 6 min, 0.015 to 0.1 μg/ml for 60 min, or to 0.01 μg/ml for 0 to 5 h and plotted survival as a function of c x t (Fig. 5). All exposure periods produced similar survivals for equivalent c x t values with a slight protective effect being observed for aerobic over hypoxic exposures. While the aerobic protection was small, it was completely absent in similar exposures to MMC (data not shown). Thus, the lack of a differential toxicity for air and hypoxia for UV-20 cells observed previously (10) was not due to the low levels of drug utilized.

DISCUSSION

On the basis of their enhanced toxicity towards hypoxic cells, bioreductive alkylating agents might be useful adjuncts to radiotherapy. Their effectiveness is dependent upon the oxygen dependency of cytotoxicity and their ability to penetrate to hypoxic regions of tumors. The cytotoxicity of PM, much like that of MMC, increased as the oxygen concentration in the exposure solution decreased below 0.1% (Fig. 1), with PM being less toxic than MMC under completely aerobic conditions and significantly more toxic under extremely hypoxic conditions. Based on these observations PM might demonstrate a higher therapeutic ratio than MMC.

To illustrate the potential effectiveness of combining bio-

---

### Fig. 3. Relative percentage of survival of AA8-4 cells as a function of exposure to 1 μg/ml of PM in the presence or absence of 2.84 mM ASC under hypoxic and aerobic conditions. ASC (2.84 mM) in the absence of drug had no effect on plating efficiency (data not shown). Different symbols represent separate experiments.

### Fig. 4. Relative percentage of survival of AA8-4 cells after various exposures to PM under either aerobic (open symbols) or hypoxic (closed symbols) conditions. Exposures were to various PM concentrations for 1 (circles) or 16 (squares) h, or to 1.0 μg/ml for various times (triangles). Repeat experiments are indicated by separate points.
The applicability of such an analysis will be influenced by the control such populations since examination of the oxygen dependence of the cytotoxic activity of PM or MMC was made (Fig. 6). Cell survival in environments of greater than 1.0% oxygen or less than 0.01% oxygen was effectively reduced by radiation or drug, respectively. However, this analysis suggests that the combined effects of these agents may not be sufficient to eliminate cells at intermediate oxygen concentrations as effectively. In fact, there might be a slight decrease in the ability of PM, compared to MMC, to control such populations since examination of the oxygen dependency of these compounds (Fig. 2) reveals that PM was not as toxic as MMC at oxygen concentrations greater than 0.02%. While this may serve to decrease normal tissue damage, it may also reduce control of tumor cells existing at 0.02 to 1.0% O2. The applicability of such an analysis will be influenced by the proportions of tumor cells at the various oxygen concentrations, but it suggests that an examination of survival at either extreme of oxygenation (≥1% or <0.01% O2) may not accurately reflect the effective therapeutic potential of such agents against tumor cells under intermediate oxygen concentrations (13).

MMC introduces monoadducts and interstrand DNA cross-links (14), with increased formation of such cross-links being associated with increased toxicity under hypoxic conditions (4, 15). Back-oxidation of activated forms of MMC by molecular oxygen generates reactive oxygen species which are also able to damage DNA (16, 17). The decreased aerobic cytotoxicity of PM compared to MMC might, therefore, arise from a general decrease in the formation of alkylating species, decreased cross-link formation, or reduced generation of reactive oxygen species such as superoxide, hydroxyl radical, or hydrogen peroxide. PM was found previously to introduce slightly fewer DNA-DNA cross-links than an equivalent dose of MMC under aerobic conditions (15), but no evidence has been presented which would support or exclude the involvement of toxic oxygen species. Thus, the mechanism(s) of the increased differential toxicity of PM compared to MMC remains undefined.

The enhancement by ASC of aerobic cytotoxicity (Fig. 3) could result from either increased formation of alkylating species under aerobic conditions, or increased oxidative damage from reactive oxygen species. The first possibility would require that ASC enhance the activity of an enzyme, such as diaphorase, which is capable of aerobicly reducing quinone compounds (18, 19). ASC is also known to inhibit the activity of catalase, a cellular enzyme which catalyzes the breakdown of H2O2 to H2O and O2 (6). ASC may, therefore, exacerbate the oxidative stress applied to the cell by aerobic reduction of PM. The present data cannot exclude either possibility.

When utilized clinically, MMC is primarily delivered as a single dose rather than in a fractionated regimen or as a continuous infusion (20). As a result, plasma concentrations of drug reach a short-lived peak (roughly 1 μg/ml) followed by a longer period of lower drug concentrations (<0.1 μg/ml) (21). If the degree of selective hypoxic cytotoxicity is dependent on concentration, or time of exposure to drug, it may be possible that therapeutically useful doses are not achieved or maintained for sufficient periods after bolus injection. This question was addressed in the present in vitro system by determining the relative aerobic and hypoxic cell survival for various concentrations and times of drug exposure. The data (Figs. 4 and 5) show that 1-h, 5-h, and 16-h (1 cell doubling time) exposures, when converted to c x t drug exposures, demonstrate very little difference in survival for either aerobic or hypoxic cells. Similarly, Rockwell et al. have found that cell survival, tumor growth, and host toxicity were not significantly different for single injection, infusion, or relatively short (1-wk) fractionation schedules in vivo (22). Thus, both in vitro and in vivo data are consistent with therapeutic effects of both MMC and PM, depending on a c x t relationship.

The present results suggest that the lethal damage produced by these agents is introduced linearly with time and concentration of exposure. It also appears that the total amount of damage produced, rather than the rate of damage production, determines survival. While the clinical situation is more complex and will involve such parameters as cellular pharmacology and drug delivery and clearance, these conclusions are supported by observations that the dose-limiting myelosuppression caused by MMC is cumulative (20), and that fractionated or infused MMC produces the same therapeutic benefit as larger, less frequent doses (20, 22). The present data also suggest that
PM and MMC cytotoxicities are not strongly dependent on cell cycle. It has previously been reported that HeLa cells in the G1 phase of the cell cycle are more sensitive to MMC than are cells in the remainder of the cycle (23). This discrepancy with the present work may arise from the fact that the G1 sensitivity is slight or absent in CHO compared to HeLa cells. Complete analysis of this question will require examination of synchronized cell populations in specific stages of the cell cycle.

The results obtained with wild-type AA8-4 cells suggest that repair is not dependent on the rate of damage production, but rather on the total amount of damage produced, represented in this investigation by c × t. In support of this, similar results were also obtained with UV-20, a CHO mutant cell line which is deficient in the incision step of cross-link repair (12), and extremely sensitive to the cytotoxic effects of MMC (9). Unlike AA8-4, though, there was very little aerobic/hypoxic differential for PM cytotoxicity. The absence of any differential in aerobic and hypoxic survival in UV-20 was suggested previously to result from the extreme sensitivity of these cells to MMC and the correspondingly low levels of drug required to kill these mutants (4). This investigation demonstrated that this hypothesis was incorrect and that the absence of hypoxic selectivity may be a result of the repair deficiency.

Monofunctional alkylations (bulky adducts) produced by MMC under aerobic conditions outnumber cross-links by at least a factor of 10 (24). In the presence of a functional repair system in AA8-4 cells, bulky adducts may be efficiently repaired, leaving interstrand DNA cross-links as the limiting lethal lesions. Under hypoxic conditions the number of cross-links increases, leading to increased cytotoxicity (4, 15). However, in the absence of bulky adduct repair in UV-20 cells, monofunctional alkylations may persist and become the limiting lethal lesion, rather than cross-links. Thus, while the formation of cross-links may increase under hypoxic conditions, the number of monofunctional alkylations may not increase correspondingly, so that the level of limiting lethal lesions in UV-20 cells, the monoadducts, is similar under both aerobic and hypoxic conditions. This would lead to the equivalent survival observed in UV-20 cells exposed to MMC or PM under either aerobic or hypoxic conditions. This possibility is consistent with a molecular model for the role of oxygen in MMC activation recently proposed by Tomasz et al. (11), in which oxygen can act both at the level of drug activation and at the level of cross-link formation in DNA. The possibility that monofunctional bulky adducts become the limiting lethal lesion in the absence of excision repair is currently under further investigation.

REFERENCES


Oxygen and Exposure Kinetics as Factors Influencing the Cytotoxicity of Porfiromycin, a Mitomycin C Analogue, in Chinese Hamster Ovary Cells

Raymond S. Marshall and A. Michael Rauth