Modification of the Cytotoxic Activity of Mitomycin C by Oxygen and Ascorbic Acid in Chinese Hamster Ovary Cells and a Repair-deficient Mutant

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ABSTRACT

The cellular and molecular damage produced by mitomycin C (MMC) in Chinese hamster ovary cells, AA8-4, and a repair deficient mutant of this line, UV-20, was studied by utilizing a system in which oxygen levels could be altered and monitored in solution during acute drug exposures. The cytotoxic activity of MMC decreased from hypoxic conditions to 1% oxygen in solution, while from 1 to 20% there was little change. The relative level of DNA cross-linking in cells was examined under these conditions by alkylated elution and found to increase as cell survival decreased. Utilizing a cell-free assay which detects formation of alkylating species it was confirmed that, while alkylolation was observed under aerobic conditions, overall levels increased in the absence of oxygen. The presence of ascorbic acid in the exposure medium increased the aerobic but not the hypoxic cytotoxicity of MMC. This resulted in a diminished differential toxicity for cells exposed under aerobic versus hypoxic conditions in the presence of ascorbic acid. When ascorbic acid was present, net alklylation increased under aerobic conditions but was unchanged under hypoxic conditions. One interpretation of these results is that at least two mechanisms of activation of MMC to toxic intermediates may be present in these cells.

INTRODUCTION

MMC is an alkylating agent considered to be a prototype of a class of antitumor drugs the cytotoxic activity of which is dependent on reductive activation to a cytotoxic species (1). DNA-DNA cross-links are thought to be the lethal lesion resulting from these cytotoxic species (2), but the production of DNA-protein cross-links, DNA monoadducts, alkylated proteins, and cytotoxic oxygen species has also been reported (2, 3). Activation is thought to require reduction of the quinone group to a semiquinone (4) or dihydroquinone (5) species, a process which may be reversed by oxygen (6–8). This activation scheme has been supported by observations that, in a cell sonicate system, the production of alkylating species is enhanced under hypoxic conditions and MMC toxicity is increased for hypoxic cells in vitro (1, 9, 10). However, cytotoxicity is still seen under aerobic exposure conditions in vitro (9–11).

Whether MMC is activated by a single mechanism which varies as a continuous function of oxygen tension or whether there are two or more components to the activation of MMC, one oxygen independent and one oxygen dependent, is not known. To examine these possibilities, CHO cells growing in suspension culture have been exposed to MMC under various measured levels of oxygen in solution and assayed for cell survival. In addition, a mutant cell line deficient in the excision of DNA-DNA cross-links (12, 13) has been included in this study to investigate the possibility that the type of lesion produced is dependent on the oxygen tension. At the molecular level, cell-free preparations of MMC have been studied for their ability to activate MMC under aerobic and hypoxic conditions using an alkylation assay described previously by Kenedy et al. (14) which detects net formation of alkylating species. Alkaline elution was utilized to examine the formation of DNA-DNA cross-links under various states of oxygenation.

MATERIALS AND METHODS

Cells. A cloned subline of CHO cells, AA8-4, and a repair-deficient mutant of this subline, UV-20, 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 α-minimal essential medium with 10% fetal calf serum (Microbiological Associates, Walkerville, MD), with typical doubling times of 12–14 h. Both cell lines were tested for Mycoplasma and found to be negative. Cell densities of 10^6 cells per ml were obtained by centrifuging appropriate volumes of cell suspensions at 240 × g for 5 min and resuspending the cells in 10 ml of fresh growth medium. Since ASC was found to alter the cytotoxic activity of MMC, most experiments were conducted in α-medium minus ASC. Cell concentration was determined using an electronic particle counter (Coulter Electronics, Hialeah, FL). Cell colony-forming ability was determined by plating cells in 5 ml of growth medium in 60-mm plastic Petri dishes (Falcon Plastics, Oxnard, CA), incubating at 37°C in a humidified 5% CO2:95% air atmosphere for 8 days, staining the cells with methylene blue, and counting colonies. Plating efficiency of control cells was routinely greater than 90% for both AA8-4 and UV-20 cells.

Chemicals. Mutamycin (Bristol-Myers, Ottawa, Canada) containing 5 mg drug and 10 mg mannitol was reconstituted with 10 ml sterile, deionized water prior to dilution into growth medium to give the desired concentration for cell exposure. Stock drug was either made up immediately prior to use or stored at 4°C for no longer than 2 weeks before use. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase (type XII), and L-ascorbic acid were obtained from Sigma Chemical Co. (St. Louis, MO). Proteinase K was obtained from BDH Chemicals Limited (Toronto, Ontario, Canada), and tetrabutylammonium hydroxide was purchased from Fisher Scientific Co. Ltd. (Toronto, Canada). Other chemicals were obtained from standard chemical sources.

Drug Exposures. Drug exposures were conducted in suspension culture in the type I container described in a previous publication (15). It was a 40-ml glass polyshell vial (John's, Scientific, Inc., Toronto, Canada) fitted tightly with a silicon stopper through which a gas inlet and outlet were inserted. Cells were routinely exposed to drug at a concentration of 10^6 cells per ml in a total volume of 10 ml at the temperature maintained at 37°C. The cell suspensions were continually stirred at a constant rate of 180 rpm with a magnetic stir bar. Gases of known oxygen concentration were flowed over the surface of the suspension at a rate of 10 liters/h. All gases were prehumidified and contained various analyzed levels of oxygen, 5% CO2 and the balance nitrogen (Gas Dynamics, Toronto, Canada). Oxygen tensions in solution were determined utilizing a sensitive oxygen sensor. Cell suspensions were allowed to equilibrate with gas for 45 min, after which the drug was added in a volume of 0.1 ml growth medium. Samples were removed as a function of time after drug addition and assayed for colony forming ability. Sampling did not alter the oxygen tension.

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3 The abbreviations used are: MMC, mitomycin C; α-MEM, alpha minimum essential medium; FCS, fetal calf serum; CHO, Chinese hamster ovary; PBS, phosphate buffered saline; ASC, ascorbic acid.

tension in solution (data not shown).

Measurement of Cell Respiration. Measurements of respiration rates were made using a Clark-type O_2-sensing electrode (Model 5331 Yellow Springs Instruments Co., Yellow Springs, OH) in combination with a YSI Model 53 biological oxygen monitor and a YSI Model 5301 Bath-Stirrer Assembly. The amplified current output of the sensor was recorded on a Linear model 285 chart recorder (Linear Instruments Corp., Irving, CA). To determine the respiration rate of the cells in the absence of MMC, aerobic cell suspensions at a density of 5 x 10^6 cells per ml were placed directly into a measuring chamber in the bath-stirrer assembly, which was then quickly sealed with a Lucite plunger containing the oxygen sensor. Measurements of oxygen consumption were initiated immediately and continued until the cells had consumed approximately 90% of the oxygen initially present. The O_2 consumption rates were calculated from the initial slopes observed within the first 5 min of consumption to avoid problems arising from respiration rate decreases at low oxygen tensions. The effect of MMC on cellular respiration was determined by incubating the cells for up to 5 h under either aerobic or hypoxic (<10 ppm O_2) conditions in the presence of MMC (10 or 0.1 μg/ml) for AA8-4 and UV-20, respectively, reoxygenating the suspension quickly, and then transferring the cells immediately into the respiration apparatus.

Alkylation Studies. A procedure described previously by Kennedy et al. (14) was utilized to examine alkylating capacity of reactive MMC species. Suspensions of AA8-4 at a cell concentration of 1.5 x 10^6 cells/ml were centrifuged, sonicated, and examined microscopically to confirm complete cellular disruption. These preparations were diluted with 0.1 M Tris-HCl buffer (pH 7.4) to a final protein concentration of 3.0 mg/ml, as determined with a Sigma Total Protein Diagnostic kit. This final protein concentration corresponded to approximately 1 x 10^7 cells per ml. The reaction mixture consisted of 2.5 mg protein, 5.0 μmol glucose-6-phosphate, 0.72 μmol NADP, 5.0 μmol MgCl_2, and 1.25 units glucose-6-phosphate dehydrogenase in 0.1 M Tris-HCl buffer (pH 7.4) in a total volume of 1.0 ml. The mixture was exposed to gas containing an analyzed level of oxygen for 30 min while it was held on ice. NADPH was then generated by shaking the mixture at 37°C for 5 min, after which the reaction was started by the addition of 0.1 mg MMC in 40 μl of acetone (100 μg/ml, final concentration) and 10 μl of a 10% acetone solution of 4-(p-nitrobenzyl)pyridine. The reaction was stopped after 1 h by the addition of 2.0 ml of acetone and 1.0 ml of 1 N NaOH, and the sample was immediately extracted in subdued light with 2.0 ml of ethyl acetate. This mixture was centrifuged at 1000 x g for 2 min, and the organic layer was removed and its absorbance was read immediately at 540 nm.

Alkaline Elution Analysis. The alkaline elution technique was similar to that described by Kohn et al. (16). AA8-4 cells in exponential growth at an initial concentration of 4 x 10^6 cells per ml were incubated in α-minimal essential medium minus nucleosides with [14C]thyminidine (0.005 μCi/ml; 60 mCi/mmol) for 24 h prior to assay. Two h before the drug treatment the radioactive medium was replaced with medium containing unlabeled thymidine. Cells were treated with MMC as described above, removed from the MMC-containing medium, washed, and resuspended in ice cold phosphate-buffered saline. Single strand breaks were introduced into the DNA by irradiating with 600 rads in a 60Co source at a dose rate of 400 rads/min. Cells were kept on ice during and after irradiation to reduce repair of the single strand breaks and then gently loaded onto polycarbonate filters (25 mm diameter; 2 μm pore size; Millipore Corp., Bedford, MA) and washed with 10 ml of cold phosphate-buffered saline (15). The cells were then treated with lysis solution [2% sodium dodecyl sulfate:0.025 M EDTA:0.1 M glycine: Proteinase K (0.5 mg/ml)], the solution passing through the filter by gravity and being collected in a scintillation vial. A peristaltic pump was then connected, and DNA was eluted from the filters in the dark with 2% tetrapropylammoniumhydroxide:0.02 M EDTA (pH 12.1) at a constant flow rate of 2.0 ml/h. Fractions were collected every 1.5 h, and the radioactivity per fraction and that remaining on the filter at the end of the elution were determined by liquid scintillation counting.

RESULTS

A high degree of variability in survival was originally noted between aerobic, but not hypoxic, exposures of AA8-4 to MMC (1 μg/ml). It was ultimately determined that aerobic drug exposure in freshly prepared medium resulted in more cell killing by MMC than exposure in medium which had been stored for several weeks before use, suggesting the involvement of an unstable component. Three potential unstable medium components were selected for study: glutamine, pyruvate, and ASC. The presence or absence of glutamine or pyruvate had no effect on aerobic MMC toxicity, but ASC did have an effect which partially accounted for the variation seen. Fig. 1 demonstrates that ASC at 0.284 mM, the level present in fresh growth medium, significantly increased toxicity under aerobic conditions but, even at levels of 2.84 mM, had no effect on hypoxic cell survival when present during MMC exposure. ASC concentrations up to 2.84 mM were not toxic to AA8-4 cells in the absence of drug under aerobic or hypoxic conditions. However, aerobic exposures to MMC (1 μg/ml) in the presence of 2.84 mM ASC reduced survival by at least a factor of 100 after 5 h. As a result of these experiments and due to the fact that ASC is unstable in growth medium (17, 18), ASC-free medium was utilized for all subsequent acute MMC exposures. Without ASC, protection from MMC toxicity by oxygen was always seen, and the variability observed in the aerobic survival curves was controlled.

To study the effects of oxygen on the cytotoxic activity of MMC on the parental cell line, AA8-4, and a repair-deficient mutant, UV-20, it was necessary to know the oxygen tension in solution. At the cell density used, 1 x 10^6 cells per ml, and at low oxygen tensions, cellular respiration significantly reduces the oxygen content of the solution (15). This reduction in oxygen content can be accounted for theoretically (15) if the drug itself has no effect on respiration. Cells were exposed to MMC (0.1 to 10.0 μg/ml) for times up to 5 h under hypoxic or aerobic conditions. Cell respiration rates were then measured. No effect of MMC on cell respiration was detected under these conditions in either cell line, a rate of 3.5 x 10^-17 mol/cell/s being consistently obtained. Thus, oxygen tension in solution could be calculated for various oxygen concentrations in the gas phase. Direct measurements of oxygen tension confirmed these calculations (data not shown).

When exposed to MMC (1.0 μg/ml), AA8-4 demonstrated decreased survival with decreasing oxygen tension (Fig. 2). The...
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Fig. 2. Survival of AA8-4 cells as a function of time of exposure to MMC (1.0 μg/ml) under various oxygen tensions in solution.

Fig. 3. Survival of UV-20 cells as a function of time after addition of MMC (0.01 μg/ml) under aerobic and hypoxic conditions.

Fig. 4. Relative percentage of survival of AA8-4 cells after 5 h of exposure to MMC (1.0 μg/ml) under various oxygen tensions in solution. Different symbols indicate separate experiments.

Fig. 5. Comparative measurement (±SD) of the formation of alkylation species by AA8-4 cell sonicates plus or minus MMC under hypoxic or aerobic conditions in the presence or absence of 2.84 mM ASC. Results are expressed relative to the formation of alkylation species observed after a 1-h exposure under hypoxic conditions.

Extreme sensitivity of UV-20 to MMC required that acute exposures with this cell line be conducted at MMC (0.01 μg/ml). In contrast to the result with wild-type parent, the repair-deficient mutant UV-20 showed no difference in its sensitivity under aerobic or hypoxic conditions (Fig. 3). In both cell lines air or hypoxia in the absence of MMC had minimal effect on cell survival over 5 h of exposure. The oxygen dependency of cell survival of AA8-4 is shown in Fig. 4, where survival after 5 h of drug exposure at 1.0 μg/ml is presented as a function of oxygen concentration. The protective effect of oxygen appeared to saturate at oxygen levels above 0.1 to 1.0%.

Kennedy et al. (14) have shown that the metabolic activation of MMC to alkylating species by cell free preparations is enhanced in the absence of oxygen and have suggested that this is the basis for selective toxicity of MMC toward hypoxic cells. The formation of alkylating species under aerobic or hypoxic conditions, as demonstrated by the alkylation assay, is shown in Fig. 5. The amount of alkylation obtained with MMC (1.0 μg/ml) under N₂ (<10 ppm O₂) for 1 h was taken as 100%. About 50% of this level of alkylation was seen under air-equilibrated conditions. Little alkylation was seen in nitrogen or air in the absence of MMC. This pattern was similar to that seen for cell survival. When ASC was added to the reaction mixtures, a small decrease in alkylation was seen under hypoxic conditions, but an increase was seen in air. Again, this result is qualitatively similar to the survival data, where ASC had little or no effect on hypoxic toxicity but increased the aerobic toxicity of MMC. Negligible alkylation of the trapping reagent (roughly 5–10%) was observed in experiments in which either MMC or cell sonicates were omitted.

It was shown previously (13) that cross-linking of DNA in cells by MMC could be detected using the alkaline elution technique of Kohn et al. (16). These studies were done under aerobic exposure conditions. The formation of interstrand cross-links in AA8-4 cells was investigated under both aerobic and hypoxic exposure conditions. Cells were exposed to MMC...
elution procedure described previously. In Fig. 6 the result of this analysis is presented as the percentage of DNA retained on the filter plotted as a function of elution time. Cross-link formation is observed as increased retention of DNA on the filter in comparison to DNA from cells not treated with drug. While retention of DNA was seen by previous workers (13) under aerobic conditions, these experiments demonstrated that this retention was enhanced after hypoxic exposure. Again, the increase in cross-link formation at intermediate and low oxygen tensions was qualitatively similar to the results seen for cell survival (Fig. 2). In contrast, preliminary data suggest that the addition of ASC to cells during aerobic or hypoxic exposure did not result in alterations in cross-link formation (data not shown), while still decreasing cell survival (Fig. 1). Thus, the effect of ASC at the molecular level was to increase net alkylation, Fig. 5, without specifically increasing DNA-DNA cross-link formation.

DISCUSSION

Variability in MMC toxicity under aerobic exposure conditions has been observed previously at high drug concentrations and short exposure times with mouse L cells (19), the source of this variation being identified as pH differences between exposures. Some impurities have also been discovered in certain batches of MMC (20), resulting in variations in the observed response to this agent. The variations in aerobic toxicity observed in the present series of experiments were not attributable to such causes. The observation that use of older batches of medium resulted in decreased toxicity and the known instability of ASC in medium (17, 18) led to the identification of this compound as a modulator of aerobic MMC toxicity. Indeed, in aqueous solutions transition metals such as copper can catalyze ASC oxidation in the presence of molecular oxygen (21). Changes in the level of aerobic toxicity caused by MMC were virtually eliminated with the exclusion of ASC from the medium and the careful storage and use of fresh medium in subsequent experiments.

The mechanism of ASC modification of MMC cytotoxicity is not understood. Concentrations of ASC up to 2.84 mM in the absence of MMC were not in themselves toxic (Fig. 1). Furthermore, exposure of AA8-4 cells to MMC in the absence of ASC with subsequent plating in the presence of ASC did not increase toxicity (data not shown). Thus, MMC and ASC must be present together to result in increased toxicity. Toxicity toward the wild-type cells under hypoxic conditions was not altered by the presence of ASC, suggesting that ascorbic acid was modulating or mediating the production of damage produced under aerobic conditions. Toxicity toward a repair-deficient mutant, UV-20, was not altered by ASC under either hypoxic or aerobic conditions (data not shown). However, MMC levels were 100-fold lower than those utilized for wild-type exposures. At this concentration, activation may be limited by the low levels of drug present so that any increase in reducing capacity may not result in increased toxicity. Alternatively, this result may be a reflection of the inability of UV-20 to repair mono- or bifunctional alkylations produced by MMC.

The alkylation assay demonstrated increased formation of alkylating species in the presence of ASC under aerobic but not hypoxic conditions, suggesting that ASC increased drug activation under aerobic conditions (Fig. 5). Ascorbic acid did not produce alkylating species in the absence of the NADPH generating system, demonstrating that it was not acting by directly reducing MMC. It has been demonstrated previously that liver microsomes from animals which had been deprived of ASC showed significant decreases in the content of cytochrome P-450, NADPH cytochrome P-450 reductase, and NADPH cytochrome c reductase (22), potential reducers and modulators of the enzymatic reduction of MMC (11). The increased toxicity observed in the presence of ASC may thus be due to increased production of an enzyme(s) capable of reducing MMC to alkylating species or compound(s) capable of modulating the reduction process. However, the limited time of exposure to ASC may not be sufficient to alter initial cellular enzyme levels prior to drug addition.

Non-toxic concentrations of sodium ascorbate have been reported to potentiate the effects of 5-fluorouracil, X-irradiation, bleomycin, prostaglandin E, and sodium butyrate on mouse neuroblastoma cells (23). These effects were postulated to result from intracellular accumulation of H2O2 and the subsequent production of hydroxyl radicals. Preliminary data indicate that the presence of catalase and superoxide during aerobic exposure of AA8-4 to MMC (1 μg/ml) does not alter MMC toxicity (data not shown). While this suggests that extracellular H2O2 and superoxide are not responsible for the ASC-induced enhancement, it does not eliminate the possible intracellular accumulation of such species as a potential mechanism. Aerobic reduction of MMC to the semiquinone or dihydroquinone state, followed by reoxidation to the parent compound, may result in the intracellular production of superoxide and other related toxic species (24), a result which would not be observed under hypoxic conditions. Further, the presence of transition metals such as copper may enhance aerobic H2O2 production by ASC (21). Increased levels of such species, as may occur in the presence ASC, might overload the cells' protective mechanisms, thereby leading to increased toxicity. To test this possibility it will be necessary to look for intracellular production of the superoxide anion or the hydroxyl radical after ASC treatment.

It is interesting to note that cross-link formation in AA8-4 cells, as determined by alkaline elution, does not appear to increase when aerobic exposures of MMC are conducted in the presence of ASC (data not shown). However, the alkaline elution procedure utilized in these experiments only measures...
bifunctional cross-links and not monofunctional adducts. One possible interpretation of these results is that ASC enhances the action of an enzyme which produces predominantly monofunctionalizing rather than bifunctional (cross-linking) species.

Keyes et al. (11) have reported that MMC demonstrated almost no preferential toxicity toward hypoxic as compared to aerobic Chinese hamster ovary cells after 3.5-h drug exposures and have seen low levels of production of reactive species after incubation of MMC with CHO cell sonicates in the alklyation assay. Our results indicate that, while the preferential toxicity is less striking after short exposure times, significant enhancement is observed under hypoxic conditions. Also, the CHO wild-type subline, AA8-4, was able to activate MMC to alkylating species under aerobic conditions and demonstrated enhanced formation of alkylating species under hypoxic conditions. These alkylations resulted seemed to support the survival data. The variations observed between the data of Keyes et al. (11) and the present data may be explained on the basis of cell line variation or perhaps an ascorbic acid effect. The wild-type CHO cell line utilized in these experiments (AA8-4) was found to be more sensitive to MMC than was another wild-type CHO cell line also carried in our laboratory (data not shown). Large differences in response to this agent have also been documented between different cell lines (9, 11). It is possible, then, that variations exist between different sublines of wild-type CHO with regard to levels and activities of enzymes and modulators of enzyme activity which may be involved in the reductive activation of MMC.

While oxygen did not completely inhibit MMC toxicity, decreased oxygen tensions in solution resulted in decreased survival. MMC exposure in the presence of oxygen levels greater than 1.0% resulted in approximately 2 logs of kill after 5-h drug exposures, indicating that toxic species were being produced under such exposure conditions. Cross-link formation was also observed after such aerobic MMC exposures. This indicated that MMC was activated to a bifunctional alkylating species, a process which has been demonstrated previously to depend upon enzymatic reduction (2). The aerobic component of MMC toxicity therefore seems to result from enzymatic activity which is not completely inhibited by the presence of oxygen. It is also apparent that aerobic MMC toxicity is not solely the result of the production of toxic oxygen species. The data demonstrate that toxicity and cross-link production increase as oxygen tension in solution is decreased below 1.0%. The increased activity observed under hypoxic conditions demonstrates the involvement of an oxygen-sensitive pathway which is capable of activating MMC to an alkylating species.

The significant aerobic toxicity and increased hypoxic toxicity observed in these experiments could result from the activity of a single enzyme, one which is more efficient at reducing MMC in the absence of oxygen but which is still able to activate the drug, although not as efficiently, under aerobic conditions. In this model the effect of ASC might be to enhance the intracellular production of hydrogen peroxide, leading to production of hydroxyl radicals. A second model to explain the present results is that more than one enzyme is involved in MMC activation. It has been shown by others that both microsomal and nuclear enzymes are able to activate MMC in cell-free systems (14). The fact that ASC increased MMC toxicity and alkylating activity under aerobic conditions but did not alter the response obtained under hypoxic conditions or enhance cross-link formation suggests that it may be mediating the activity of one enzyme without altering the activity of the other. The shape of the curve for the oxygen dependence of MMC toxicity would also be consistent with a two component mechanism of drug activation, one of which is sensitive to oxygen and one resistant. The present results suggest that the activity or levels of these different enzymes are affected in different ways by the presence or absence of ASC and/or oxygen. Such independent manipulation of two separate components of MMC activation may lead to enhanced kill of hypoxic cells, an approach ultimately aimed at improving the selective toxicity of such clinically utilized drugs. Further testing of the effects of ASC on enzyme activity and H2O2 production in cell-free systems may be one approach to testing these two possible models.

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