Nuclear Overexpression of NADH: Cytochrome b5 Reductase Activity Increases the Cytotoxicity of Mitomycin C (MC) and the Total Number of MC-DNA Adducts in Chinese Hamster Ovary Cells*

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Mitomycin C (MC),¹ a natural product antibiotic with anti-tumor activity, has been used successfully to eradicate hypoxic tumor cells in head, neck, and cervix cancers in combination with ionizing radiation (1–4). As a prodrug, MC can be chemically and enzymatically converted into a reactive species capable of alkylating a variety of nucleophilic molecules, including genomic DNA (5). Specifically, the quinone moiety of MC is reduced to give unstable intermediates that are capable of further transformations to generate a reactive molecule with two electrophilic carbon centers (6). Both monofunctional and bifunctional adducts are formed between MC and DNA (7, 8).

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The abbreviations used are: MC, mitomycin C; FpD, NADH:cytochrome b₅ reductase; CHO, Chinese hamster ovary; NLS, nuclear localization signal.

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ate the relatively nontoxic MC prodrug. Studies focusing on NAD(P)H dehydrogenase are to a large extent attributable to experiments that identified higher levels of this enzyme in tumor cells compared with normal cells, which might account for preferential activation of MC in neoplastic cells (30–32). However, attempts to correlate NAD(P)H dehydrogenase activity with drug sensitivity have led to conflicting results (31, 33–35).

In contrast to NAD(P)H dehydrogenase and NADPH:cytochrome P-450 reductase, FpD is present in fairly consistent, relatively low levels in both normal and tumor cells (30, 31). Apparently for this reason, FpD has received considerably less attention. However, this enzyme has been shown to play a role in the activation of MC (19). The purified enzyme has been shown to reduce MC to a species capable of alkylating DNA in vitro. In addition, it has been demonstrated that overexpression of FpD in the cytoplasm of CHO cells by removal of the membrane binding domain enhances sensitivity to MC relative to the parental line under both aerobic and hypoxic conditions (36). To further investigate the importance of the subcellular site of bioactivation to the cytotoxicity of MC, FpD, an enzyme found predominantly in the outer mitochondrial membrane of CHO cells, was overexpressed in the nucleus of these cells. The effects of nuclear localization of FpD on the sensitivity of CHO cells to MC and on the formation of DNA adducts of the alkylating agent were then assessed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture—CHO-K1/dhfr**-, the CHO-K1 cell line deficient in dihydrofolate reductase, was obtained from American Type Culture Collection (Rockville, MD) and maintained in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum, 2 mM glutamine, 0.1 mM hypoxanthine, 0.01 mM thymidine, and antibiotics (penicillin, 100 units/ml; streptomycin, 100 mg/ml). Transfected cell lines were maintained in the same medium with the addition of G418 (1 mg/ml) to select for the expression vector. Cells were grown as monolayers at 37 °C under an atmosphere of 95% air, 5% CO2. Cultures (2 ml) were gassed for 2 h prior to trypsinization and then rescreened under selection conditions. Cells under aerobic conditions were treated in an identical manner in a humidified atmosphere of 95% air, 5% CO2. Surviving fractions were calculated using the plating efficiencies of hypoxic and aerobic vehicle-treated controls.

**Nuclear Localization of FpD and MC Cytotoxicity**

**Enzymatic Assays—**Trypsinized cells were washed and resuspended in phosphate-buffered saline for sonication. Spectrophotometric assays were performed on crude cell sonicates at 37 °C as described previously (36). Briefly, FpD activity was measured by reduction of potassium ferricyanide at 420 nm. NADPH:cytochrome c (P-450) reductase and rotenone-insensitive NADPH:cytochrome c reductase activities were monitored by the rate of ferricytochrome c reduction at 550 nm. NAD(P)H dehydrogenase activity was measured as the dicumarol-inhibitable reduction of dichloroindophenol measured at 600 nm. Total protein was determined with bicinchoninic protein assay reagent (Pierce Chemical Co.).

**Aerobic/Hypoxic Experiments—**Cells (2.5 × 10^6) were plated in microtiter plates and allowed to grow at 37 °C under an atmosphere of 95% air, 5% CO2 (in a humidified incubator for 72 h). To establish minimally growing cultures were gassed through rubber septums fitted to the glass bottles for 2 h at 37 °C with 95% N2, 5% CO2 (<1 ppm O2) to induce hypoxia. Graded concentrations of MC (0, 2.5, 5, and 10 μM) were injected through a rubber septum in volumes less than 75 μl. After 1 h of treatment, cells were harvested by trypsinization and assayed for their ability to form macroscopic colonies. Cells under aerobic conditions were treated in an identical manner in a humidified atmosphere of 95% air, 5% CO2. Surviving fractions were calculated using the plating efficiencies of hypoxic and aerobic vehicle-treated controls.

**I[3H]Mitomycin C Assay for Total DNA Adducts—**Suspension cultures were prepared by trypsinization of exponentially growing monolayers and were cultured at a density of 1 × 10^6 cells/ml in glass vials fitted with rubber septums and stir bars. Cultures (2 ml) were gassed for 2 h with 95% N2, 5% CO2 to induce hypoxia or with 95% air, 5% CO2 to produce aerobic conditions. Cells were then exposed to 10 μM I[3H]MC (0.18 μCi/μmol) for 2 h under hypoxic or aerobic conditions. Total genomic DNA was extracted from 1 × 10^6 cells using the PUREGENE DNA purification system (Gentra Systems, Minneapolis, MN) following the manufacturer guidelines. Briefly, cell lysates were treated with proteinase K (100 μg/ml) overnight followed by RNase A (20 μg/ml) for 1 h at 37 °C. Proteins were removed by precipitation. DNA was then precipitated, washed twice with 70% ethanol to reduce radioactive counts in the wash to background levels, and then dissolved in 10 μM Tris-HCl, 1 mM EDTA, pH 7. Radioactivity in the samples was normalized to the total DNA concentration, as determined by absorbance measurements at 260 nm.

**RESULTS**

**Isolation and Analysis of Stable CHO Cell Line Transfectants Overexpressing NADH-Cytochrome b5 Reductase—**Considerable difficulty was encountered in obtaining stable transfectants of CHO cells that overexpressed FpD either in its normal subcellular environment of the mitochondria and endoplasmic reticulum or in the nucleus. Several hundred clones were screened before a few were found that minimally overexpressed the enzyme compared with the parental cell line. Important oxidoreductase activities of the parental line (CHO-K1/dhfr−) and the transfectants expressing either a cytosolic membrane-bound enzyme (CHO-FpD-5) or a nuclear localized form (CHO-NLS-FpD-3) are summarized in Table I. Based on the NADH: ferricyanide oxidoreductase activities in crude cell lysates, clone CHO-NLS-FpD-3 expressed the FpD activity 3-fold over that of the parental line, whereas clone CHO-FpD-5 expressed the enzyme activity 5-fold over that of the parental line. The rotenone-insensitive NADPH:cytochrome c reductase assay measures the reduction of cytochrome c via endogenous cytochrome b5. Thus, the higher activity in this assay for clone CHO-FpD-5 provides support for FpD overexpression in the cytosolic membranes of the mitochondria and endoplasmic reticulum. Compared with the parental line, the levels of other oxidoreductase activities implicated in MC bioreduction (NADPH:cytochrome P-450 oxidoreductase and NAD(P)H dehydrogenase) remained unchanged in the two transfected cell lines. The activities of xanthine oxidase and xanthine dehydrogenase, two additional enzymes known to reduce MC, were not determined because we have previously shown that the CHO-K1/dhfr− cell line does not express either enzyme (27). A clone transfected with empty vector (pRC/CMV) was also character-
ized in terms of the various oxidoreductase activities, and was similar to the parental line (results not shown).

**Immunofluorescence Staining of Overexpressed FpD in CHO Cell Line Transfectants**—The muscle actin epitope was included at the carboxyl terminus of the FpD gene in the plasmid constructs to allow for visual detection of the subcellular localization of the enzyme in transfected cells. Cells of the CHO-FpD-5 transfectant that expressed a form of FpD unaltered in terms of localization exhibited primarily cytosolic staining, as shown in Fig. 1A. These results are in agreement with subcellular fractionation studies performed previously in our laboratory, which indicated that FpD is localized primarily in the mitochondria of CHO cells, with a smaller amount in the endoplasmic reticulum (36). In contrast, CHO-NLS-FpD-3 cells that expressed the FpD gene fused with the SV40 large T antigen nuclear localization signal sequence displayed fluorescence intensity that was concentrated in the nucleus (Fig. 1B).

**Survival Data for the Parental Line and the Stable CHO Cell Line Transfectants Expressing FpD under Both Aerobic and Hypoxic Conditions**—Survival curves for the parental line (CHO-K1/dhfr−), the stably transfected cell line overexpressing FpD activity in cytosolic particles (CHO-FpD-5), and the stably transfected cell line overexpressing FpD activity in the nucleus (CHO-NLS-FpD-3) are shown in Fig. 2, A and B, for aerobic and hypoxic conditions, respectively. The CHO-K1/dhfr− cell line is highly resistant to exposure to 10 μM MC for 1 h under aerobic conditions but sensitivity increases ∼10-fold at 10 μM MC under hypoxia. The survival curves for the CHO-FpD-5 transfectant, overexpressing FpD activity by 5-fold, are virtually superimposable with those of the parental line under both aerobic and hypoxic conditions. In contrast, the nuclear transfectant, CHO-NLS-FpD-3, overexpressing FpD activity by 3-fold, exhibits a small, but significant increase in MC sensitivity under aerobic conditions when compared with the other two cell lines. Furthermore, the nuclear transfectant is markedly more sensitive to MC under hypoxia, producing a readily observable oxic/hypoxic differential.

Analysis of the survival data in Fig. 2B was made by comparing the drug concentrations required for equivalent levels of survival in the transfected cell lines. The concentration of MC for 10% survival under hypoxia is ∼5.5 μM MC for the transfectant overexpressing mitochondrial/endoplasmic reticulum-localized FpD, and ∼3.8 μM MC for the transfectant overexpressing nuclear-localized FpD, to yield a sensitivity ratio of ∼1.4. The same analysis performed at 1% survival yields an identical sensitivity ratio. This comparison is made between cell lines that overexpress FpD activity at different levels relative to the parental cell line (3-fold overexpression for CHO-NLS-FpD-3 cells versus 5-fold overexpression for CHO-FpD-5 cells). Thus, the oxic/hypoxic differential represented by the sensitivity ratio should be expected to increase with equivalent overexpression of FpD activity in the nucleus and the mitochondrial/endoplasmic reticulum.

**Total [3H]MC-DNA Adducts in Drug-treated Cell Lines**—

Table II shows the amount of total [3H]MC-DNA adducts produced in cell lines treated with 10 μM [3H]MC under aerobic and hypoxic conditions. In these experiments, the majority of the radioactivity remained in the medium or associated with cellular debris and proteins, resulting in total MC-DNA adducts that were in the range of 3–12-fold over background. Under aerobic conditions, no statistically significant differences were found between the adduct levels of the three cell lines that were tested. In contrast, a small, but statistically significant difference in adduct levels was observed under hypoxia. Under hypoxia, the genomic DNA isolated from the nuclear transfectant, CHO-NLS-FpD-3, yielded almost twice the amount of radioactivity as the DNA from the parental line or the CHO-FpD-5 transfectant. Similar numbers of MC-DNA adducts occurred in DNA samples from the parental line and the

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**Table I**

Oxidoreductase activities of CHO-K1/dhfr− parental cells and NADH-cytochrome b5 reductase-transfected cell lines expressing either a mitochondrial/endoplasmic reticulum (CHO-FpD-5) or a nuclear (CHO-NLS-FpD-3) rat NADH-cytochrome b5 reductase cDNA

| Enzyme activitya | CHO-K1/dhfr− | CHO-FpD-5 | CHO-NLS-FpD-3 |
|------------------|--------------|-----------|---------------|
| NADH:ferricyanide oxidoreductase | 19.960 (100) | 9.460 (470) | 5.990 (410) |
| Rotenone-insensitive NADH-cytochrome c oxidoreductase | 18 (14) | 43 (20) | 14 (20) |
| NADPH:cytochrome P-450 oxidoreductase | 15 (6) | 16 (3) | 9 (3) |
| NAD/P dehydrogenase | 19 (6) | 16 (3) | 13 (3) |

a Enzymatic activities in cell sonicates were determined spectrophotometrically as described under “Experimental Procedures.”

b Values shown are the means of 3–12 determinations with the standard deviations given in parentheses.
prior to the determination of radioactivity.

Little has been done to characterize the impact of bioactivation by FpD on the cytotoxicity of MC, although previous studies by workers in our laboratory (36) have shown that a cytosolic form of FpD alters the sensitivity of CHO cells to MC under both aerobic and hypoxic conditions. To demonstrate that the intracellular location of the enzyme modulates cytotoxicity, we generated CHO cell lines that overexpressed FpD activity in its normal location (i.e. mitochondria and endoplasmic reticulum) and in the nucleus, without altering the levels of other oxidoreductases implicated in MC bioactivation (Table I). Only minimal levels of overexpression of FpD activity were achieved regardless of the enzyme location. Thus, CHO-NLS-FpD-3 overexpressed the nuclear-localized enzyme activity by 3-fold whereas CHO-FpD-5 expressed the cytoplasmic membrane-bound form by 5-fold over that of the parental line, CHO-K1/dhfr<sup>−</sup>.

**DISCUSSION**

Whereas MC is a highly potent anticancer agent that is clinically used in combination with radiation and chemotherapy regimens to treat several cancers, it is extremely toxic to normal tissues. There are also several factors that limit the efficacy of the drug. The toxic interstrand cross-link between MC and DNA must compete with monoalkylation products, as well as with the less damaging oxygen radicals (i.e. superoxide, hydrogen peroxide, and/or hydroxyl radical) produced in redox cycling reactions. Furthermore, the drug, once activated, is highly electrophilic and reacts readily with a host of nucleophilic cellular components including water. Consequently, only a very minor fraction of the activated drug reaches nuclear DNA. As part of an ongoing investigation to address this last issue, the subcellular site of MC bioactivation was modified by overexpression of bioreductive enzymes in the nucleus, close to the proposed target producing cytotoxicity, nuclear DNA.

**Table II**

**Determination of total [3H]MC-DNA adducts in cell lines treated under aerobic and hypoxic conditions**

| Cell line          | Cpm/μg DNA<sup>a</sup> | Aerobic | Hypoxic |
|--------------------|-------------------------|---------|---------|
| CHO-K1/dhfr<sup>−</sup> parental | 0.55 (0.10) | 0.77 (0.05) |
| CHO-FpD-5          | 0.54 (0.06) | 0.79 (0.07) |
| CHO-NLS-FpD-3      | 0.52 (0.04) | 1.40 (0.39) |

<sup>a</sup> DNA samples (200 μl) were mixed with glacial acetic acid (50 μl) prior to the determination of radioactivity.

<sup>b</sup> Three to four determinations were made for each cell line. Standard deviations are given in parentheses.

CHO-FpD-5 transfectant under hypoxia. Based upon the survival data obtained under hypoxia, shown in Fig. 2B, the drug sensitivity at 10 μM MC increased more than 10-fold in CHO-NLS-FpD-3 cells compared with CHO-FpD-5 cells, even though the level of overexpressed FpD activity in the nucleus of CHO-NLS-FpD-3 cells was less than the level of overexpressed FpD activity in the mitochondria and endoplasmic reticulum of CHO-FpD-5 cells. Thus, the approximate 1.8-fold increase in the total number of MC-DNA adducts at 10 μM MC for the nuclear FpD transfectant relative to both the parental cell line and the mitochondrial/endoplasmic reticulum FpD transfectant is much less than the approximate 10-fold increase in sensitivity estimated from the survival data at the same concentration (see Fig. 2B).

**Fig. 2. Survival curves under aerobic (A) and hypoxic (B) conditions for the parental line (CHO-K1/dhfr<sup>−</sup>, ▼), the stably transfected cell line overexpressing FpD activity in the mitochondria/endoplasmic reticulum (CHO-FpD-5, ○), and the stably transfected line overexpressing FpD activity in the nucleus (CHO-NLS-FpD-3, □). Cell lines were treated for 1 h with graded concentrations of MC under aerobic conditions (95% air, 5% CO<sub>2</sub>) or hypoxic conditions (95% N<sub>2</sub>, 5% CO<sub>2</sub>). Points are an average of three to four independent determinations. Standard deviations are shown where larger than the points.**

Despite the relatively low level of nuclear overexpression of FpD activity, transfectant CHO-NLS-FpD-3 caused an increase, albeit modest, in the cytotoxicity of MC under aerobic conditions and a more pronounced increase in the cytotoxicity of MC under hypoxia when compared with the cytoplasmic membrane-bound form of FpD expressed at a somewhat higher level in the mitochondria and endoplasmic reticulum. The slight but statistically significant increase in MC cytotoxicity under aerobic conditions in the transfectant expressing nuclear FpD activity most likely reflects the production of the reactive alkylating species closer to its DNA target. Under hypoxia, the marked increase in cytotoxicity for CHO-NLS-FpD-3 not only reflects the production of the activated species near nuclear DNA, but also the increased efficiency of the reactive species because of the absence of redox cycling reactions that occur from the one-electron reduction of MC under aerobic conditions. Whereas the increase in cell kill under hypoxia by nuclear overexpression of FpD activity is substantial, the total magnitude of the cell death is comparable with that achieved with cytosolic overexpression of the enzyme activity in a pre-
vious study in our laboratory (36). However, because a greater level of overexpression of enzyme activity (12-fold) was achieved in the previous study, a direct comparison between these CHO transfectants cannot be made. Nonetheless, given that a 3-fold increase in nuclear localized FpD activity produces a 10-fold increase in MC cytotoxicity, an even greater increase in cytotoxicity would be expected if greater overexpression of this enzyme activity could be attained in the nucleus.

The survival curves for the cell line expressing the enzyme activity in its normal intracellular location also generates some results that conflict with our previous findings. We have previously shown that overexpression of membrane-bound FpD in CHO cells by 9-fold results in a slight decrease in MC sensitiv-
ity under aerobic conditions, whereas the sensitivity under hypoxic conditions remains equal to that of the parental cell line (36). This protective effect was postulated to be because of the sequestering of MC electrophiles in the CHO cells, thereby decreasing nuclear DNA alkylations and reducing cytotoxicity.

In the present study, no apparent change in MC cytotoxicity occurred in the transfectant overexpressing FpD activity in the mitochondria/endoplasmic reticulum under either aerobic or hypoxic conditions relative to the parental cell line. Perhaps the protective effect demonstrated previously in CHO cells overexpressing FpD activity by 9-fold was not observed because the enzyme was overexpressed at a lower level of 5-fold in the present investigation. Alternatively, cell line drift may be responsible for the difference in MC sensitivities occurring in these FpD transfectants. In any case, the findings imply that activation of MC in these cytoplasmic sites limits the ability of the reactive MC hydroquinone electrophilic species to reach and interact productively with nuclear DNA. Thus, site-specific activation in the mitochondrion may lead to the alkylation of mitochondrial DNA or mitochondrial membrane components. Consequently, the alkylating species interacts with other molecules or macromolecules to produce less cytotoxic events, minimizing its diffusion into the nucleus to target DNA.

Previous studies have demonstrated a correlation between cell survival and MC-DNA cross-links, considered to be the primary cytotoxic lesion (37). In the present study, total MC-DNA adducts, monoalkylations and cross-links, were measured using radiolabeled [3H]MC. Inconsistent with the MC survival assays under aerobic conditions, no measurable differences in total MC-DNA adducts were found for the nuclear FpD transfectant relative to the other cell lines. It is likely that the identical number of MC-DNA adducts produced in parental cells, CHO-FpD-5 cells, and CHO-NLS-FpD-3 cells under aerobic conditions is a reflection of the degree of activation by endogenous NAPD(P)H dehydrogenase, a two-electron reducing system not susceptible to the redox cycling that occurs in air with the one-electron generating reducing systems NADPH:cytochrome P-450 oxidoreductase and FpD. Because DNA alkylations by MC are rare events, small differences in the number of total adducts may be undetectable in the radioactivity assay, yet produce an observable effect in survival assays. Alternatively, nuclear activation of MC in aerobic cells overexpressing FpD would produce increased levels of oxygen radicals in close proximity to DNA resulting in increased DNA damage that could result in increased cell kill.

The increase in total MC-DNA adducts under hypoxia in parental cells and in CHO-FpD-5 cells presumably reflects the increased generation of MC hydroquinone through one-electron reductants over that produced by NAPD(P)H dehydrogenase. The statistically significant increase in total [3H]MC-DNA adducts detected under hypoxia for the transfectant overexpressing nuclear FpD activity relative to the parental line and the transfectant overexpressing cytoplasmic membrane-bound FpD is assumed to be because of activation of MC to reactive electrophiles by nuclear FpD. The finding that a small increase in total adducts is capable of producing a significant increase in cell kill is not unreasonable given that a single cross-link per bacterial genome is sufficient to produce lethality (14) and very few cross-links are required to cause marked cell kill of mammalian cells (15). Thus, incremental changes in the level of DNA alkylations produced by MC, and in particular, the covalent MC-DNA cross-link, can have pronounced effects on the cytotoxicity of this drug.

As part of a larger investigation by our laboratory to improve the efficacy of the anticancer agent MC, this study has examined the role of the intracellular location of NADH:cytochrome b5 oxidoreductase on the bioactivation of the prodrug MC. The results of these experiments indicate that the efficacy of MC is increased significantly when bioactivation by FpD occurs in the nucleus, in close proximity to nuclear DNA. Thus, a relatively minor 3-fold increase in FpD activity in the nucleus leads to a marked increase in drug sensitivity and in total MC-DNA adducts under hypoxia. These findings, which have been determined in CHO cells, suggest that modest changes in the bioactivation of MC can significantly increase the cytotoxicity of this agent in tumor cells. Thus, FpD may play an important role in the bioactivation of MC if a method is developed to increase the expression of the enzyme in the nucleus of tumor cells.
Nuclear Localization of FpD and MC Cytotoxicity

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