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Nuclear Localization of NADPH:Cytochrome *c* (P450) Reductase Enhances the Cytotoxicity of Mitomycin C to Chinese Hamster Ovary Cells

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ABSTRACT

Overexpression of endoplasmic reticulum-localized NADPH: cytochrome *c* (P450) reductase (NPR) in Chinese hamster ovary cells increases the hypoxic/aerobic differential toxicity of the mitomycins. Because considerable evidence indicates that DNA cross-links are the major cytotoxic lesions generated by the mitomycins, we proposed that bioactivation of the mitomycins in the nucleus close to the DNA target would influence the cytotoxicity of these drugs. The simian virus 40 large T antigen nuclear localization signal was fused to the amino-terminal end of a human NPR protein that lacked its membrane anchor sequence. Immunofluorescent imaging of transfected cell lines expressing the fusion protein confirmed the nuclear location of the enzyme. Regardless of the oxygenation state of the cell, mitomycin C (MC) cytotoxicity was enhanced in cells with overexpressed NPR localized to the nuclear compartment compared with cells overexpressing an endoplasmic reticulum localized enzyme. Enhanced cytotoxicity in cells treated under hypoxic conditions correlated with increases in genomic DNA alkylations, with more MC-DNA adducts being formed when the enzyme was expressed closer to its DNA target. No change was observed in the hypoxic/aerobic differential toxicity as a function of enzyme localization. These findings indicate that drug efficacy is increased when the subcellular site of drug activation corresponds to its site of action.

Bioreduction of the anticancer prodrug mitomycin C (MC) by cellular enzymes results in the generation of highly reactive alkylating intermediates that are capable of cross-linking genomic DNA and causing cytotoxicity (Sartorelli et al., 1993, 1994; Tomasz, 1995). A key feature of the clinically used mitomycins is their ability to exploit the unique hypoxic, acidic, and nutritionally deprived environments within poorly vascularized solid tumors, where they are preferentially activated to cytotoxic species (Rockwell and Sartorelli, 1990). Hypoxic cells within solid tumors are resistant to ionizing radiation, slowly cycling or quiescent, located in poorly perfused regions that drugs may not reach, and subjected to an environment that enhances the selection of mutations that cause the progression of the neoplasm toward an increasingly aggressive phenotype (for reviews, see Rockwell and Knisely, 1997; Belcourt et al., 1998a). Therefore, the hypoxic cellular population is also refractory to many chemotherapeutic regimens (Sartorelli, 1988). The ability of MC to undergo bioactivation in hypoxic regions of solid tumors, and thereby preferentially target hypoxic malignant cells, has led to its clinical evaluation as an adjuvant to radiation therapy, where it has shown improved cause-specific survival in both head and neck cancer (Haffty et al., 1997) and cervical cancer (Roberts et al., 2000) patients.

Enzymes capable of bioreductively activating the mitomycins include NADPH:cytochrome *c* (P450) reductase (NPR; EC 1.6.2.4), NAD(P)H:quinone oxidoreductase (NQO1; DT-diaphorase; EC 1.6.99.2), and NADH:cytochrome b_5 reductase (NBR; EC 1.6.2.2) (for reviews, see Sartorelli et al., 1994; Belcourt et al., 1998a). NPR activates the mitomycins through a one-electron reductive mechanism, producing a very short-lived semiquinone anion radical intermediate with a half-life in the microsecond range in the presence of

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ABBREVIATIONS: MC, mitomycin C; NPR, NADPH:cytochrome *c* (P450) reductase; NQO1, NAD(P)H:quinone oxidoreductase; NBR, NADH: cytochrome b_5 reductase; CHO, Chinese hamster ovary; SV40, simian virus 40; NLS, nuclear localization signal; PCR, polymerase chain reaction.

oxygen (Kalyanaraman et al., 1980; Halliwell and Gutteridge, 1989). The semiquinone anion radical intermediate is susceptible to redox cycling, reacting with oxygen to regenerate the parent molecule. Thus, treatment of NPR-transfected CHO cells overproducing NPR with the mitomycins results in an oxygen-sensitive differential toxicity to these drugs, with hypoxic cells being more sensitive than oxygenated cells (Belcourt et al., 1996b).

NQO1 reduction of the mitomycins proceeds by a two-electron reductive mechanism, producing the longer lived hydroquinone intermediate with a half-life of approximately 15 s (Penketh et al., 2001). CHO cells overproducing this enzyme are sensitized to MC regardless of the degree of oxygenation, indicating that this intermediate is not subject to redox cycling (Belcourt et al., 1996a; Seow et al., 2004a,b).

NBR activates the mitomycins through a one-electron reductive pathway (Hodnick and Sartorelli, 1993) and, when overproduced as a cytoplasmic protein in CHO cells, sensitizes cells differentially to the mitomycins, with greater kill of hypoxic cells than aerobic cells (Belcourt et al., 1998b; Holtz et al., 2003). MC metabolism by the membrane-bound and soluble forms of this enzyme proceeds with similar kinetics (Belcourt et al., 1998b). These findings are consistent with the production of the semiquinone anion radical by NBR and the subsequent redox cycling of this intermediate in the presence of oxygen. NPR resides in the endoplasmic reticulum, whereas NBR is known to localize predominantly in the mitochondrial outer membrane (Pietrini et al., 1992) and NQO1 is located in the cytosol (Seow et al., 2004a). The locations of major MC-activating enzymes suggest that activation of this antineoplastic agent in a cellular region distal to its presumed cytotoxic target, nuclear DNA, may not result in optimal cell killing. We have shown that nuclear overexpression of NBR (Holtz et al., 2003) or NQO1 (Seow et al., 2004b) close to its DNA target results in enhanced cell kill. The present report provides comparable findings for NPR, arguably the most important of the one-electron MC-activating enzymes, by demonstrating that localization of NPR-overexpressed activity to the nucleus of CHO cells enhances MC sensitivity relative to cells expressing a similar overexpressed level of this enzyme activity in its normal subcellular location, the endoplasmic reticulum.

Materials and Methods

Materials. MC was contributed by the Bristol-Myers Squibb Co. (Wallingford, CT). NADH, NADPH, NAD^+, chloroquine, rotenone, and HEPES were purchased from Sigma-Aldrich (St. Louis, MO). Glutamine, hypoxanthine, thymidine, G418 (Geneticin), trypsin, penicillin, and streptomycin were purchased from Invitrogen (Carlsbad, CA). Tissue culture flasks and 60-, 100-, and 150-mm tissue culture dishes were acquired from Costar (Cambridge, MA). Dicumarol, potassium ferricyanide, and ethanol were obtained from Aldrich Chemical Co. (Milwaukee, WI). 2-Mercaptoethanol was from Bio-Rad (Hercules, CA). $Na_2HPO_4 \cdot 2H_2O$, dextrose, $CaCl_2$, glycerol, Tris, EDTA, KH_2PO_4, KCl, and NaCl were obtained from J. T. Baker (Phillipsburg, NJ).

Cell Culture. The cell line used in this study is a variant of the CHO-K1 cell line termed CHO-K1/dhfr$^-$ and was obtained from the American Type Culture Collection (Manassas, VA). This cell line is deficient in dihydrofolate reductase. The cells were maintained in Iscove's modified Dulbecco's medium (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone Laboratories, Logan, UT), 2 mM glutamine, 0.1 mM hypoxanthine, 0.01 mM thymidine, and antibiotics (100 units/ml penicillin, 100 μg/ml streptomycin). Transfected lines were maintained in the identical medium supplemented with 1 mg/ml G418 to provide for selection of the expression vector. Cells were grown as monolayers in tissue culture flasks, petri dishes, or glass milk dilution bottles at 37°C under an atmosphere of 95% air, 5% CO_2 in a humidified incubator. The doubling time of CHO-K1/dhfr$^-$ cultures is 19 h.

Plasmid Constructions. To generate a chimeric protein capable of localizing NPR to the nucleus, sequences encompassing the SV40 large T antigen nuclear localization signal (NLS) (Kalderon et al., 1984; Vancurova et al., 1994) were fused to a truncated human *NPR* gene (Yamano et al., 1989) lacking its membrane anchor sequences using PCR methodologies with the following oligonucleotides: 5′ oligonucleotide, 5′-CGCGGTACC*AAGCTT*GCCACC**ATG**GATAAAGTTTTAAACAGAGAGGAATCTTCTAGTGATGATGAGGCTACTGCTGACTCTCAACATTCTACTCCTCCAAAAAAGAAGAGAAAGGTAGAAGACCCCGCTAGCGTCCCCGAGTTCACCAAAATTCAG-3′; 3′ oligonucleotide, 5′-CGCGGATCC*TCTAGA*CTAAATACTTGGCCCTGCTTCATCA TATTCTTGTTTGGATATCCACATCCCGCTCCACACGTCCAGGGAGTAGCG-3′.

The 5′ oligonucleotide encodes a HindIII restriction site (italicized), the translational initiation codon (bold), the SV40 sequences encompassing the nuclear localization signals (underlined), and sequences complementary to the NPR gene beginning at nucleotide 151 of the coding sequence (double underlined). The 3′ oligonucleotide encodes an XbaI site (italicized) and a 15-amino acid region encoding the muscle actin epitope (underlined) (McHugh and Lessard, 1988), recognized by the HUC1-1 antibody (MP Biomedicals, Irvine, CA) (Montecucco et al., 1995), fused in frame to sequences complementary to the carboxy-terminal end of the *NPR* gene (double underlined). An unaltered *NPR* gene, except for a fusion of the 15-amino acid muscle actin epitope to the final NPR amino acid, was generated by PCR using the 3′ oligonucleotide depicted above and the following 5′ oligonucleotide: 5′-CGCGGATCC*AAGCTT*GGTACCTGGCCACC**ATG**GGAGACTCCCACGTGGA-3′. The 5′ oligonucleotide encodes a HindIII restriction site (italicized), the translational initiation codon (bolded), and sequences complementary to the *NPR* gene beginning at nucleotide 1 of the coding sequence (double underlined).

PCR was performed using these oligonucleotides and a cDNA encoding the full-length *NPR* gene. The amplified PCR product was extracted with phenol/chloroform, precipitated with 2.5 volumes of ethanol, and resuspended in 50 μl of Tris-EDTA (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). After digestion of 5 μl of the amplified cDNA with HindIII and XbaI (Roche Diagnostics, Indianapolis, IN), the fragment was subcloned into the eukaryotic expression plasmid pRC/CMV (Invitrogen), and recombinants were screened by restriction analysis. The plasmid, designated pRC/CMV-NLS-NPR, contains the promoter sequences from the immediate early gene of the human cytomegalovirus and the appropriate sequences for polyadenylation and selection (neomycin resistance); it inserts stably into the genome of transfected cell lines.

Transfections. Transfections were performed by the $Ca_3(PO_4)_2$-DNA coprecipitation method essentially as described by Sambrook et al. (1989) and modified by Belcourt et al. (1998b). Single colonies were introduced into wells of a 24-well plate using sterile cotton-tipped applicators (General Medical Corp., Richmond, VA). After expansion, the isolates were screened for expression of the NPR cDNA gene. Isolates having elevated enzyme activity were cloned by flow cytometric single cell sorting, and the resulting clones were rescreened. Vector-transfected control clones were CHO-K1/dhfr$^-$ cells transfected with the plasmid without a cDNA insert.

Assays of Enzyme Activity. Exponentially growing cells (approximately 5×10^6 total cells) were harvested by trypsinization, washed in cell culture medium containing 10% fetal bovine serum to inactivate the trypsin, washed with phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4, pH 7.4), and then resuspended in 2 ml of the same buffer. Cells were disrupted by sonication using a Branson sonicator (Branson Ultrasonics Corp., Danbury, CT) with three 15-s bursts at a setting of 25 with 1 min of cooling on ice between each sonication burst. Cell disruption was confirmed microscopically. NQO1 activity was measured as the dicumarol-inhibitable reduction of dichlorophenol indophenol, measured at 600 nm with a Beckman model 25 UV/Vis spectrophotometer (Beckman Coulter, Fullerton, CA) using an extinction coefficient of 21 mM^{-1} cm^{-1} at 30°C (Ernster, 1967). The final concentration of dicumarol was 100 μM. NPR activity was assayed in cell extracts by monitoring the rate of ferricytochrome *c* reduction at 550 nm (extinction coefficient of 21 mM^{-1} cm^{-1}) at 30°C (Yasukochi and Masters, 1976). NBR activity was measured as NADH:ferricyanide reductase at 420 nm (extinction coefficient of 1.02 mM^{-1} cm^{-1}) at 30°C essentially as described by Yubisui and Takeshita (1982) but using a final concentration of 0.34 mM NADH. Protein concentrations were assayed using the bicinchoninic protein assay reagent (Pierce Chemical, Rockford, IL) (Smith et al., 1985).

Aerobic/Hypoxic Experiments. Exponentially growing monolayers of CHO-K1/dhfr$^-$, CHO-NLS-NPR-9A, CHO-NPR-8A, and CHO-NPR-16 cells were seeded in glass milk dilution bottles at 2×10^5 cells per bottle and were used in mid-exponential phase (approximately 3 to 4 days of growth). Hypoxia was induced by gassing the cultures with a humidified mixture of 95% N_2, 5% CO_2 (<10 ppm O_2) at 37°C for 2 h through a rubber septum fitted with 13-gauge (inflow) and 18-gauge (outflow) needles. After induction of hypoxia, cells were exposed to 2.5, 5, 7.5, and 10 μM MC for 1 h; drugs were injected through the septum without compromising the hypoxia. Cells under aerobic conditions were treated with MC in an identical manner for 1 h in a humidified atmosphere of 95% air, 5% CO_2 at 37°C. Treated cells were washed, harvested by trypsinization, and assayed for survival by measuring their ability to form macroscopic colonies (Rockwell, 1977). Both aerobic and hypoxic vehicle controls (70% ethanol) were included in each experiment; the surviving fractions were normalized using these vehicle controls. The plating efficiencies (colonies/100 plated cells; means ± standard deviations) for CHO-K1/dhfr$^-$, CHO-NPR-16, CHO-NPR-8A, and CHO-NLS-NPR-9A cells were 85 ± 5, 74 ± 5, 84 ± 1, and 77 ± 3, respectively. The surviving fractions for the aerobic vehicle-treated controls (means ± standard deviations) were 1.07 ± 0.09, 1.01 ± 0.03, 1.02 ± 0.04, and 0.95 ± 0.05, whereas the surviving fractions for the hypoxic vehicle-treated controls were somewhat lower, reflecting the toxic effects of the hypoxia: 0.58 ± 0.05, 0.55 ± 0.03, 0.54 ± 0.04, and 0.45 ± 0.02 for CHO-K1/dhfr$^-$, CHO-NPR-16, CHO-NPR-8A, and CHO-NLS-NPR-9A cells, respectively.

Immunofluorescence Microscopy. Cells were seeded on poly-D-lysine-coated glass slides and cultured for 2 days as described above. After a 15-min fixation in 20% formaldehyde, the cells were permeabilized with cold acetone for 5 min and then incubated with a 1:128 dilution of an anti-muscle actin monoclonal antibody (HUC1-1; MP Biomedicals), washed, and then incubated with a fluorescein isothiocyanate-conjugated anti-mouse IgG (Sigma-Aldrich) diluted to 1:128. Cells were counterstained using 10 μg/ml Hoechst 33342 (Molecular Probes, Eugene OR). Cells were visualized and photographed at 40× magnification using a Zeiss Axioskop fluorescence microscope (Carl Zeiss, Jena, Germany) equipped with appropriate excitation/emission filters and a SPOT charge-coupled device camera (Diagnostic Instruments, Sterling Heights, MI).

Total [^{3}H]MC-DNA Adducts. Cells in suspension (1×10^7 cells/ml) were treated with 10 μM [^{3}H]MC (0.18 mCi/mmol; donated by Kyowa Hakkao Kogyo Co, Tokyo, Japan) for 2 h under aerobic or hypoxic conditions that were established by continuously gassing the cells with either 95% air, 5% CO_2 or 95% N_2, 5% CO_2, respectively, starting 2 h before drug exposure. Genomic DNA was isolated from 1×10^7 cells using the PURGENE DNA purification system (Gentra Systems, Inc., Minneapolis, MN) as described by the manufacturer and reported previously (Holtz et al., 2003; Seow et al., 2004b). In brief, cells were lysed and treated with 100 mg/ml proteinase K overnight followed by 20 mg/ml RNase A for 2 h at 37°C. Isolated DNA was washed two times with 70% ethanol to remove noncovalently bound [^{3}H]MC, and the DNA was resuspended in 10 mM Tris-HCl, 1 mM EDTA, pH 7.0. DNA samples with A_{260}/A_{280} ratios of 1.8 to 1.9 were isolated by this methodology. An aliquot was used to quantify the number of [^{3}H]MC-DNA adducts using a Beckman scintillation spectrometer, and DNA concentration was determined spectrophotometrically at A_{260}. Radioactivity in the sample was normalized to the total DNA concentration.

Results

CHO cells were transfected with plasmids encoding a wild-type NPR cDNA that produced a protein localized to its normal subcellular compartment, the endoplasmic reticulum (CHO-NPR-16), an NPR cDNA with a 15-amino acid muscle actin epitope carboxyl-terminal fusion (CHO-NPR-8A), or a truncated NPR cDNA with the membrane anchor sequence deleted and the region of the SV40 large T antigen sequence, which localizes this protein to the nucleus, added (CHO-NLS-NPR-9A). This latter construct also contained a carboxyl-terminal fusion to the 15-amino acid muscle actin epitope to allow visualization of the NPR protein by immunofluorescence. Cells transfected with these constructs were cloned and screened for NPR enzymatic activity. Three clones with relatively similar levels of NPR enzyme activity, CHO-NLS-NPR-9A, CHO-NPR-8A, and CHO-NPR-16, overexpressing NPR by 9-, 8-, and 16-fold, respectively, were changed only in the levels of expression of NPR specific activity, with no significant change in two other enzyme systems important for MC bioreduction in these cell lines (i.e., NQO1 and NBR), compared with parental cells (Table 1). CHO-NPR-8A is included to control for any effects on enzyme-specific activity of the carboxyl-terminal fusion of the 15-amino acid actin epitope.

The subcellular location of the NPR enzyme in cells transfected with the NLS fusion protein was examined by indirect immunofluorescence microscopy and confirmed by colocalization with DNA stained with the Hoechst 33342 dye in the same cells. After incubation with the HUC1-1 monoclonal antibody specific for the 15-amino acid muscle actin epitope of the fusion protein, CHO-NLS-NPR-9A cells exhibited highly fluorescent nuclear staining (Fig. 1A) that colocalized with the nuclear DNA staining identified by the Hoechst 33342 dye (Fig. 1C), indicating the successful localization of this enzyme to the nuclear compartment. Cells of the CHO-NPR-16A transfectant that expressed a form of NPR that does not partition into the nucleus exhibited primarily cyto-

TABLE 1

Oxidoreductase activities of parental and NPR-transfected cell lines with the enzyme localized to either the nucleus or the endoplasmic reticulum

All enzyme activities were determined by standard assays as described under *Materials and Methods*. Values are means ± S.E. of three to seven determinations. There were no significant differences between the values for parental and transfected cell lines, except for those comparisons marked as [a] (paired Student's *t* test; $P > 0.05$). No detectable xanthine oxidase or xanthine dehydrogenase activity was observed in any cell line.

Enzyme	Activity			
	CHO-K1/dhfr$^-$	CHO-NLS-NPR-9A	CHO-NPR-16	CHO-NPR-8A
	nmol/min/mg protein			
NPR	9 ± 1	84 ± 18[a]	143 ± 21[a]	75 ± 23[a]
NBR	1930 ± 180	1620 ± 58	1700 ± 7	2570 ± 500
NQO1	10 ± 3	9 ± 1	26 ± 10	24 ± 7

[a] Value significantly different from parental CHO-K1/dhfr$^-$, $P < 0.05$.

solic staining, as shown in Fig. 1B, and did not colocalize with the nuclear DNA observed in these cells counterstained with Hoechst 33342 dye (Fig. 1D). In CHO-K1/dhfr$^-$ parental cells incubated with the HUC1-1 monoclonal antibody (Fig. 1C), no fluorescence was observed, demonstrating that the antibody was specific for the epitope. In these cells, fluorescence was observed when visualized using the Hoechst filter set that served as a control (Fig. 1E). We have shown previously that the wild-type NPR enzyme, containing the amino terminal membrane anchor sequence (retained in CHO-NPR-16 and CHO-NPR-8A), localizes to the microsomal fraction of CHO-K1/dhfr$^-$ cells (Belcourt et al., 1996b).

Using a clonogenic assay, the cytotoxicity of MC to the parental cell line and the three NPR-overexpressing cell lines was measured under both aerobic and hypoxic conditions. Parental cell sensitivity to MC is shown in Fig. 2A, which demonstrates slightly greater cell kill under hypoxic conditions; these results are in agreement with our findings in a previous report (Belcourt et al., 1998b). Elevation of the wild-type NPR enzyme activity by 16-fold resulted in the CHO-NPR-16 cell line becoming markedly more sensitive to MC, with greater increases in cytotoxicity occurring under hypoxia than under aerobic conditions (Fig. 2A). This result is consistent with previous studies (Belcourt et al., 1996b) and presumably indicates the increased generation of the semiquinone anion radical intermediate of MC by the NPR enzyme, with redox cycling and consequent inactivation of most of this intermediate under aerobic, but not hypoxic, conditions. The control cell line CHO-NPR-8A, containing an NPR cDNA clone modified only through the addition of a carboxyl-terminal fusion of the 15-amino acid muscle actin epitope to the NPR enzyme, yielded an increase in MC sensitivity under hypoxia comparable with CHO-NPR-16, with no increase in the cytotoxicity under aerobic conditions over that produced in CHO-NPR-16 cells (Fig. 2B).

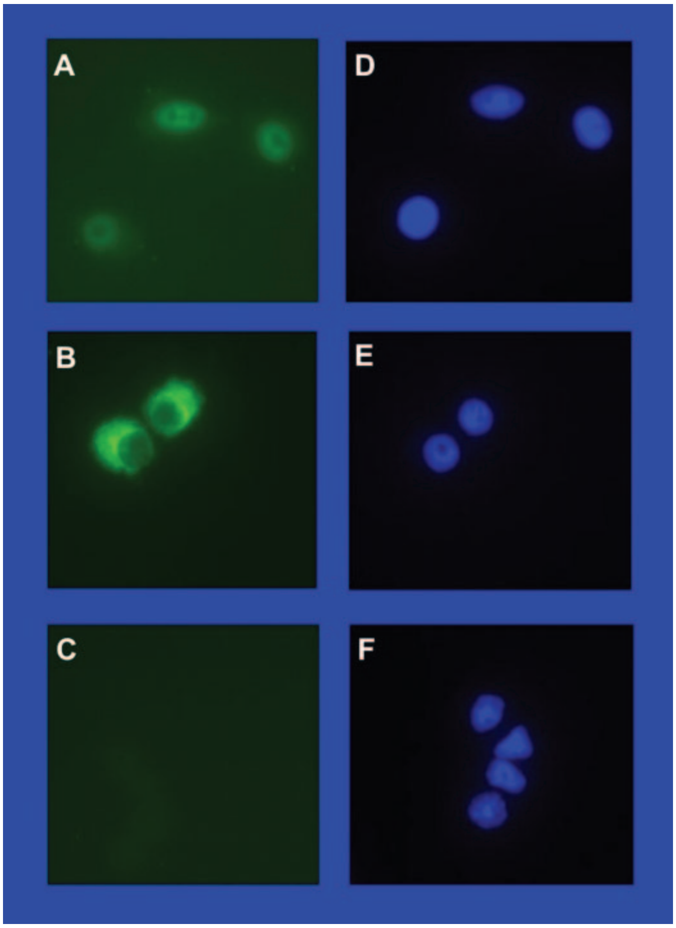

Fig. 1. Immunofluorescence microscopy of CHO-NLS-NPR-9A (A and D), CHO-NPR-16A (B and E), and parental CHO-K1/dhfr$^-$ (C and F) cells expressing NPR. Cells were grown as described under *Materials and Methods*, stained with the HUC1-1 antibody specific for the muscle actin epitope, counterstained for DNA using Hoechst 33342 dye, and subsequently photographed using a fluorescence microscope. Cellular fluorescence associated with the recognition of the actin epitope tag was observed using a fluorescein isothiocyanate filter set and are shown in A to C. Identical cells visualized using a Hoechst filter set are shown in D and E.

Nuclear localization of the NPR enzyme resulted in even greater sensitivity of CHO cells to MC, compared with the endoplasmic reticulum-localized enzyme, regardless of the oxygenation state of the cells (Fig. 2C). No change was observed, however, in the aerobic/hypoxic differential toxicity as a function of enzyme localization. Despite expressing only 60% of the amount of NPR enzyme activity present in CHO-NPR-16 cells (Table 1), the CHO-NLS-NPR-9A cells were at least 18- and 25-fold more sensitive to MC at 7.5 μM under aerobic and hypoxic conditions, respectively (Fig. 2C). Compared with parental cells treated with 10 μM MC, the CHO-NLS-NPR-9A cell line exposed to 7.5 μM drug was 29- and 840-fold more sensitive under aerobic and hypoxic conditions, respectively. A cell line that spontaneously reverted from an overexpressed NLS-localized NPR enzyme to the parental level and distribution of NPR activity resulted in a loss of hypersensitivity to MC (data not shown).

By treating cells with 10 μM [^{3}H] MC for 2 h under hypoxic conditions, striking increases in the number of genomic DNA alkylations were observed (Table 2). Thus, in cells that overexpressed NPR activity in the endoplasmic reticulum, the number of MC-DNA adducts increased by 35% over that of wild-type parental cells. Overexpression of NPR activity by 9- and 13-fold in the nucleus produced increases in MC-DNA adducts of 54 and 116%, respectively, in hypoxia over that occurring in parental cells exposed to [^{3}H]MC under identical conditions. Furthermore, nuclear overexpression of NPR activity by 9- and 13-fold under hypoxia produced increases in MC-DNA adducts of 14 and 87%, respectively, over that occurring in cells overexpressing NPR activity by 16-fold in the endoplasmic reticulum. In contrast, overexpression of NPR activity in aerobic cells in either the endoplasmic reticulum or the nucleus did not produce significant increases in the number of MC-DNA adducts over that of parental cells, reflecting the fact that NPR activity does not participate in the activation of MC under aerobic conditions because of redox cycling. CHO parental and the CHO-NPR-16, CHO-NLS-NPR-9A, and CHO-NLS-NPR-13A transfectants exposed to MC under conditions of hypoxia exhibited increases of 53, 94, 200, and 330% in the production of MC-DNA adducts over corresponding aerobic cells, reflecting preferential alkylation of DNA by [^{3}H]MC under hypoxic conditions.

Discussion

The bioreduction of MC can occur through either a one- or a two-electron reduction mechanism, producing intermediates with markedly different reaction kinetics. The product of the two-electron reduction of MC, the hydroquinone intermediate, has a half-life of approximately 10 s in aqueous buffer at physiological temperature and pH (Penketh et al., 2001). Moreover, relative to the one-electron reduction intermediate of MC, the semiquinone anion radical, the hydroquinone is not subject to reoxidation by molecular oxygen (Ross et al., 1994), and thus is a highly reactive electrophile that is probably the major precursor of the MC-DNA cross-links in living cells (Butler et al., 1985). Considering the 15-s half-life of the hydroquinone, its subcellular distribution should not be diffusion limited. On the other hand, the semiquinone anion radical intermediate, produced by NPR, reacts with oxygen at a rate comparable with diffusion-controlled reactions, regenerating the nontoxic parental compound (Kalyanaraman et al., 1980; Halliwell and Gutteridge, 1989). In the presence of oxygen, the half-life of the semiquinone intermediate is estimated to be approximately 2×10^{-8} s (Butler et al., 1985). Thus, in the presence of oxygen, the semiquinone anion radical is essentially innocuous. However, under reducing conditions, the generation of the semiquinone anion radical does result in a highly reactive alkylating species capable of cross-linking DNA (Cera et al., 1989). Some studies suggest that the mitosenyl semiquinone anion radical is a better alkylating and cross-linking agent than is the mitosenyl hydroquinone (Egbertson and Danishefsky, 1987), although more compelling evidence implies that disproportionation of the semiquinone anion radical to the hydroquinone intermediate must occur for the activation cascade to proceed to the formation of DNA cross-links (Hoey et al., 1988; Machtalere et al., 1988; Suresh Kumar et al., 1997). Given the potential importance of NPR in the activation of MC, coupled with the greater reactivity and shorter half-life of the semiquinone anion radical, we sought to explore whether the subcellular site of MC bioreduction by NPR, which generates the semiquinone anion radical through a one-electron reduction of MC, could affect the sensitivity of CHO cells to this drug. Previous studies by this laboratory have shown that nuclear localization of the one-electron reducing system NBR (Holtz et al., 2003) and the two-electron reducing enzyme NQO1 (Seow et al., 2004b) markedly increased the cytotoxicity of MC.

CHO cell clones were developed that overexpressed NPR activity from cDNA constructs encoding either an endoplasmic reticulum (overexpression of NPR by 16-fold for CHO-NPR-16 and 8-fold for CHO-NPR-8A) or nuclear (overexpression of NPR by 9-fold for CHO-NLS-NPR-9A and 13-fold for CHO-NLS-NPR-13A) localized form of NPR. In each of the transfected clones, the levels of other known oxidoreductases present in these cells, capable of bioreductively activating MC, were similar to those of the parental cell line. Immunofluorescence microscopy demonstrated that in CHO-NLS-NPR-9A cells the chimeric NPR protein localized to the nucleus. Subcellular fractionation studies with wild-type NPR established that this protein colocalizes with the cellular microsomal fraction (Belcourt et al., 1996b).

Compared with parental cells, elevating the expression of the unmodified, endoplasmic reticulum-localized human NPR protein in CHO-NPR-16 caused significant increases in cell sensitivity to MC under both aerobic and hypoxic conditions, with a markedly greater increase in cell kill occurring under hypoxia. This resulted in an increase in the hypoxic/aerobic differential toxicity of MC, as we observed previously in NPR-transfected cells (Belcourt et al., 1996b). A similar result was observed with CHO-NPR-8A cells, indicating that the fusion of the 15-amino acid actin epitope to the carboxy terminus of NPR did not adversely affect the ability of this enzyme to reduce MC. Because the NPR-catalyzed one-electron reduction product of MC, the semiquinone anion radical, is known to be highly reactive and short-lived, we reasoned that activation of MC close to the site of its presumed target for its cytotoxic action, nuclear DNA, might increase the efficacy of the drug. Numerous studies have demonstrated the ability of the mitomycins to cross-link complementary DNA strands at the two-amino function of guanine residues in the sequence 5′-CpG-3′ (for review, see Tomasz, 1995). These lesions are so lethal that a single genomic DNA interstrand cross-link will kill susceptible bacterial cells (Szybalski and Iyer, 1964). In mammalian cells, the degree of cytotoxicity of several mitomycins, including MC, correlates with the number of DNA cross-links formed, supporting the concept that the critical lesion responsible for the cytotoxicity of the mitomycins is DNA cross-linking (Keyes et al., 1991). Moreover, a panel of DNA repair-deficient CHO cells has

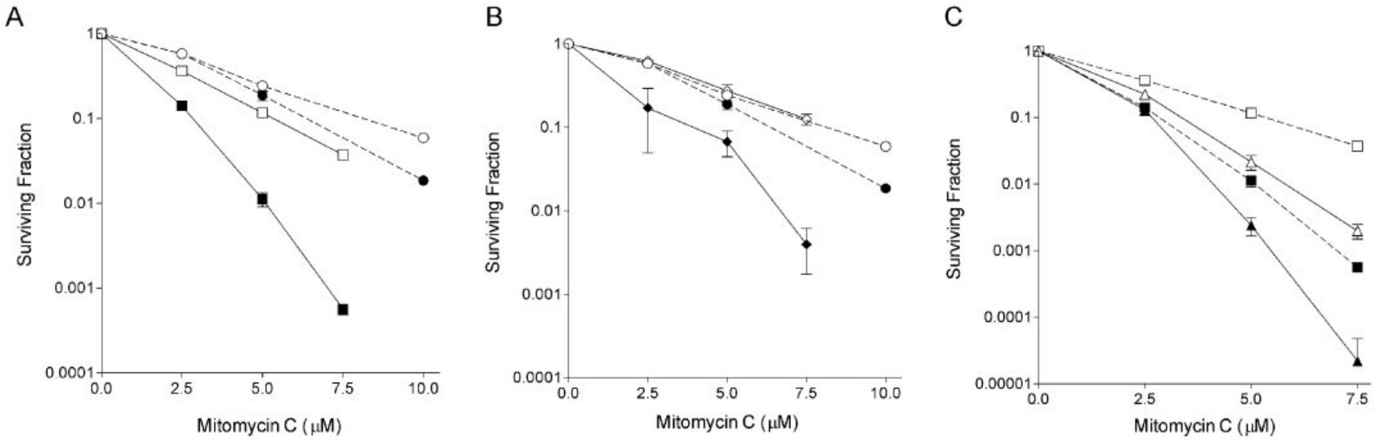

Fig. 2. Survival curves for CHO-K1/dhfr⁻, CHO-NPR-16, CHO-NPR-8A, and CHO-NLS-NPR-9A cells treated for 1 h with graded concentrations of mitomycin C under aerobic (open symbols) or hypoxic (filled symbols) conditions. A, comparison of CHO-K1/dhfr⁻ parental cells (○, ●) and CHO-NPR-16 cells (□, ■). B, comparison of CHO-K1/dhfr⁻ parental cells (○, ●) and CHO-NPR-8A cells (◇, ◆). C, comparison of CHO-NPR-16 cells (□, ■; from A) and CHO-NLS-NPR-9A cells (△, ▲). Surviving fractions were calculated using the plating efficiencies of the aerobic and hypoxic vehicle-treated controls. Points are geometric means of three to seven determinations; S.E. are shown where larger than the points.

been used to genetically implicate mitomycin-induced DNA cross-links as a major cytotoxic lesion (Hughes et al., 1991).

To test the hypothesis that MC cytotoxicity is increased when the subcellular site of drug activation is close to its site of action, the cell line CHO-NLS-NPR-9A and CHO-NLS-NPR-13A, overexpressing the nuclear-localized form of NPR activity, was tested for changes in sensitivity to MC relative to a cell line, CHO-NPR-16, overexpressing an endoplasmic reticulum-localized form of the enzyme. Despite expressing only 56 and 80% of the level of NPR activity as CHO-NPR-16 cells, CHO-NLS-NPR-9A and CHO-NLS-NPR-13A cells, respectively, were markedly more sensitive to MC than CHO-NPR-16 under both aerobic and hypoxic conditions; the hypoxic/aerobic differential was similar in both cell lines. A considerable increase in cytotoxicity is apparent under aerobic conditions, notwithstanding the increased generation of the redox-sensitive semiquinone anion radical. The generation of the semiquinone anion radical in the nucleus possibly allows it to react with nuclear DNA before reacting essentially completely with oxygen and recycling back to parental MC. However, recent evidence suggests that the biological effects of the semiquinone anion radical may be caused by its disproportion to the hydroquinone (Suresh Kumar et al., 1997). It is therefore more likely that the increased intracellular concentration of the semiquinone anion radical allows greater disproportionation to the hydroquinone intermediate under aerobic conditions, resulting in increased cytotoxicity. Moreover, the activation of MC in the cytoplasm has been suggested to result in the alkylation of nucleophiles other than nuclear DNA, with the resulting loss of active drug, as the reactive hydroquinone electrophile migrates toward its nuclear DNA target (Seow et al., 2004b). In addition, the proximity of the hydroquinone intermediate to nuclear DNA has been suggested to be important for mitomycin toxicity because this prevents the shunting of the hydroquinone into an alternative, noncytotoxic pathway, culminating in the formation of 2,7-diaminomitosene (Palom et al., 1998). Previous results with NBR (Holtz et al., 2003) and NQO1 (Seow et al., 2004b) and the present findings with NPR clearly indicate that the subcellular site of MC bioactivation can influence the cytotoxicity of this antibiotic.

TABLE 2

Quantity of [^{3}H]MC-DNA adducts formed by parental and NPR-transfected cell lines with the enzyme localized to either the nucleus or the endoplasmic reticulum treated under aerobic and hypoxic conditions

[^{3}H]MC-DNA was isolated from cells treated with 10 μM [^{3}H]MC for 2 h as described under *Materials and Methods*. Values shown are the means of three to four determinations ± S.E. Significant differences between groups were determined using the paired Student's *t* test. There were no significant differences between the values for any cell line treated under aerobic conditions. Significant differences are found for comparisons marked a to e.

	[^{3}H]MC-DNA adducts	
	Aerobic	Hypoxic
	cpm/μg of DNA	
CHO Parental	0.37 ± 0.01	0.78 ± 0.01^{a}
CHO-NPR-16	0.40 ± 0.04	1.05 ± 0.01^{a,b}
CHO-NLS-NPR-9A	0.40 ± 0.02	1.20 ± 0.03^{a,c,d}
CHO-NLS-NPR-13A	0.39 ± 0.01	1.69 ± 0.10^{a,d,e}

[a] Values are significantly different from aerobic counterparts, $P < 0.0001$.
[b] Values are significantly different from the parental cells, $P = 0.0001$.
[c] Values are significantly different from the parental cells, $P < 0.001$.
[d] Values are significantly different from ER-NPR, $P < 0.05$.
[e] Values are significantly different from the parental cells, $P < 0.02$.

Because reduction of MC by NPR and NBR generates the one-electron reduction intermediate, the semiquinone anion radical, we hypothesized that agents that generate short-lived, highly reactive alkylating intermediates may require activation close to their targets to be maximally effective. Drug activation in the cytosol by NPR and NBR allows the reduced mitomycin intermediates to generate cytotoxicity under hypoxic conditions, presumably because they can now reach and cross-link nuclear DNA before spontaneously rearranging and reacting with other macromolecules or water. Likewise, the nuclear localization of NPR enhances the cytotoxicity of MC relative to that of wild-type, endoplasmic reticulum localized NPR, suggesting that, at the subcellular level, proximity to the DNA target is important for the degree of cell kill by the semiquinone anion radical and hydroquinone intermediates.

An important clinical implication from this work is that, depending upon the nature of the therapeutic agent, the subcellular location of an activating protein delivered by gene therapy methodologies should be considered when designing the constructs. Drug activation at the wrong subcellular location could lead to resistance to the therapeutic agent of interest. Likewise, it may be possible to exploit this concept to overcome natural or acquired cellular resistance to an agent by relocating an activating enzyme to a more appropriate subcellular site.

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