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Radiosensitization of Hypoxic Tumor Cells by Depletion of Intracellular Glutathione

Abstract. *Depletion of glutathione in Chinese hamster ovary cells in vitro by diethyl maleate resulted in enhancement of the effect of x-rays on cell survival under hypoxic conditions but not under oxygenated conditions. Hypoxic EMT6 tumor cells were similarly sensitized in vivo. The action of diethyl maleate is synergistic with the effect of the electron-affinic radiosensitizer misonidazole, suggesting that the effectiveness of misonidazole in cancer radiotherapy may be improved by combining it with drugs that deplete intracellular glutathione.*

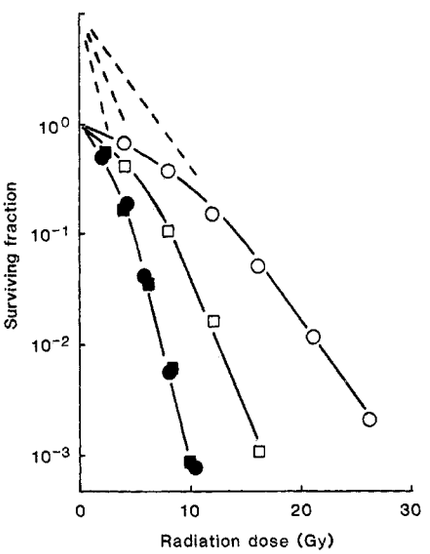
It is well known that hypoxia protects cells from the cytotoxic effects of radiation. Hypoxic cells in solid tumors are therefore considered a problem in the treatment of cancer by radiotherapy, and several methods have been tried to overcome this problem. These include giving radiation in small multiple fractions, treating patients in high-pressure oxygen chambers, using densely ionizing radiations such as neutrons or π^- mesons, and, more recently, administering electron-affinic agents that act like oxygen to enhance radiation-induced damage (*1*). One of these electron-affinic radiosensitizers, the 2-nitroimidazole misonidazole (MIS), is now undergoing extensive clinical trials (*2*).

Another approach to sensitization of hypoxic tumor cells would be to decrease their endogenous radioprotective capacity. Radiation induces the formation of free radicals on DNA either directly by ionization or indirectly by the action of radiolytic products. Within milliseconds of the initial radiolytic event, DNA damage is either restored by hydrogen donation or rendered nonrestorable by reaction with a sensitizer (*3*). Perturbation of this process would be expected to have a dose-modifying effect, that is, to change the slope of the radiation-survival curve. The nonprotein thiol glutathione has been regarded as the main endogenous reducing agent responsible for restoration of radiation-induced lesions by hydrogen donation (*4*).

We used diethyl maleate (DEM) as a reagent to deplete intracellular glutathione in order to examine the hypothesis that depletion of endogenous nonprotein thiol should sensitize hypoxic cells selectively. This possibility is strongly suggested by the observation (*4*) that cells which are genetically deficient in glutathione synthesis have a reduced differential in radiosensitivity between oxygenated and hypoxic conditions.

Diethyl maleate effectively depletes glutathione in Chinese hamster ovary (CHO) cells in vitro without cytotoxicity (no effect on cloning efficiency at $2 \times 10^{-4}M$ DEM for 8 hours at 37°C) and without significant effects on protein thiol content (*5*). When DEM-pretreated cells are irradiated, their sensitivity to radiation is increased in a dose-modifying manner (Fig. 1). The enhancement ratio (ER) of 1.7 ± 0.2—that is, the ratio of the slope of the exponential portion of the survival curve of cells treated with DEM to that of control cells—seen when glutathione is reduced to 1 percent of control levels by $2 \times 10^{-4}M$ DEM is well above that which can be achieved by a clinically tolerable dose of MIS. Oxygenated cells were not radiosensitized by DEM.

In previous work on the importance of endogenous thiols in radioresistance, other reagents such as *N*-ethylmaleimide (*6*) and diamide (*7*) were used; however, these reagents not only decrease intracellular reduced glutathione concentrations but also cause a loss of protein thiol, which could affect enzymatic repair (*5*). The data obtained with *N*-ethylmaleimide and diamide also showed a reduction in the initial shoulder region of



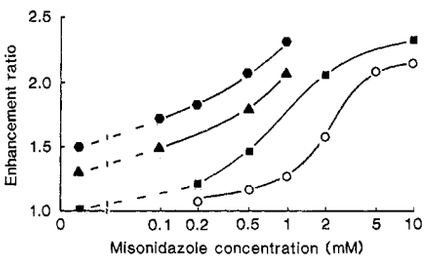


Fig. 1 (left). Effect of glutathione depletion by DEM on the radiosensitivity of CHO cells in vitro. CHO cells were grown in suspension culture (*5*) and preincubated for 1 hour at 37°C with $2 \times 10^{-4}M$ DEM under 5 percent CO_2 in N_2 at 5×10^6 cells per milliliter (□) or 5 percent CO_2 in air at 1×10^6 cells per milliliter (■) in Eagle's minimum essential medium without serum. Cells were irradiated in suspension with a 250-kV x-ray machine. Survival was determined by a clonogenic assay (*8*). The ER for DEM under hypoxia was 1.8 in the experiment shown; the ER for DEM in air (2.8) was identical to that of air alone (●). Survival of controls irradiated under 5 percent CO_2 in N_2 is indicated by open circles. Results are from a representative experiment. One gray (*Gy*) equals 100 rads. Fig. 2 (right). Potentiation of misonidazole radiosensitization of hypoxic CHO cells in vitro by DEM. CHO (HA-1) cells (*5*) were incubated at 25°C for 1 hour with DEM [$2.5 \times 10^{-5}M$ (■), $5 \times 10^{-5}M$ (▲), and $2 \times 10^{-4}M$ (●)] and irradiated in monolayer culture in tissue culture plates under 5 percent CO_2 in N_2 (*8*). MIS was added concurrently with DEM. Controls are indicated with open circles. Glutathione (GSH) was assayed enzymatically (*9*). No effect on the shoulder of the survival curve was seen with any drug combinations. Enhancement ratios for DEM alone were 1.0 for $2.5 \times 10^{-5}M$ at 35 percent of control GSH (no enhancement), 1.3 for $5 \times 10^{-5}M$ at 20 percent of control GSH, 1.5 for $2 \times 10^{-4}M$ at 5 percent of control GSH, and 2.1 for $1 \times 10^{-3}M$ at less than 1 percent of control GSH (data not shown). Data shown are averages from two to four experiments.

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the survival curve, rather than a strictly dose-modifying effect of glutathione removal as seen in the present study.

Since endogenous reducing agents (the main one is glutathione) act in competition with electron-affinic radiosensitizers in the cell, a modest depletion of intracellular glutathione might make the hypoxic cells more susceptible to radiosensitization by these electron-affinic agents. We tested this hypothesis by depleting glutathione in hypoxic CHO cells to varying degrees with DEM and irradiating in the presence of various concentrations of misonidazole. In Fig. 2 the open symbols show the ER produced by different concentrations of MIS alone in hypoxic CHO cells. The closed symbols show that the radiosensitization of hypoxic cells by MIS is enhanced by glutathione depletion: less MIS is needed to produce equivalent radiosensitization in glutathione-depleted cells. Even a concentration of DEM ($2.5 \times 10^{-5} M$) which depletes glutathione to 35 percent of control values, and which itself produces no radiosensitization, reduces the MIS concentration necessary to achieve a given ER by a factor of approximately 3. This factor increases to approximately 15 at $5 \times 10^{-5} M$ DEM, which depletes glutathione to 10 to 15 percent of control values.

We carried out similar experiments in vivo with EMT6 tumors in BALB/c mice (*5*). An ER of 1.5 ± 0.2 for hypoxic EMT6 tumor cells in vivo was achieved by glutathione depletion to 20 percent of control values with DEM (720 mg/kg). This is comparable to the in vitro results with CHO cells at the same degree of glutathione depletion. The combination of DEM and MIS (25 mg/kg) in hypoxic EMT6 tumors in vivo resulted in an ER of 2.0 ± 0.2. This dose of MIS alone did not produce any significant radiosensitization (ER = 1.08 ± 0.05). For MIS alone to produce an ER of 2.0, a dose of 250 mg/kg is required—that is, ten times the dose used in the combination.

The clinical effectiveness of MIS has been severely limited by its neurotoxicity (doses are limited to 20 to 30 mg/kg when given with daily fractionated radiotherapy) (*2*). Our results suggest that the effectiveness of MIS in the treatment of cancer by radiation may be improved by combining it with drugs that deplete intracellular glutathione.

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Eumelanins and Pheomelanins: Characterization by Electron Spin Resonance Spectroscopy

Abstract. *Synthetic dopa melanin and cysteinyldopa melanin have different electron spin resonance spectra. Data are reported for mixtures of these melanins and for dopa-cysteinyldopa copolymers, which are spectroscopically similar. A simple parameterization of the spectra allows estimation of the relative amounts of (i) dopa melanin and cysteinyldopa melanin in mixtures and of (ii) dopa and cysteinyldopa incorporated into copolymers. Several natural eumelanins and pheomelanins have been characterized and shown to be copolymers.*

Mammalian melanin pigments can be classified as either eumelanins or pheomelanins. Both classes are amorphous, heterogeneous polymers (*1*). The major units in eumelanins are thought to be derived from tyrosine through dopa (*2*), while those in pheomelanins are apparently derived from the dopa metabolite 5-*S*-cysteinyldopa (*3*). However, since the sulfur content of many natural melanins is intermediate between that of pure dopa and cysteinyldopa melanins (*4*), it has been argued (*5*, *6*) that copolymers of dopa and 5-*S*-cysteinyldopa are widespread. In support of this, cysteinyldopa-derived units have been chemically identified among the degradation products of eumelanins extracted from mammalian eyes (*7*). The chief criterion for distinguishing between pheomelanins and eumelanins is their solubility in alkali: eumelanins are insoluble in dilute alkali, whereas pheomelanins are alkali-soluble. Some other means of characterizing melanins that reflects the copolymeric nature of most of these materials is desirable.

We showed previously (*8*) that pure cysteinyldopa melanin has a characteristic electron spin resonance (ESR) spectrum associated with a novel kind of biological free radical, probably a semiquinonimine. This spectrum, which has three distinct features (associated with interaction with a nitrogen atom), is different from the single-line, featureless

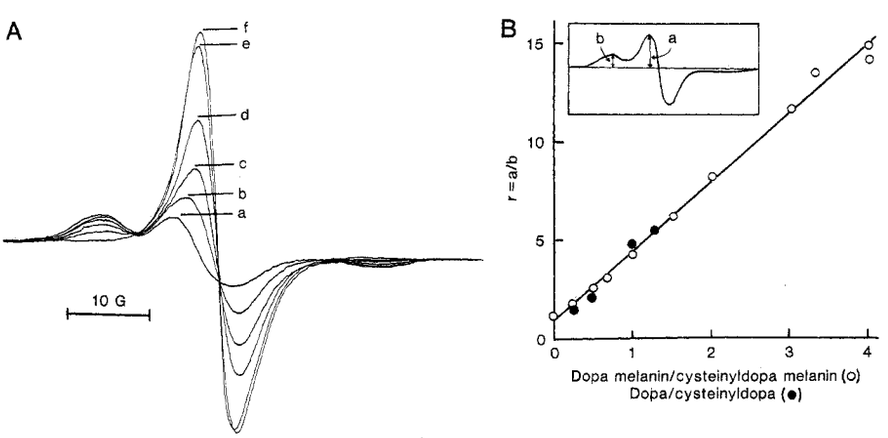

Fig. 1. (A) ESR spectra of mixtures of cysteinyldopa and dopa melanins. Spectra *a* to *f* correspond to mixtures containing 100, 80, 60, 40, 20, and 0 percent (by weight) cysteinyldopa melanin, respectively. Spectra were obtained as previously described (*8*) from suspensions (~ 1 mg/ml) containing 3 m*M* Zn^{2+} (*p*H 4.5) at −196°C. Cysteinyldopa was prepared by the method of Agrup *et al.* (*16*) and melanins by the method of Ito *et al.* (*13*). Melanins were obtained and stored as aqueous suspensions. They were not subjected to drying. (B) Characterization of ESR spectra of mixtures (○) and copolymers (●) in terms of *r* values (see inset).

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