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DNA DAMAGE IN NORMAL AND NEOPLASTIC MOUSE TISSUES AFTER TREATMENT WITH MISONIDAZOLE *IN VIVO**

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Abstract—Alkaline elution has been used to examine the integrity of DNA isolated from various tissues from mice treated with misonidazole (MISO). High doses (1–3 mg/g) of MISO caused extensive DNA strand breakage in cells isolated from two fibrosarcoma tumors that were known to contain hypoxic cells, and also in cells from certain normal tissues (liver and kidney in particular). The incidence of strand breaks gives further support to the suggestion that MISO can be metabolically nitroreduced beyond the singly reduced nitro radical-anion in some normal tissues as well as in hypoxic tumor cells, generating DNA-reactive species. Nitroreductases must therefore be able to compete successfully with molecular oxygen for the MISO nitro radical-anion in such tissues.

The potential of using MISO‡ to enhance the therapeutic effects of radiation or chemotherapeutic agents, or of directly exploiting its selective toxicity toward hypoxic cells, is severely limited by its toxicity toward normal tissues, particularly peripheral neuropathy [1, 2]. Thus, the development of improved hypoxic-cell sensitizers is dependent upon a more complete understanding of the mechanisms of their various biological activities [3]. With the exception of the metabolism-independent, O_2-mimetic component of radiosensitization, the effects of MISO *in vitro* have been shown to require prior reduction of the nitro group [3–6], although little is known about the subsequent interaction of these metabolites with cellular components. Binding of nitroreduced MISO metabolites to the DNA of hypoxic cells has been suggested as a possible cytotoxic event [7], and recent studies have also demonstrated intracellular binding in certain mouse tissues *in vivo* [8, 9]. However, Brown and co-workers [6, 10] have considered such adducts to be relatively nontoxic, since the enhancement of MISO toxicity after glutathione (GSH) depletion by diethyl maleate was not accompanied by increased levels of bound metabolites. Rather, these authors suggested that cytotoxicity may be the result of H-atom abstraction from critical cellular targets by free radicals generated during MISO metabolism. Several studies have, in fact, shown that MISO produces DNA strand breaks in hypoxic mammalian cells *in vitro* (e.g. Refs. 11–13). Palcic and Skarsgard [11] have reported a correlation between cytotoxicity and DNA damage in three different cell lines, while Olive [12] has shown that both cytotoxicity and strand breaks correlate with the redox potential of a series of nitroheterocyclic sensitizers. However, to our knowledge, such effects have not been demonstrated in any *in vivo* systems.

In the present study, we used the technique of alkaline elution to examine the effect of MISO on the integrity of DNA isolated from various mouse tissues treated *in vivo*. MISO at high doses caused extensive DNA strand breakage in cells from two fibrosarcoma tumors that were known to contain hypoxic cells, and also in cells from certain normal tissues (especially those from liver and kidney). Our results support a previously proposed hypothesis that MISO can be metabolically nitroreduced in normal tissues.

MATERIALS AND METHODS

Mice, drug treatments and tissue suspensions. Details of the specific pathogen-free C_3H/Kam mouse colony and of the NFSa [14] and FSa [15] fibrosarcomas have been described elsewhere. Tumors were grown by injecting 5×10^5 cells subcutaneously into the hind legs of recipient mice and used when they reached a mean diameter of 10–12 mm. Tumor-free mice were used for those experiments involving the response of normal tissues, although the presence of either type of tumor appeared to have no effect on the resulting DNA damage in normal tissues (data not shown). MISO (Hoffman-La Roche Inc., Nutley, NJ) was dissolved in saline and injected intraperitoneally at doses between 1 and 3 mg/g. Animals were killed either 1.5 or 6.5 hr after injection. The relevant tissues were quickly removed, immersed in ice-cold Puck's saline A (PSA) containing EDTA (5 mM), and par-

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‡ Abbreviations: MISO, misonidazole; GSH, reduced glutathione; and SSF, strand scission factor.

tially minced to facilitate rapid chilling; a total of less than 20 sec was required for this operation. Single-cell suspensions for the estimation of DNA damage were prepared as described elsewhere [16] and were maintained on ice at all times to prevent the repair of strand breaks and also to minimize the possibility of continuing drug metabolism after tissue removal which has been reported in other studies [8, 17]. Despite these precautions, some metabolism during tissue preparation cannot be completely ruled out, particularly in the liver [8]. Based on these previous studies [8, 17], we tried rapid freezing of liver and kidney samples on dry ice as a possible alternative; unfortunately, this procedure was accompanied by significant levels of DNA degradation and could not be used in conjunction with sensitive DNA damage assays such as alkaline elution.

Alkaline elution. DNA strand breaks were determined using the technique of alkaline elution [18] adapted for use with animal tissues [16]. Briefly, between 6 and 8×10^6 cells were impinged onto a 47 mm diameter, 0.8 μm pore polycarbonate filter, washed with cold PSA, and lysed as described previously [16]. The samples were eluted overnight in the dark with 0.1 M tetrapropylammonium hydroxide–0.02 M Na_4EDTA (pH 12.1) at a flow rate of 0.04 ml/min. Fractions were collected every 90 min. The DNA concentration in each fraction, as well as that remaining on the filter or in the elution barrels at the end of the experiment, was determined using the fluorescent dye Hoechst 33258 (Aldrich Chemical Co., Milwaukee, WI). A 1-ml aliquot was withdrawn from each fraction, transferred to a 13×100 mm glass culture tube and neutralized with 1 ml of 0.08 M KH_2PO_4. Finally, 1 ml of Hoechst dye (1.5×10^{-6} M in standard saline citrate) was added, and the resulting fluorescence was measured using an Aminco SPF-125 spectrofluorometer ($\lambda_{excitation}$ = 350 nm, $\lambda_{emission}$ = 460 nm). In each experiment, a control elution was run with no cells added, and the fluorescence from these controls was used to correct for background emission.

The SSF was determined from: $SSF = \log(f_x/f_o)$ where f_o and f_x are the proportion of DNA retained on the filter after an eluted volume of 17.5 ml for the untreated control and for the MISO-treated animal respectively. Thus, a strand scission factor of zero indicates no DNA strand breakage. All data presented are averaged from at least three separate experiments.

RESULTS

Typical alkaline elution profiles for DNA isolated from either NFSa or spleen cells 1.5 hr after mice were treated with MISO (1–3 mg/g) are shown in Fig. 1, panels a and b, respectively. The main effect apparent from these profiles is that DNA from NFSa cells isolated from treated animals eluted much faster than that from untreated control animals, which indicates a dose-dependent formation of DNA strand breaks. The rate of elution of spleen-cell DNA, however, was relatively unaffected even at the highest dose of MISO. SSFs were calculated from elution profiles such as those in Fig. 1 for NFSa and spleen, and from similar experiments with FSa, liver and kidney (data not shown). Dose responses relating the observed degree of strand scission, measured 1.5 hr after injection, to the dose of MISO are shown in Fig. 2 for each of these five tissues. The degree of strand scission is expressed in rad-equivalents of X-ray damage; the reference system was mouse spleen cells irradiated *in vivo* [16].

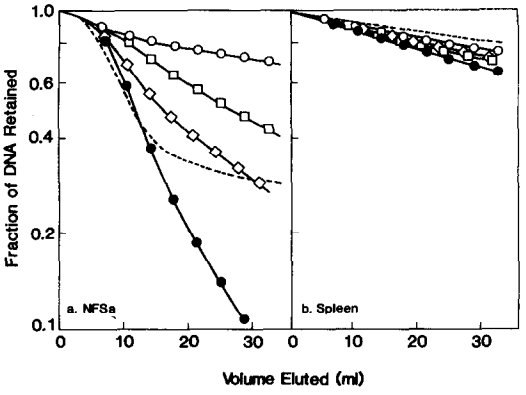

Fig. 1. Typical alkaline elution profiles for DNA isolated from NFSa and spleen cells, either from untreated control animals (○) or 1.5 hr after treatment of mice with various doses of MISO: 1 mg/g (□), 2 mg/g (◇) or 3 mg/g (●). The dashed line shows a typical profile for each tissue obtained 6.5 hr after injection with the lowest dose (1 mg/g) of MISO.

Treatment with 1 mg/g of MISO, a dose comparable to that used in previous studies of radio- and chemosensitization, produced no measurable strand

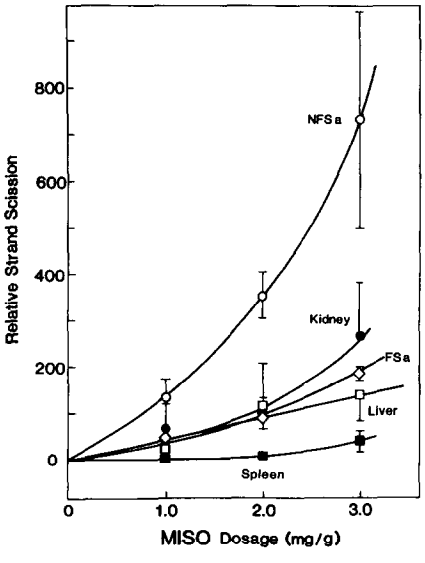

Fig. 2. Dose–response relationships relating the degree of DNA strand scission to the dose of MISO administered intraperitoneally to C_3H mice. Data are shown for two murine fibrosarcomas: NFSa (○) and FSa (◇); and for several normal mouse tissues: spleen (■), liver (□) and kidney (●). DNA strand breaks were estimated by alkaline elution and are expressed as rad-equivalents of X-ray damage, the reference system being mouse spleen cells irradiated *in vivo* (see Materials and Methods).

breaks within experimental error in DNA from FSa, spleen, liver or kidney cells 1.5 hr after injection. However, in the case of NFSa cells, some breaks were apparent: NFSa was thus the only studied tissue to show detectable DNA breaks at this dose of MISO. The extent of these breaks was relatively unaffected by the inclusion of a proteinase K digestion step in the alkaline elution procedure (data not shown), indicating the absence of additional protein-concealed strand breaks; in all of the present data, proteinase K was included routinely in the lysis buffer.

Increasing the dose of MISO to 2 or 3 mg/g (Fig. 2) resulted in some strand breakage in both neoplastic and normal tissues, with the exception of spleen (Fig. 2) and brain (data not shown): in these two tissues, few breaks were apparent even at the higher doses. In those tissues showing strand break induction, the dose responses were nonlinear, with the number of breaks increasing more rapidly at the higher doses. NFSa always showed considerably greater levels of breaks than any other tissue, including FSa. The large variation in the NFSa data after MISO at 3 mg/g may be related to the high toxicity expressed in tumor-bearing mice. Some of these animals died during the 1.5-hr treatment, but even survivors showed severe side effects of the drug, including marked neurological dysfunction. Tumor-free animals invariably showed less severe side effects, and none of them died in the first 1.5 hr at any drug dose.

To determine whether strand breaks produced by low doses of MISO were reparable, we examined the effect of a suitable post-treatment repair period on the level of breaks in each tissue. Typical elution profiles obtained for NFSa and spleen 6.5 hr after injection with MISO at 1 mg/g are shown by dashed lines in Fig. 1, a and b. By this time, considerable further fragmentation of the DNA from NFSa was apparent (relative to the situation after 1.5 hr), with the elution profile assuming a biphasic character. Spleen-cell DNA, on the other hand, remained intact. All other tissues, including FSa, showed little evidence of this "delayed" strand breakage up to 6.5 hr after injection with MISO at 1 mg/g (data not shown). It was not possible to determine the time course of strand breakage after higher doses because of the extreme toxicity that was seen particularly in the tumor-bearing animals, as noted above. After the lower dose (1 mg/g) of MISO, however, no deaths occurred during the first 6.5 hr.

DISCUSSION

In addition to radiosensitization, nitroimidazoles exhibit a wide variety of biological effects, including direct toxicity to hypoxic cells, depletion of intracellular GSH and enhancement of the effect of various chemotherapeutic agents (e.g. see recent reviews by Brown [6] and Rauth [3]). In view of data obtained with hypoxic cell cultures, all three of these activities appear to require reduction of the nitro group, inhibition of the reaction of the MISO nitro radical-anion with oxygen [4] being particularly important. However, such *in vitro* measurements are usually made under conditions of either severe hypoxia or atmospheric oxygen tension, while animal tissues *in vivo* may well experience intermediate levels of oxygenation that may result in both quantitative and qualitative differences in MISO metabolism compared to these extreme situations [3]. In recent studies of the oxygen dependence of MISO cytotoxicity [19, 20] and chemosensitization [20], where measurements have been made over a range of dissolved O_2 concentrations, both effects have been shown to be described by similar K-curves, with toxicity and sensitization being inhibited by relatively low O_2 levels.

The observation that nitroreduction-mediated events do not require complete hypoxia, but can occur even at low levels of oxygenation, has potentially important implications to the action of MISO *in vivo*, since there is now considerable evidence that some normal tissues may be hypoxic to the extent that they can be sensitized to the effects of ionizing radiation by O_2 or hypoxic-cell sensitizers (e.g. see Refs. 21 and 22). In fact, we have shown recently that variations in the level of X-ray-induced DNA strand breaks in different mouse tissues can be explained by assuming that both tumors and normal tissues exist at oxygen tensions intermediate between aerobia and anoxia [16]. However, there may be some important differences in those factors determining the response of tissues to MISO and X-rays at the molecular level. For example, while kidneys appear to be relatively well oxygenated in terms of their response to X-rays [16], they showed considerable strand breakage (Fig. 2), GSH depletion and enhanced levels of nitrogen mustard cross-links [23], after treatment with MISO. Each of these processes requires hypoxia-mediated nitroreductive metabolism *in vitro*, suggesting that the metabolism of MISO by normal tissues may not necessarily correlate with their degree of radiobiological hypoxia. Indeed, while oxygenation is likely to be the major determinant of X-ray damage to DNA, Taylor and Rauth [19] have suggested that additional factors controlling the redox environment of cells or tissues may also be critical determinants of reductive metabolism and MISO cytotoxicity. Several recent studies of MISO metabolism in rodents have established that normal tissues, in particular liver, are important sites of nitroreduction. Varghese *et al.* [24] have detected nitroreduced MISO metabolites in mouse liver but not in other tissues. Chin and Rauth [8] have reported extensive metabolism and retention of [^{14}C]MISO in mouse liver, and to a lesser extent in kidney, whereas little evidence of metabolism was seen in brain or spleen. Garrecht and Chapman [9] have also observed retention of [^{14}C]MISO by mouse liver and by hypoxic regions of tumors.

Several possible mechanisms have been suggested to explain the DNA strand breakage observed in hypoxic cells exposed to MISO *in vitro*. Palcic and Skarsgard [11] have speculated that strand breaks may result indirectly from the binding of reduced MISO metabolites to DNA and the subsequent action of DNA repair enzymes. Brown [6] has proposed that MISO cytotoxicity is related to metabolically generated neutral radicals that can abstract H-atoms from critical cellular targets such as DNA. Edwards and co-workers (e.g. see Ref. 25) have

shown recently that electrolytically reduced nitroimidazoles directly induce breaks in *Escherichia coli* DNA via a mechanism that does not involve the binding of the drug to DNA, but which involves the transfer of an electron from DNA to a transient, reduced intermediate of MISO. These *in vitro* studies therefore provide a precedent for actual MISO-mediated DNA damage and suggest that DNA strand breaks observed *in vivo* after 1.5 hr (Figs. 1 and 2) may have a similar origin, although it was not possible to discount the possibility that these breaks were, in fact, a result of lysosomal disruption and resulting nuclease activity, as observed in mouse mammary tumors even after low doses (0.5 mg/g) of MISO [26]. However, the 1.5-hr elution profiles obtained from NFSa were essentially first order and had a similar shape to profiles obtained after X-irradiation [16], suggesting a single population of randomly damaged DNA molecules. On the other hand, the 6.5-hr profiles were biphasic (Fig. 1a, dashed line), suggesting the existence of two populations of molecules, one with DNA eluting at essentially the same rate as the untreated control (suggesting little residual damage) and a second early-eluting population in which the DNA has been progressively degraded. These data therefore support the idea that breaks observed at 1.5 hr are actually MISO-induced breaks that are probably reparable. However, possible subsequent DNA degradation associated with dying cells precludes the clear determination of repair processes. This interpretation is supported by data obtained with hypoxic cells *in vitro* [11]; in cells receiving supralethal exposures to MISO, DNA degradation increased progressively up to 24 hr after treatment, while cells destined to survive the treatment repaired DNA breaks effectively.

An interesting aspect of the dose responses shown in Fig. 2 is their apparent curvilinearity. One possible explanation for such behavior is that DNA damage (or cell-killing) may be mediated via a free-radical mechanism, as discussed above, and therefore moderated by GSH levels. Since MISO itself depletes GSH in hypoxic cells *in vitro* [10, 27], these dose responses may reflect both an increased production of radicals and a simultaneous GSH depletion with increasing dose of MISO, thereby reducing the proportion of these radicals that would be inactivated; these data are consistent with the idea expressed by Brown [6] that MISO is essentially "self-sensitizing" because of its effect on GSH levels. Furthermore, the dose dependence of GSH depletion is itself non-linear, since at lower doses of MISO the resynthesis of GSH can compensate for its removal [6]. An alternative explanation for such curvilinear dose responses is suggested by pharmacological studies [28] which have shown that the initial plasma half-life of MISO in mice increased with increasing MISO dosage over the range employed in the present study, and this could therefore result in increased effective MISO dosage at the higher dose. In either case, these data obtained after high MISO doses may not, therefore, be relevant to the situation after lower doses.

Finally, it is interesting to consider the possible relationship between the relative radiobiological hypoxic fractions reported for the two tumors and their response to MISO (Fig. 2). FSa has been reported to contain 27% hypoxic cells based on TCD_{50} (the X-ray dose that cured the tumor in 50% of the animals) data [29], although a value closer to 50% has been estimated using a lung colony assay.* A much lower hypoxic fraction of *ca.* 10% has been reported for NFSa, again based on lung colony assay data [30]. The apparent lack of a correlation between the relative hypoxic fractions and the degree of MISO-induced strand breakage for these two tumors may well reflect their very different morphologies, and in particular the heterogeneity of distribution and relative hypoxia within this hypoxic fraction. FSa contains significant regions of necrosis, as well as populations of non-viable or metabolically inactive cells which may not be accessible to certain cytotoxic drugs (whereas hypoxic fraction is determined on the basis of radiation response which is not affected by such pharmacological factors). Furthermore, it should be remembered that the underlying biochemical mechanisms governing the O_2-dependence of radiosensitivity and the response to drugs which require reductive metabolism to express their activity are themselves different. It may therefore be inappropriate to predict specific aspects of a response to drugs such as MISO at the DNA damage level based on radiobiological observations.

In summary, the incidence of DNA strand breaks produced by high doses of MISO in mouse tissues appears to support the hypothesis that certain normal tissues, particularly liver and kidney, exhibit appreciable capacity to nitroreduce MISO beyond the singly reduced nitro radical-anion, and therefore that nitroreductases can compete successfully with molecular oxygen for this short-lived species in such tissues. This situation apparently results in the occurrence *in vivo* of three processes—DNA strand breakage (Fig. 2), GSH depletion and chemosensitization [23]—which only occur under hypoxic conditions *in vitro*.

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