Therapeutic Analysis of Melphalan-resistant Human Rhabdomyosarcoma Xenograft TE-671 MR

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ABSTRACT

Investigations with the melphalan-resistant human rhabdomyosarcoma xenograft TE-671 MR were carried out to identify patterns of cross-resistance and collateral sensitivity and to define the mechanism(s) mediating melphalan resistance. TE-671 MR was cross-resistant to thio-TEPA, mitomycin, vincristine, and cisplatin, and partially resistant to chlorambucil and cyclophosphamide. TE-671 MR and the parent line TE-671 were both resistant to 1,3-bis(2-chloroethyl)-nitrosourea and expressed similar levels of O6-alkylguanine-DNA alkyltransferase. TE-671 MR retained full sensitivity to actinomycin D and demonstrated enhanced sensitivity to VP-16 compared to TE-671. Treatment of TE-671 MR with melphalan plus VP-16 resulted in greater than additive growth delays. The frequency of hypoxic regions was similar in TE-671 MR and TE-671, respectively. Measurement of tumor-to-plasma levels at 180 min following i.p. administration of melphalan at 0.5 of the 10% lethal dosage showed mean tumor-to-plasma ratios of 3.81 in TE-671 MR and 7.38 in TE-671, respectively. The lower drug levels in TE-671 MR may be contributing to the resistance to melphalan and thus indicate the need for further studies to define the reasons for these differences in tumor drug level.

INTRODUCTION

The development of drug resistance with subsequent treatment failure is a major obstacle to the successful treatment of cancer. Selection of new therapies effective in bypassing or overcoming clinically relevant drug resistance may be facilitated by identification of patterns of cross-resistance and collateral sensitivity and by definition of mechanisms mediating drug resistance (1). Identification of cross-resistance and collateral sensitivity allows the use of agents to which the tumor is still sensitive, while avoiding those drugs to which the tumor has developed cross-resistance. Definition of mechanisms mediating drug resistance may allow for modulations effective in overcoming resistance. Despite the activity of bifunctional alkylating agents against a broad spectrum of human neoplasms, resistance to these agents frequently develops, with subsequent relapse and death. The majority of laboratory models of alkylator resistance have resulted from in vitro exposure of cell lines to these agents (2). However, drug resistance may develop through mechanisms that are only operational in vivo (3). The establishment of a human rhabdomyosarcoma xenograft, TE-671 MR, with melphalan resistance generated in vivo, has provided a model for the therapeutic analysis of melphalan resistance in a human neoplasm (4).

The current studies were conducted to define the response of TE-671 MR to a broad spectrum of chemotherapeutic agents and to attempt to identify both the mechanisms of resistance of TE-671 MR to melphalan and the modulations effective in overcoming this resistance. In addition to the determination of the sensitivity of TE-671 MR to a variety of agents in vivo, the resistant tumor was compared with the sensitive line TE-671 with respect to O6-alkylguanine-DNA alkyltransferase levels, tumor oxygenation levels, and ability of the tumor to transport and retain melphalan. Since combinations of melphalan and VP-16 had shown synergistic activity in the TE-671 line, this combination was also examined in the resistant line. The data suggest that altered melphalan delivery or efflux may be a contributing factor to resistance to the drug.

MATERIALS AND METHODS

Animals. Male or female athymic BALB/c mice (nu/nu genotype, 6 weeks or older) were used for all studies and were maintained as previously described (5).

Xenograft Transplantation and Tumor Lines. TE-671, a subline of the human rhabdomyosarcoma-derived continuous cell line RD, and the melphalan-resistant line TE-671 MR, growing as s.c. xenografts, were used for all studies. The establishment and characterization of TE-671 and TE-671 MR have been described previously (4, 6-8). Xenograft transplantation s.c. was performed as previously described with inoculation volumes of 30 µl (9).

Tumor Measurements. Tumors were measured every 3-4 days with vernier calipers (Scientific Products, McGaw Park, IL) as previously described (9).

Drugs for Therapy Studies and Drug Toxicity. All drugs were purchased from commercial sources except chlorambucil and melphalan, which were provided by Burroughs Wellcome, Research Triangle Park, NC, and bleomycin, which was provided by Bristol-Myers, Wallingford, CT. The lethal toxicity of individual drugs was assessed by probit analysis (10). A minimum of 4 dose levels with 10 animals/dose was used to calculate the LD100 of each drug. The LD50 for the individual drugs was found to be as follows: actinomycin D, 0.5 mg/m2; BCNU, 100 mg/m2; bleomycin, 330 mg/m2; chlorambucil, 130 mg/m2; cisplatin, 32.5 mg/m2; cyclophosphamide, 1391 mg/m2; ifosfamide, 205 mg/m2; melphalan, 71.3 mg/m2; mitomycin, 15.7 mg/m2; thio-TEPA, 61.8 mg/m2; vincristine, 9.8 mg/m2; and VP-16, 170 mg/m2. The dose administered in each single-agent experiment was 100% of the calculated LD50. In combination studies with VP-16 and melphalan, the doses used were 0.50 LD50 of VP-16 with varying fractions (0.10, 0.25, 0.375, 0.50, and 0.75) of the LD50 of melphalan. Drugs were given as a single dose on Day 1 except for daily doses of actinomycin D, on Days 1-5; VP-16 on Days 1, 4, and 7; and bleomycin on Days 1, 4, and 7. When used in combination with VP-16, melphalan was given on Day 1 and VP-16 was given on Days 1, 4, and 7. Drugs were administered by i.p. injection in a volume of 90 ml/m2. All drugs were dissolved in 0.9% saline except melphalan, which was dissolved in 17% dimethyl sulfoxide; BCNU, which was dissolved in 0.12% ethanol; and chlorambucil, which was dissolved in 17% dimethyl sulfoxide.

Tumor Therapy. Groups of 8-10 randomly assigned mice bearing TE-671 or TE-671 MR tumors were treated by i.p. injection with chemotherapeutic compounds according to the doses and schedules described above when the median tumor volume exceeded 200 mm3.

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3 The abbreviations used are: LD50, dose lethal to 50% of treated animals; BCNU, 1,3-bis(2-chloroethyl)-nitrosourea.
Groups of matched animals served as controls and received the drug vehicle on Day 1.

Assessment of Response. Response of xenografts was assessed by growth delay (T - C) being the difference in days between the median time for the tumors of treated (T) and control (C) animals to reach a volume of 5 times greater than the volume at the time of treatment), and by treated versus control tumor regressions. Statistical analyses were performed as previously described (11).

Melphalan Levels. Athymic nude mice bearing s.c. TE-671 MR xenografts were given i.p. injections of melphalan at 0.5 of the LD sub 10 when the median tumor volumes exceeded 200 mm 3. Groups of 3 mice each were anesthetized with halothane; blood was obtained by cardiac puncture; and the xenografts were resected at 30, 60, 120, 180, and 240 min following injection of the drug. Plasma obtained from the blood samples was frozen (~70°C) for subsequent measurement of melphalan levels. The harvested xenografts were homogenized in a Brinkman Polytron in 10% perchloric acid (5:1, v/w), and the homogenates were frozen (~70°C) for subsequent melphalan assays. Melphalan measurements were performed by high pressure liquid chromatography as previously described (12). Mean tumor-to-plasma melphalan ratios were calculated from the ratios obtained in individual mice.

O6-Alkylguanine-DNA Alkyltransferase. Two TE-671 xenografts and two TE-671 MR xenografts were resected and snap-frozen in liquid nitrogen for subsequent determination of O6-alkylguanine-DNA alkyltransferase levels. Enzyme levels were determined as previously described (13).

Tumor Oxygenation Studies. Oxygen tension was measured in TE-671 and TE-671 MR tumors by using a polarographic technique (14). Briefly, the methodology relies on ionization of molecular oxygen at a polarizing voltage of 0.7 V. The amount of current which flows at the polarizing voltage is directly proportional to the oxygen concentration near the sensing electrode. Clark style microelectrodes (Diamond General Corp., Ann Arbor, MI) (15), with tip diameters ranging from 50 to 150 µm, were used for this study. All measurements were made in a radiofrequency-shielded enclosure which was securely grounded. This was done to prevent stray electromagnetic radiation from interfering with the measurements. Electrodes were calibrated in saline and bubbled with gas mixtures certified for 0, 5, 10, and 21% oxygen prior to and after each experiment. The calibration temperature was 37°C. Calibrations were also performed in vivo. Measurements over exposed, resting muscle in a film of saline were assumed to be equivalent to room air (21% oxygen). At the end of each experiment, the animals were euthanized with a barbiturate overdose. Measurements taken in deep thigh muscle 10-30 min after euthanasia were assumed to be at 0% O2. The calibrations performed in vivo did not always fit the in vitro curves, which was likely due to electrolyte differences between the saline and the animal. Thus, all data were fit to the in vitro curves, and the in vitro calibrations were used to verify that the electrode was functioning properly before and after each experiment. The electrode sensitivities averaged 4.9 ± 3.3 (SD) pA/mm Hg.

Animals bearing 5- to 7-mm s.c. tumors in the lateral flank were anesthetized by using pentobarbital sodium (40 mg/kg i.p. injection). The animals were placed on a constant temperature pad (37°C) and covered to prevent hypothermia. An incision was made over a lateral surface of the tumor. The oxygen microelectrodes were inserted through the skin and fascia incision and into the tumors, using the tip for blunt dissection. Before a measurement was taken, the tip was retracted slightly to prevent pressure effects on the microvessels. Two to 4 radial profiles were made in each tumor at 1-mm intervals. After each profile was taken, the tip of the electrode was rinsed with 0.1 N HCl followed by saline to prevent buildup of protein on the tip. All resultant data from these experiments were converted to mm Hg O2.

RESULTS

Drug Toxicity. Twenty-two deaths in 326 tumor-bearing treated animals resulted from drug toxicity at the doses given in “Materials and Methods” with the following distribution: cyclophosphamide, 4 of 20; vincristine, 4 of 10; actinomycin D, 1 of 20; ifosfamide, 3 of 20; chlorambucil, 3 of 30; VP-16 (1.0 LD sub 10), 2 of 17; melphalan (1.0 LD sub 10), 3 of 28; melphalan plus VP-16 (0.75 LD sub 10 ± 0.5 LD sub 10), 2 of 9. Mean nadir weight loss was less than 15% in all groups except cyclophosphamide (19.7%), vincristine (15.7%), actinomycin D (19.1%), bleomycin (24.9%), and melphalan plus VP-16 (0.75 LD sub 10 ± 0.50 LD sub 10) (19.6%).

Single-Agent Therapy. The response of TE-671 MR and TE-671 to chemotherapy is summarized in Table 1. Results of previously published experiments showing the response of the parent line, TE-671, to chlorambucil, cyclophosphamide, ifosfamide, thio-TEPA, and vincristine are shown for comparative purposes (4, 16). In addition to resistance to melphalan, TE-671 MR demonstrated a similar degree of resistance to the alkylating agents chlorambucil, cyclophosphamide, and ifosfamide. Three other alkylating agents active in the parent line, thio-TEPA, cisplatin, and mitomycin, were ineffective in TE-671 MR. BCNU, which was inactive against TE-671, was similarly inactive against TE-671 MR. TE-671 MR was cross-resistant to bleomycin, retained sensitivity to actinomycin D, and demonstrated collateral sensitivity to VP-16.

Combination Therapy. The response of TE-671 MR to VP-16 (0.50 LD sub 10) alone, to varying fractions of the LD sub 10 of melphalan alone, or to VP-16 (0.50 LD sub 10) plus melphalan at various fractions of the LD sub 10 was summarized in Table 2. The combination of melphalan and 0.50 LD sub 10 VP-16 was more than additive, with the greatest combined effect found at the 0.25-0.50 LD sub 10 dose range of melphalan. These results are similar to the effects seen with the same drug combinations against TE-671, where a synergistic interaction was shown previously (17).

Melphalan Levels. Melphalan levels in the tumors and plasma of mice bearing TE-671 MR were compared with those previously found in mice bearing tumors of the parent line TE-671. The tumor-to-plasma melphalan ratio for the TE-671 MR line was not significantly different from that obtained in the TE-671 line at 30, 60, and 120 min post-melphalan injection, but was significantly lower than that of TE-671 at 180 and 240 min postinjection (3.18 ± 0.967 versus 7.38 ± 1.72 at 180 min; undetectable versus 4.67 ± 3.40 at 240 min) (Table 3).
The mean and SD of pO2 values for TE-671 was 49.2 ± 46 mm Hg. The large standard deviations are indicative of large variations in pO2 values for TE-671 MR tumors was 32.9 ± 21.0 mm Hg. The precise mechanism of this cross-resistance to mitomycin C remains unclear. Although Dean et al. (21) have demonstrated that enhanced nitroreduction cytotoxicity in a melphalan-resistant Walker 256 carcinosarcoma is mediated by a decrease in O6-alkylguanine-DNA alkyltransferase levels, this was not true in TE-671 MR, which demonstrated BCNU resistance and O6-alkylguanine-DNA alkyltransferase levels virtually identical to the parent line.

Bleomycin is an antineoplastic antibiotic which requires the presence of a reducing agent, such as glutathione, for cytotoxic action (22, 23). Since a glutathione-rich human ovarian carcinoma cell line was previously shown to be bleomycin sensitive (24), TE-671 MR, which also exhibits increased glutathione levels relative to the parent line (4), was treated with bleomycin to determine if this xenograft demonstrated collateral sensitivity to bleomycin. Bleomycin was not, however, effective against TE-671 MR. Patterns of cross-resistance and collateral sensitivity are, therefore, poorly predictable and may possibly depend on the specific tumor histiotpe. TE-671 MR demonstrated collateral sensitivity to VP-16 with growth delays approximately twice that observed in the parent line. VP-16 is an epipodophyllotoxin with cytotoxic properties due to inhibition of topoisomerase II activity. High understanding of patterns of cross-resistance, collateral sensitivity, and mechanisms of resistance is, therefore, essential for the optimal use of current drugs, as well as for the development of modulations effective in overcoming resistance.

Exposure to a single chemotherapeutic agent may result in cross-resistance to other drugs with different structures and mechanisms of action. This phenomenon has generally been seen with the use of natural products and their derivatives and is often associated with the presence of P-glycoprotein, which functions as an efflux pump (18). The development of resistance to an alkylating agent has typically not been shown to lead to cross-resistance to other alkylating agents (2, 19, 20). The melphalan-resistant xenograft TE-671 MR retained sensitivity to actinomycin D, exhibited collateral sensitivity to VP-16, but demonstrated complete or partial cross-resistance to a series of alkylating agents. The marked cross-resistance to mitomycin C was not mediated by changes in tumor oxygenation since TE-671 MR was not more oxygenated than the parent line. Indeed, the tumor oxygenation measurements tended to be lower in TE-671 MR than in TE-671, as shown by lower pO2 profiles and lower average median values (Fig. 1). The precise mechanism of this cross-resistance to mitomycin C remains unclear. Although Dean et al. (21) have demonstrated that enhanced nitroreduction cytotoxicity in a melphalan-resistant Walker 256 carcinosarcoma is mediated by a decrease in O6-alkylguanine-DNA alkyltransferase levels, this was not true in TE-671 MR, which demonstrated BCNU resistance and O6-alkylguanine-DNA alkyltransferase levels virtually identical to the parent line.

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levels of topoisomerase II are associated with increased sensitivity to the effects of VP-16 (25). Furthermore, a nitrogen mustard-resistant Burkitt’s lymphoma cell line, Raji-HN2, was found to becollaterally sensitive to VP-16 by virtue of elevated expression and transcription of the topoisomerase gene (26, 27). Previous studies with the parent line TE-671 have demonstrated the synergistic interaction between VP-16 and melphalan (17), and greater than additive growth delays were seen with this combination against TE-671 MR.

Multiple mechanisms of alkylator resistance are documented and include increased glutathione content, increased or altered activity of glutathione-S-transferase, increased repair of drug-induced DNA damage, and decreased uptake of drug (2). Reported mechanisms of resistance to melphalan have included increased levels of glutathione or glutathione-S-transferase, increased repair of DNA cross-links, and decreased melphalan transport (28–31). Previous studies have shown that melphalan resistance in TE-671 MR is not associated with an altered karyotype, increased expression of mdrl, or increased glutathione-S-transferase (4). A 2-fold increase in glutathione content in TE-671 MR was observed, but this alteration cannot yet be directly implicated as responsible for the observed resistance, since buthionine sulfoxime-mediated glutathione depletion enhances melphalan activity in both TE-671 and TE-671 MR xenografts (4). Our current studies show lower concentrations of melphalan into resistant tumors compared with the parent line and suggest that decreased melphalan delivery (as a consequence of altered tumor blood flow or intracellular drug transport) or enhanced melphalan efflux may be contributing to melphalan resistance in TE-671 MR.

The extent to which TE-671 MR illustrates clinically relevant mechanisms of melphalan resistance and cross-resistance is presently undefined. The general heterogeneity of tumors and the wide spectrum of mechanisms mediating alkylating agent resistance in various tumor histiotypes suggests that identification of the mechanisms most responsible for clinical drug resistance may be difficult. Nevertheless, studies of the characteristics and mechanisms of melphalan resistance (specifically the factors responsible for the decreased melphalan uptake in TE-671 MR) in this and other tumor models provide a greater understanding of alkylator resistance generally and may lead to useful clinical applications in some tumors.

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