ABSTRACT

Polar solvents, which induce differentiation in murine and human tumor cells, enhance the effect of ionizing radiation on cultured mouse mammary and human colon cancer cells. To determine whether this enhancement occurs in vivo, DLD-2 human colon carcinoma xenografts in nude mice were treated with combinations of 6 MV photon irradiation, the polar solvent N-methylformamide (NMF), or combinations of the two agents. Nude mice bearing 300- to 600-mg s.c. implants of DLD-2 tumors were treated i.p. with 150 mg NMF/kg daily for 19 days. Local tumor irradiations were administered as graded single doses or as fractionated doses, daily for 4 days, following the third NMF injection. The growth-inhibiting effect of the radiation treatment for both single dose and fractionation protocols was enhanced by the polar solvent. NMF alone increased the time required for a doubling of initial tumor volume by 1.7 days, compared to control tumors. Initial tumor volume doubling times compared to untreated controls were increased by 3.6 and 7.6 days by photon doses of 10.0 and 13.75 Gy, respectively, whereas NMF plus 10.0 or 13.75 Gy increased the DLD-2 regrowth delay time by 7.5 or 12.9 days. NMF caused essentially equivalent enhancements, whether split-dose schedules of 2.5 Gy daily for 4 days, and 3.44 Gy daily for 4 days, or single doses of 10.0 and 13.75 Gy were used; therefore, radiation enhancement was not due to effects on sublethal damage repair. The results support the use of NMF, currently in Phase 1-Phase 2 clinical trials, with radiation in the therapy of selected human neoplasms.

INTRODUCTION

The ability of polar solvents to induce differentiation in cultured murine and human tumor cells has been extensively documented (2-4, 14, 16, 23), and has been particularly well studied in Friend cultured mouse mammary and human colon cancer cells. To determine whether this enhancement occurs in vivo, DLD-2 human colon carcinoma xenografts in nude mice were treated with combinations of 6 MV photon irradiation, the polar solvent N-methylformamide (NMF), or combinations of the two agents. Nude mice bearing 300- to 600-mg s.c. implants of DLD-2 tumors were treated i.p. with 150 mg NMF/kg daily for 19 days. Local tumor irradiations were administered as graded single doses or as fractionated doses, daily for 4 days, following the third NMF injection. The growth-inhibiting effect of the radiation treatment for both single dose and fractionation protocols was enhanced by the polar solvent. NMF alone increased the time required for a doubling of initial tumor volume by 1.7 days, compared to control tumors. Initial tumor volume doubling times compared to untreated controls were increased by 3.6 and 7.6 days by photon doses of 10.0 and 13.75 Gy, respectively, whereas NMF plus 10.0 or 13.75 Gy increased the DLD-2 regrowth delay time by 7.5 or 12.9 days. NMF caused essentially equivalent enhancements, whether split-dose schedules of 2.5 Gy daily for 4 days, and 3.44 Gy daily for 4 days, or single doses of 10.0 and 13.75 Gy were used; therefore, radiation enhancement was not due to effects on sublethal damage repair. The results support the use of NMF, currently in Phase 1-Phase 2 clinical trials, with radiation in the therapy of selected human neoplasms.
from their nude mice hosts and were carefully minced. The mince from nonneoplastic tissue from all 5 tumors was pooled to provide a collection of 1-cu mm fragments, which were implanted into all the mice used in a given experiment.

Animals were inoculated in the upper hip region with 1-cu mm fragments of the DLD-2 carcinoma; palpable tumors appeared in about 7 days. After tumors had reached a size of about 5 x 5 mm, mice were carefully matched into groups of 10 animals each, and were ear tagged so that host mice could be followed individually. Tumors were irradiated when they had attained approximately a size of 8 x 10 mm (about 300 mg).

Measurement of Tumor Size. As we have described before (8), DLD-2 tumors were measured by calipers in 2 orthogonal diameters, and the tumor volumes were calculated using the formula for a prolate ellipsoid:

\[ V = \frac{L \times W^2}{2} \]

where L and W are the major and minor diameters (in mm), respectively. The average tumor volumes for each irradiation group were then plotted as a function of time postirradiation to obtain tumor growth curves.

Irradiation of Solid Tumors. Tumors were irradiated using a Siemens’ 6 MV linear accelerator at the Rhode Island Hospital Department of Radiation Oncology. The mice were lightly anesthetized with methoxyflurane (“Metofane”; Pitman-Moore, Inc., Washington Crossing, NJ), and were then placed on a 1.5-cm-thick slab of Lucite. Mice were restrained in place by tape and allowed to recover from the anesthesia. Two mice were irradiated at a time and were positioned with the tumors in a 3 x 3 x 3 cm irradiation field, with the photon beam traversing the Lucite slab from above to obtain proper electronic equilibrium at the tumor. A source-to-target distance of 100 cm was used, and the mice were irradiated at ambient temperature at a dose rate of 2.0 Gy/min. Tumors were approximately 300 mg when they were irradiated.

Tumor Regrowth Delay Study. Following X-ray treatment, mice were followed for radiation effects on tumor size. Tumor dimensions were obtained at least 3 times weekly for a period of about 3 weeks following photon irradiation, and tumor sizes in cu mm were estimated, using the formula given previously.

As an index of the sensitivity of these xenografted carcinomas to photon irradiation, or NMF or to the combination, we calculated the time (in days) after irradiation needed for the tumors to grow to twice the volume at the time of irradiation. As this time will increase with increasing drug and/or radiation dose, the calculation allows the construction of dose-response curves for the different treatment groups. The curves can then be used to intercompare the relative sensitivities of the DLD-2 tumors to single or combination modalities.

NMF Treatment. Mice were treated i.p. with NMF (150 mg/kg) (1.5% NMF in 0.9% NaCl solution) daily for 19 days. This was one-half the 300-mg/kg dose of NMF administered on the same schedule in an earlier study, which caused a significant inhibition of tumor growth (8). The 150-mg/kg dose was shown in preliminary experiments to have a modest effect on DLD-2 tumor growth. Therefore, it was concluded that this lower dose would allow a better demonstration of interactive effects with radiation, compared to a higher NMF dose with a significant tumor growth-inhibiting effect alone.

NMF-Radiation Combination Treatment. Nude mice bearing approximately 300-mg s.c. implants of DLD-2 tumors were treated i.p. with NMF (150 mg/kg) daily for 19 days. Local tumor irradiations were administered as graded single doses or as fractionated doses (daily for 4 days, to study repair of radiation damage), following the third NMF injection. Therefore, polar solvent treatment both preceded and followed the radiation exposures, which were administered on Day 3 (single doses), or on Days 3 to 6 (fractionated doses) of NMF injections. Altogether, 4 independent experiments were conducted. Each experiment included groups of control mice (0.9% NaCl solution only), NMF treated mice only, radiation-only treated mice (graded and/or fractionated doses), and mice treated with NMF plus radiation (graded and/or fractionated doses). Each group contained 10 mice.

In Vitro X-irradiation Studies. As a complement to the in vivo studies on the responses of DLD-2 xenograft tumors in nude mice, parallel studies on the DLD-2 tumor line in vitro were performed. Exponentially growing DLD-2 cells were cultured in RPMI Medium 1640 containing 15% fetal bovine serum (Grand Island Biological Co., Grand Island, NY). These cultures were irradiated with graded doses of X-rays (Philips 200/250 therapeutic X-ray machine, Eindhoven, The Netherlands) under operating conditions of: 250 kVp, 15 ma, dose rate, 1 Gy/min, with a 0.4-mm Thoraxus filter. Exposure doses were corrected using appropriate factors to obtain the absorbed doses (in Gy). After irradiation, the tumors were detached from the surface of 25-sq cm plastic flasks (Falcon Products, Oxnard, CA), using 0.03% trypsin-EDTA (Grand Island Biological Co.). The cells were rinsed once with trypsin-EDTA, and then were incubated with a second application of trypsin-EDTA for 7 min at 37°. The cells were then added to an equal volume of complete medium containing serum, mixed, and centrifuged at 1000 rpm for 7 min. The cells were then resuspended in complete medium, counted, and appropriate cell numbers were seeded into 60-mm dishes (Falcon Products) to obtain colonies. After 18 days of incubation at 37° in a humidified environment of 95% air-5% CO2, the medium was removed from the plates, and the colonies were rinsed gently with 0.9% NaCl solution, and then fixed and stained with a 0.5% solution of crystal violet in absolute methanol. Only colonies containing more than 50 cells were counted.

In some experiments, the ability of these cells to repair sublethal radiation injury was studied, using a split-dose technique. In these experiments, a single large dose of X-rays (4.0 Gy) was given to reduce survival to a level of about 5% that of control cultures. Then, the cultures were returned to the 37° incubator for 12 hr, at which time they were given a second graded series of X-ray exposures. Any repair of radiation injury from the first exposure will result in a displacement in the position of the survival curve of the cells receiving the 2-dose irradiations from the single-dose-response curve. The fractional amount of dose repaired can then be determined from the degree of displacement of the split-dose curve from that of cells receiving the same amount of irradiation, but in a single exposure.

RESULTS

The DLD-2 tumors used in this study were 331 ± 37 (S.E.) mg at the time of irradiation. To assess the effects of 0.9% NaCl solution, NMF, photon irradiation, or NMF plus photon irradiation on the xenografted colon cancers, the times required for tumors to double their initial volume were determined from graphs of tumor growth versus time. Control DLD-2 carcinomas (in mice receiving 0.9% NaCl solution) doubled their volume in 3.0 ± 0.3 days. DLD-2 tumors in mice treated with NMF only achieved a volume doubling in 4.7 ± 0.4 days. Thus, NMF at a dose of 150 mg/kg had a growth-inhibiting effect on these cancers; the 57% increase in doubling time is statistically significant (Student's t test; p < 0.05) (10). The growth of the DLD-2 tumor in control mice, and the effect of NMF (150 mg/kg) injected daily for 19 days on the growth of the colon carcinoma are shown in Chart 1. We have reported previously that NMF administered on the same schedule at a dose of 300 mg/kg produced a marked inhibition of the growth of DLD-2 tumors (8).

The effect of 6 MV photon irradiation on the xenografted carcinomas is summarized in Table 1. There is a clear dose response when the amount of ionizing radiation is increased from 2.5 to 13.75 Gy. Interestingly, when split-dose schedules of 2.5 Gy daily for 4 days and 3.44 Gy daily for 4 days were used (corresponding in total dosage to 10.0 and 13.75 Gy, respectively), approximately equivalent responses to the appropriate single dose were observed (Table 1), indicating little or no repair...
of sublethal radiation injury. The response of the DLD-2 tumor to 10.0 Gy photon irradiation is shown in Chart 2. The data have been normalized to an initial control tumor volume of 1.0.

We performed single- and split-dose schedule irradiations of cultured DLD-2 cells to determine whether we could corroborate the apparent inability of the solid tumor to repair sublethal radiation damage. The survival response data of cells exposed to graded doses of X-rays are shown in Chart 3, together with the survival response of cells given an initial dose of 4 Gy (sufficient to reduce survival to about 5% that of control), and 12 hr later given a second, graded-dose exposure. It can be seen that the survival response of these cells is a single exponential curve with no indication of a "shoulder" in the low-dose region of the curve. This response is in contrast to other human colon tumor lines that we have studied previously that do possess such a shoulder (19). The lack of a nonexponential region in the low-dose region of the survival has traditionally been interpreted as a failure of cells to accumulate sublethal radiation injury before death, i.e., an inability to repair radiation damage. This lack of repair ability indicated by the shape of the graded single-dose curve is supported by the results of the split-dose X-ray experiment with cultured DLD-2 cells (Chart 3). Even with a 12-hr interval between the 2 radiation exposures, there is no difference between the single-dose curve and the split-dose curve, indicating that there is no repair of radiation injury. The mean lethal inactivation (D0) dose for these DLD-2 tumor cells (i.e., the dose needed to reduce survival to 1/e of incident survival) is 1.35 ± 0.28 Gy for the single-dose exposures, and 1.19 ± 0.20 Gy for the split-dose survival curve data (mean dose ± 95% confidence limits). These D0 values are not statistically different (10). Our in vitro findings, therefore, completely corroborate the failure to repair radiation damage seen in the in vivo fractionated irradiation exposures.

<table>
<thead>
<tr>
<th>Photon dose (Gy)</th>
<th>NMF (150 mg/kg)</th>
<th>Time to reach twice tumor volume at time of radiation (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>−</td>
<td>3.0 ± 0.3*</td>
</tr>
<tr>
<td>2.5</td>
<td>−</td>
<td>3.3</td>
</tr>
<tr>
<td>5.0</td>
<td>+</td>
<td>3.9</td>
</tr>
<tr>
<td>6.25</td>
<td>+</td>
<td>4.6</td>
</tr>
<tr>
<td>10.0</td>
<td>−</td>
<td>6.6 ± 0.1</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10.5 ± 0.2</td>
</tr>
<tr>
<td>4 × 2.5</td>
<td>−</td>
<td>6.4 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>10.8 ± 0.2</td>
</tr>
<tr>
<td>13.75</td>
<td>−</td>
<td>10.6 ± 1.0</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>15.9 ± 1.2</td>
</tr>
<tr>
<td>4 × 3.44</td>
<td>−</td>
<td>11.9</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>16.4</td>
</tr>
</tbody>
</table>

* Mean ± S.E.
Effect of Radiation and NMF on Human Colon Tumors

![Graph](chart1.png)  
**Chart 3.** Fractional survival response of DLD-2 human colon tumor cells is depicted. ©, single-dose responses; O, split-dose survival responses (initial dose, 4.0 Gy; 12-hr interval, graded series of second doses). Points, mean; bars, S.E.

NMF in combination with radiation resulted in an enhanced inhibition of tumor growth at all doses studied. Furthermore, the extent of the enhancement increased as a function of the dose of radiation. When NMF was combined with 13.75 Gy, the time required for tumor volume doubling was 15.9 days, compared to an excess growth delay of 1.7 days for NMF alone, and 10.6 days for 13.75 Gy of radiation only. The additional 3.6 days represents a more than 2-fold increase in excess growth delay over what would be expected if the 2 agents interacted additively. When NMF was superimposed on the radiation fractionation protocols, the enhancement was approximately the same as was observed with the appropriate single dose of photons. (Table 1). The dose responses of DLD-2 tumors to irradiation, with and without NMF, as measured by tumor regrowth delay times, are shown in Chart 4. Also shown is the predicted response if the modalities produced an additive effect. Chart 4 illustrates that the enhancement of the radiation response caused by NMF increases with increasing radiation dose.

**DISCUSSION**

The major finding of this study is that NMF enhances the in vivo radiation response of a human colon tumor xenograft. The effect is observed when either single or fractionated doses of photon irradiation are administered during the course of NMF treatment. These data extend our earlier findings that polar solvents enhance the responses of cultured mouse mammary tumor and human colon cancer cells to X-irradiation (18, 19). The in vivo results presented here are important, because the increased radiation sensitivity of cultured carcinoma cells caused by prior exposure to polar solvents would not necessarily predict for an NMF-effected increased sensitivity of a solid tumor to ionizing radiation. The 3-dimensional architecture of the tumor tissue, the presence of an hypoxic fraction within the tumor, host-tumor interactions, environmental effects, and dose-limiting organ toxicities are factors that might affect and limit the efficacy of polar solvents as radiation-enhancing agents in vivo. However, the results of this study demonstrate that NMF does cause an enhancement of the effect of photon irradiation against a xenografted human colon tumor.

NMF is a good inducer of differentiation in the murine Friend erythroleukemia and human promyelocytic HL-60 leukemia systems (1). The polar solvent has been effective in inhibiting the growth of murine sarcomas and human colon, breast, and lung tumor xenografts (8, 21). NMF has recently entered Phase 1 clinical trials, and Phase 2 studies are anticipated (20, 22). However, there are 2 difficulties associated with the use of NMF, or other maturational agents, as single agents for the therapy of human cancers. First, it is not certain whether polar solvents are acting as cytotoxic drugs, rather than as differentiating agents against at least some types of human solid tumor xenografts. Second, various groups have reported that polar solvents and other inducers such as sodium butyrate induce the conversion of several types of cultured cancer cells to less malignant, better-differentiated phenotypes in a reversible manner (3, 12, 16). Agents that reversibly cause maturational changes in tumors might not be expected to do more than slow down disease progression for a period of time.

Because of these considerations, we have advocated for some time the use of differentiation-inducing agents in combination...
with other modalities, including radiation (4, 7, 24). The feasibility of including polar solvents in a protocol using radiation as the antitumor agent was demonstrated in our earlier studies with cultured murine and human cancer cells (18, 19). In this regard, Einspänner et al. (9) have recently reported that dimethyl sulfoxide causes an increased sensitization of Friend cells to ionizing radiation at a time when the polar solvent is inducing the commitment to terminal differentiation in these cells. The use of an agent like NMF in combination with ionizing radiation in clinical trials is further supported by the results of the study reported here. Patients might benefit from whatever efficacy drugs like NMF can confer as single agents, and the enhancement of radiation treatment by a polar solvent might further improve the effectiveness of therapy.

The mechanism whereby NMF modulates the radiation response of the DLD-2 colon tumor, or of cultured colon and mammary carcinoma cells, is not known. One possibility is that polar solvents induce differentiation in tumors, and the more benign, posttreatment tumors are more sensitive to radiation. Hill et al. (13) have reported that a better-differentiated melanoma clone was more sensitive to X-rays than was a more poorly differentiated subpopulation. Cultured mouse and human neuroblastoma cells have been induced to differentiate by serum deprivation, and the more mature cells were shown to be more responsive to UV radiation (15, 17). However, it should be pointed out that the general finding has been that normal (differentiated) tissues are often more radioresistant than are most tumors. Furthermore, there is no evidence that polar solvents induce differentiation in human solid tumor xenografts, and the responses of carcinomas to agents like NMF may be explained by toxic rather than maturational events (8). Yet another possibility is suggested by recent data showing that DMF and NMF cause a rapid, marked decrease in intracellular glutathione levels in colon cancer cells. This decrease could reduce the ability of the cells to scavenge radiation-produced free radicals, thus increasing the DNA damage.4

In summary, earlier results from our laboratory with cultured cancer cells implicated polar solvent modification of intracellular repair processes as being involved in the radiation enhancement phenomenon (18, 19). However, in this study DLD-2 cultured cells and tumors did not exhibit a significant ability to repair sublethal radiation damage. Therefore, the observed increased tumor growth inhibition is probably not mediated through this mechanism (at least in this tumor system). Our analysis of the enhancement by NMF of the effect of photon irradiation on DLD-2 tumors, based on isoeffect considerations, suggests that in this system NMF may be acting by increasing the number of radiation-induced lesions, perhaps through depletion of intracellular glutathione levels. These alternative mechanisms are being investigated in our laboratory.

REFERENCES

Enhancement by N-Methylformamide of the Effect of Ionizing Radiation on a Human Colon Tumor Xenografted in Nude Mice


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