Enhancement of Tumor Radioresponse of a Murine Mammary Carcinoma by Paclitaxel[1]

Luka Milas,[2] Nancy R. Hunter, Kathryn A. Mason, Belma Kurdoglu, and Lester J. Peters

Departments of Experimental Radiotherapy [L. M., N. R. H., K. A. M., B. K.] and Clinical Radiotherapy [L. J. P.], The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

ABSTRACT

Paclitaxel is a chemotherapeutic agent with potent microtubule stabilizing activity that arrests cells in G_2-M. Because G_2 and M are the most radiosensitive phases of the cell cycle, paclitaxel has potential as a cell cycle-specific radiosensitizer. In this study, we investigated the ability of paclitaxel to increase tumor radioresponse *in vivo* using a murine mammary carcinoma and the dependency of this response on accumulation of tumor cells in mitosis. Mice bearing 8-mm tumors were treated with paclitaxel (60 mg/kg i.v.), 9, 15, or 21 Gy of single-dose radiation, or with a regimen of both agents in which radiation was given 1, 9, or 24 h after paclitaxel. The effect of the treatments was determined by tumor growth delay. Microscopically, the percentage of mitotically arrested cells was only 4% 1 h after treatment with paclitaxel, increased to a maximum value of 30% at 9 h, and decreased to 12% 24 h after paclitaxel. Paclitaxel enhanced tumor radioresponse by factors of 1.21 to 2.49. The degree of enhancement increased with increases in both the dose of radiation and the time between paclitaxel administration and radiation delivery. Radiation efficiently destroyed mitotically arrested cells by apoptosis. The greatest enhancement of radiation response was not at the time of the highest mitotic arrest but at 1 day after paclitaxel treatment, showing that paclitaxel potentiates tumor radioresponse by mechanisms in addition to blocking the cell cycle in mitosis, possibly by tumor reoxygenation. Thus, these results show that paclitaxel is a potent *in vivo* radiopotentiating agent and has the potential to be usefully combined with radiotherapy.

INTRODUCTION

The possibility of using chemotherapeutic agents to selectively enhance radiation response in tumors is an appealing approach to improving the results of cancer treatment. Besides augmenting the cytotoxic action of radiation, combined modality treatment offers the prospect of spatial cooperation, whereby the powerful local effect of precisely directed radiation therapy on gross tumor deposits is complemented by the systemic effect of drugs on micrometastatic disease. The ideal drug for this therapeutic strategy would have potent independent anticancer action as well as the ability to sensitize radioresistant tumor cells to the lethal effects of ionizing radiation. Paclitaxel, a diterpene compound derived from the bark of the Western yew *Taxus brevifolia*, could meet both requirements; it possesses antitumor activity against a variety of common cancers (1–3) and shows a strong capability for radiosensitizing tumor cells growing *in vitro* (4, 5).

Although exactly how paclitaxel kills tumor cells is unknown, it appears that it acts by promoting inappropriate microtubule assembly and stabilizing tubulin polymer formation (6, 7). These microtubular changes affect cells mostly in G_2 and M, preventing completion of cell division, which in turn results in accumulation of cells in G_2 and M (4, 7). Many of the arrested cells are doomed to die (8); however, what proportion of the paclitaxel-arrested cells are capable of further survival is unknown.

This ability of paclitaxel to arrest cells in G_2 and M makes paclitaxel a potential cell cycle radiosensitizer because G_2 and M cells are more radiosensitive than cells within other cell cycle phases (9). Only a few studies reported thus far have tested the interaction between paclitaxel and radiation, and they were performed using tumor cells growing *in vitro* (4, 5). One report used astrocytoma cells and showed that paclitaxel-treated cells exhibited an enhanced radiosensitivity only when irradiated in G_2-M (4). In another study (5), paclitaxel was shown to have a significant radiosensitizing effect on both proliferating and nonproliferating cells of three different ovarian carcinoma cell lines. Proliferating cells were more radiosensitized than nonproliferating cells, which is consistent with paclitaxel having cell cycle effects. However, the finding that the nonproliferating cells were also significantly radiosensitized suggests that, in addition to the cell cycle perturbation, there must exist some other mechanisms by which paclitaxel potentiates cellular radioresponse.

To our knowledge, no studies thus far have addressed the radiosensitizing action of paclitaxel on tumors *in vivo*. Such studies are essential for addressing questions of practical therapeutic application as well as elucidating the mechanisms of radiosensitization. The study reported here was performed to determine whether paclitaxel enhances radioresponse of tumors growing *in situ* and whether the enhancement is dependent on the accumulation of cells in mitosis. The tumor used was a murine mammary carcinoma that responds well to the treatment with paclitaxel only (8).

MATERIALS AND METHODS

Mice and Tumors. C3Hf/Kam female mice, bred and maintained in our own specific pathogen-free mouse colony, were used. Animals used in this study were maintained in facilities approved by the American Association for Accreditation of Laboratory Animal Care and in accordance with current regulations and standards of the United States Department of Agriculture and Department of Health and Human Services. They were 3 to 4 months old at the beginning of the experiments and were housed 3–5 per cage. The tumor used was a nonimmunogenic mammary carcinoma, designated MCA-4, syngeneic to this strain of mice, which was in its fourth isotransplant generation. Solitary tumors were generated in the muscles of the right thigh of mice by the inoculation of 5×10^5 viable tumor cells. Tumor cell suspensions were prepared by enzymatic digestion of nonnecrotic tumor tissue (10), and the viability was in the range of 80–85% as determined by trypan blue exclusion and phase microscopy.

Irradiation. The tumor-bearing legs were locally irradiated with single doses of γ-radiation, ranging from 9 to 21 Gy, delivered from a dual-source ^{137}Cs irradiator at a dose rate of 7 Gy/min. During irradiation, unanesthetized mice were immobilized on a jig, and the tumor was centered in a 3-cm diameter irradiation field.

Paclitaxel. Paclitaxel was kindly supplied by the Bristol-Myers Squibb Co., Wallingford, CT. Paclitaxel was initially dissolved in absolute ethanol with an equal volume of cremophor (Sigma Chemical Co., St. Louis, MO), sonicated for 30 min, and stored at 4°C for up to 1 week. This stock solution (30 mg/ml) was further diluted 1:4 with sterile physiological saline within 15 min of injection. The paclitaxel solution was then injected i.v. at a dose of 60 mg/kg body weight.

Tumor Growth Delay Assay. Tumor growth was determined at 2- to 3-day intervals or more frequently as needed by measuring three orthogonal tumor diameters with vernier calipers. When tumors grew to 8 mm in diameter,

they were exposed to 9-, 15-, or 21-Gy radiation. Tumors in mice treated with paclitaxel were irradiated with the same doses at 1, 9, or 24 h after injection. These irradiation times were selected on the basis of our earlier study (8) to correspond with a small initial increase in mitotic arrest (1 h after paclitaxel), its peak value (9 h), or with a subsequent considerable decline in mitotic arrest (24 h) with the objective of determining to what extent radiosensitization by paclitaxel was dependent on its blockade of cells in mitosis. After treatment, tumor growth was followed until tumors reached at least 12 mm in diameter. The effect of the treatments on tumor regrowth was expressed either as absolute growth delay, which is defined as the time in days for tumors in the treated groups (paclitaxel or radiation) to grow from 8 to 12 mm in diameter minus the time in days for tumors in the untreated control group to reach the same size, or as normalized growth delay, which is defined as the time for tumors in groups treated with both paclitaxel and radiation to grow from 8 to 12 mm minus the time to reach the same size in mice treated with paclitaxel alone. Groups consisted of 7 to 14 mice each.

Histological Analysis of Mitotic Arrest and Apoptosis. At different times ranging from 1 to 96 h after treatment with paclitaxel, the mice were killed by cervical dislocation, and the tumors were immediately excised and placed in neutral-buffered formalin. The tissue was then embedded in paraffin blocks from which 2- to 4-μm sections were cut and stained with hematoxylin and eosin. Both mitosis and apoptosis were scored in coded slides by microscopic examination at ×400 magnification. The morphological features used to identify apoptotic bodies have been described and illustrated in earlier publications from our laboratory (11, 12). Five fields of nonnecrotic areas were randomly selected in each histological specimen, and in each field, the number of apoptotic nuclei and cells in mitosis were recorded as numbers per 100 nuclei and expressed as a percentage. The percentage was based on scoring 1500 nuclei, obtained from three mice per group.

RESULTS

The influence of paclitaxel on tumor radioresponse was studied in mice bearing 8-mm tumors. The mice were given 60 mg/kg paclitaxel i.v., and 1, 9, or 24 h later, their tumors were irradiated with a single dose of 15-Gy γ-irradiation. The antitumor effect of the treatments was expressed as the tumor growth delay values (Table 1). The results show that the combined treatment with paclitaxel and radiation delayed tumor growth more than the additive effect of individual treatments, implying that paclitaxel acted as a radiopotentiating agent. This radiopotentiating effect of paclitaxel was achieved by all treatment schedules; however, the degree of radiopotentiation increased as the time interval between paclitaxel administration and tumor irradiation was increased. The EFs[3] were 1.47 when irradiation was given 1 h, 1.7 when irradiation was given 9 h, and 2.49 when irradiation was given 24 h after paclitaxel administration. Therefore, the degree of radiopotentiation did not correlate with the mitotic index of tumor cells at the time of radiation delivery, which reaches its peak at 9 h (8).

The radiation dose-response of the radiopotentiating effect of paclitaxel was studied in an experiment in which the mice bearing 8-mm tumors were treated i.v. with 60 mg/kg paclitaxel and 9 or 24 h later received graded single doses of local tumor irradiation, ranging from 9 to 21 Gy. Fig. 1 shows a radiation dose-dependent delay in tumor growth in mice treated and untreated with paclitaxel. The delay was longer in mice treated with paclitaxel, and more so in mice treated with paclitaxel 24 than 9 h before irradiation. To quantify the extent of paclitaxel-induced tumor radiopotentiation, we plotted normalized tumor growth delay values (*i.e.*, time in days that tumors were required to grow from 8 to 12 mm) produced by radiation in paclitaxel-treated or untreated mice were plotted as a function of radiation dose (Fig. 2). For both 9 and 24 h, the enhancement of tumor radioresponse was larger as the radiation dose was increased. The enhancement factors at a tumor growth delay level of 6 days were 1.21

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[3] The abbreviations used are: EF, enhancement factor; TBE, tumor bed effect.

Table 1 *Effect of paclitaxel on radioresponse of MCA-4 tumor: influence of time interval between paclitaxel administration and radiation delivery*

Treatment[a]	Time in days that tumors are required to grow from 8 to 12 mm[b]	Growth delay[c]	EF[d]
None	5.2 ± 0.2		
Paclitaxel	12.4 ± 0.5	7.2	
Radiation	10.9 ± 0.3	5.7	
Paclitaxel + radiation (1 h)	20.8 ± 1.1	8.4	1.47
Paclitaxel + radiation (9 h)	22.1 ± 0.7	9.7	1.70
Paclitaxel + radiation (24 h)	26.6 ± 1.3	14.2	2.49

[a] Mice bearing 8-mm tumors in the right thighs were given i.v. 60 mg/kg paclitaxel or 15 Gy local tumor irradiation. When the two agents were combined, irradiation was given 1, 9, or 24 h after paclitaxel. Groups consisted of seven mice each.

[b] Mean ± SE.

[c] Normalized growth delay is defined as the time in days for tumors to reach 12 mm in mice treated by the combination of paclitaxel and radiation minus the time to reach 12 mm in mice treated by paclitaxel alone.

[d] EF was calculated as the ratio of normalized growth delay in mice treated with paclitaxel plus radiation over absolute growth delay in mice treated by radiation alone. Absolute growth delay is defined as the time in days for tumors in the treated groups (paclitaxel or radiation) to grow from 8 to 12 mm minus the time in days for tumors in the untreated control group to reach the same size. Normalized growth delay is defined as the time for tumors in groups treated with both paclitaxel and radiation to grow from 8 to 12 mm minus the time to reach the same size in mice treated with paclitaxel alone.

when paclitaxel was given 9 h and 1.48 when paclitaxel was given 24 h before irradiation, and at the level of 8.5 days, 1.5 and 1.96 when paclitaxel was given 9 or 24 h before irradiation, respectively (Fig. 2).

Additional groups of tumors treated with paclitaxel, irradiation, or both were histologically analyzed to determine a possible relationship of both the extent of mitotic arrest induced by paclitaxel and the magnitude of destruction of the arrested cells by irradiation to the degree of paclitaxel-induced enhancement of tumor radioresponse. These tumors were also assessed for the induction of apoptosis by these treatments to see whether this mode of cell deletion might underlie the observed potentiation of the antitumor effect of radiation by paclitaxel. Histological analysis of tumors treated with paclitaxel only was performed 1, 5, 9, 13, 24, 28, and 36 h after paclitaxel administration. Tumors treated with radiation were histologically analyzed 4 or 12 h after irradiation. According to our earlier studies, radiation-induced apoptosis in solid tumors peaks at 4 h and returns almost to the background level at 12 h after irradiation (12).

As shown in Fig. 3A, paclitaxel induced a profound accumulation of cells in mitosis that peaked at 9 h after its administration, and at which time, about 30% of cells were arrested in abnormal telophase. After this time, the percentage of mitotic cells declined, but it was still considerably increased at 24 h after paclitaxel administration (about 12%). These findings are consistent with our earlier observations (8).

Irradiation delivered 1 h after paclitaxel, at which time the percentage of mitotic cells had increased to only 4.3% from the 1% control value, prevented any further increase in mitotically arrested cells, as determined 4 and 12 h after irradiation. In tumors exposed to radiation only, virtually no mitotic cells were observed at 4 and 12 h after irradiation. Radiation delivered 9 or 24 h after paclitaxel produced a massive destruction of mitotic cells so that 4 and 12 h after irradiation, less than 3% of mitotic cells remained intact. Thus, the largest number of mitotic cells destroyed by radiation was at the 9-h postpaclitaxel treatment point. These data show no temporal correlation between the extent of destruction of mitotically arrested cells and the degree of antitumor efficacy of radiation in paclitaxel-treated mice.

Fig. 3B shows the kinetics of apoptosis induction by paclitaxel, radiation, or both. Apoptosis induced by paclitaxel did not begin until 5 h after paclitaxel administration and then increased with time until it reached a plateau of about 18% 12 h after paclitaxel. The plateau remained until 28 h after paclitaxel, at which time the percentage of apoptotic cells began to decline but still remained high (13%) at 36 h

after paclitaxel. As shown in an earlier, more detailed study by us (8), most apoptotic cells induced by paclitaxel represent morphological manifestations of mitotically arrested cells that died. Compared with paclitaxel, radiation induced more rapid and more extensive apoptosis. By 4 h after irradiation, about 27% of cells in the tumor were apoptotic, and this percentage declined to about 10% at 12 h after irradiation, findings that are consistent with our earlier observations (11, 12).

When irradiation was given 1 h after paclitaxel (Fig. 3B), there was no significant difference in the percentage of induced apoptosis compared to that induced by radiation in tumors untreated with paclitaxel. However, when irradiation was given 9 h after paclitaxel, the percentage of apoptotic cells scored 4 h later increased to about 55%, which was more than the additive effect of paclitaxel and radiation given separately. This percentage declined rapidly below that found in the paclitaxel only-treated mice. When irradiation was given 24 h after paclitaxel, the percentage of apoptotic cells was about 40%, which was equal to the sum of the percentages of apoptotic cells induced by paclitaxel and irradiation given as individual treatments. Here again, the percentage of apoptotic cells rapidly declined below that in mice treated with paclitaxel only. These data show that paclitaxel-treated tumor cells undergo massive and rapid destruction by apoptosis when exposed to radiation but only if they are mitotically arrested.

Fig. 2. Effect of paclitaxel on radioresponse of MCA-4 tumor and normalized tumor growth delay as a function of radiation dose. For treatment details, see Fig. 1, and for definition of normalized growth delay, see Table 1. O, radiation only; ●, paclitaxel and 9 h later radiation; ■, paclitaxel and 24 h later radiation. A and B arrows, the levels at the normalized tumor growth delay at which the EFs were determined. EFs were 1.21 when paclitaxel was given 9 h and 1.48 when paclitaxel was given 24 h before 15 Gy irradiation (arrow A) and 1.5 and 1.96 when paclitaxel was given 9 or 24 h before 21 Gy irradiation (arrow B), respectively.

DISCUSSION

The results of this study show that paclitaxel can increase radioresponse of a murine mammary carcinoma when given to mice i.v., demonstrating that the radiopotentiating effect of paclitaxel is not limited to in vitro settings. Both paclitaxel and radiation were administered as single doses with paclitaxel preceding irradiation by 1, 9, or 24 h. Radiation enhancement factors ranging between 1.21 and 2.49 were obtained, depending on the time interval between paclitaxel administration and irradiation and on the dose of radiation.

The interval between paclitaxel and radiation administrations was a major factor influencing the degree of radiopotentiation. Although paclitaxel enhanced tumor radioresponse at all time points tested, i.e., when given 1, 9, or 24 h before irradiation, the effect was greater as the time period between paclitaxel and irradiation was increased. This increase in the degree of tumor radiopotentiation with time did not correspond to the changes in the percentage of tumor cells mitotically arrested by paclitaxel. The percentage of arrested cells at 1 h after paclitaxel was small (only 4%), rose to its maximum of about 30% at 9 h after paclitaxel, and then declined to about 12% at 24 h after paclitaxel (Fig. 3A). These observations are not in concordance with the findings in vitro (4) that showed that tumor cells treated with paclitaxel exhibited an enhanced radiosensitivity only when irradiated in G_2-M.

Our data show that mechanisms other than the paclitaxel-induced cell cycle perturbation must exist, at least in the in vivo setting, by which paclitaxel potentiates cellular radioresponse. In this study, we addressed a possibility that paclitaxel makes tumor cells more susceptible to radiation-induced apoptosis. There is increasing evidence that various anticancer agents, including radiation (11, 12) and chemotherapeutic drugs (8, 13), induce apoptosis in tumors and that the degree of induced apoptosis correlates with the antitumor effectiveness of cytotoxic agents (13, 14). We have recently reported that paclitaxel is capable of inducing a strong apoptotic response in murine tumors, including the MCA-4 tumor used in the present study (8).
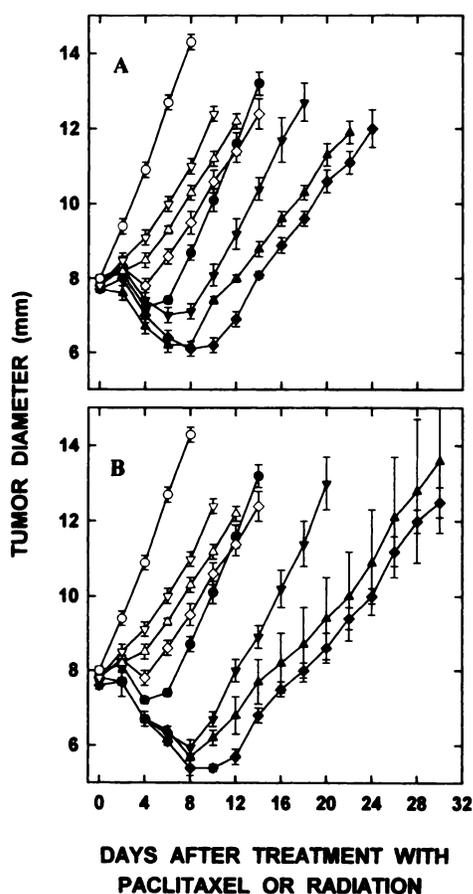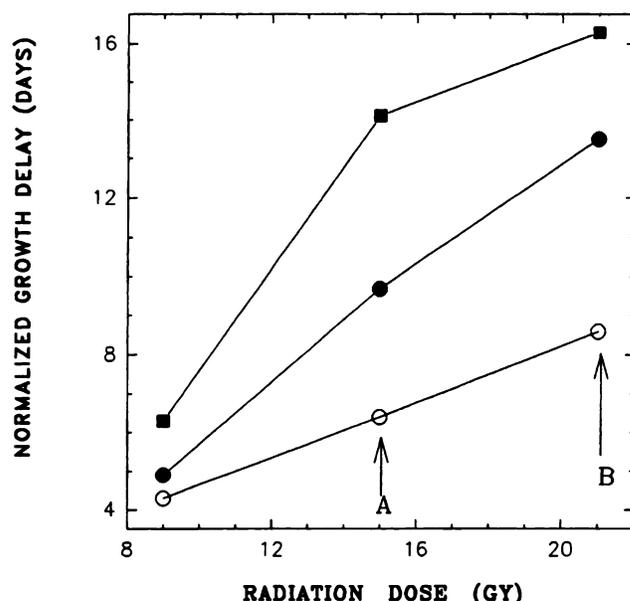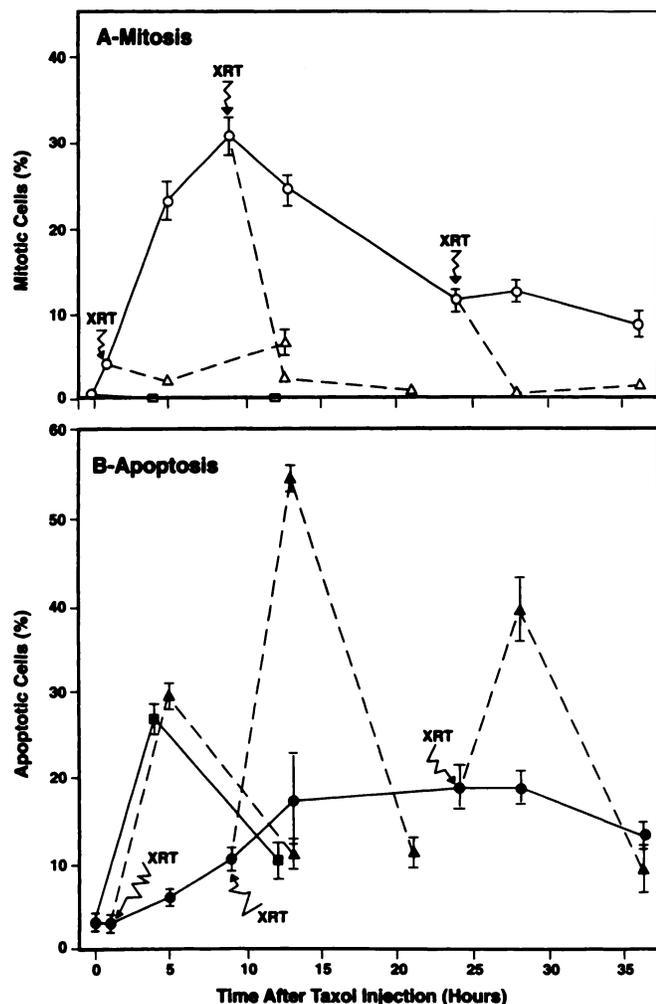

Fig. 1. Effect of paclitaxel on the radioresponse of MCA-4 tumor and the influence of radiation dose. Mice bearing 8-mm tumors in the right thighs were given i.v. 60 mg/kg paclitaxel (●) or local tumor irradiation with 9 Gy (▽), 15 Gy (△), or 21 Gy (◇); or paclitaxel plus 9 Gy (◆), 15 Gy (▲), or 21 Gy (▼). When paclitaxel was combined with irradiation, the latter was given 9 h (A) or 24 h (B) after paclitaxel. 0, untreated. Points, mean; bars, SE. Groups consisted of 7–14 mice each.

Fig. 3. Mitotic arrest (*A*) and apoptosis (*B*) in MCA-4 tumor treated with paclitaxel and radiation. For treatment details, see Table 1. (○, ●), paclitaxel only; (□, ■), radiation only; (△, ▲), paclitaxel plus radiation. *Points,* mean; *bars,* SE. Groups consisted of three mice each.

Paclitaxel-induced apoptosis developed mainly from mitotically arrested cells (8). These findings were confirmed by the present study (Fig. 3*B*) showing that a large percentage of mitotically arrested cells are doomed to die by apoptosis. Because development of apoptosis after paclitaxel treatment depended on mitotic arrest, the pattern of development of apoptosis was similar to the kinetics of mitotic arrest, with the difference being that the development of apoptosis lags several h behind that of mitotic arrest. The apoptotic response induced by paclitaxel persists for about 2 days (Fig. 3*B*; Ref. 8).

In contrast to paclitaxel, radiation induced apoptosis in MCA-4 tumors increased rapidly so that the peak in apoptotic response occurred 4 h after irradiation. Radiation-induced apoptosis rapidly declined, approaching the background level by 12 h after irradiation (Fig. 3*B*; Refs. 11 and 12). The efficacy of radiation in inducing apoptosis in tumors treated with paclitaxel depended on the time when radiation was delivered after paclitaxel administration or whether cells were in mitosis at the time of irradiation. Radiation delivered 1 h after paclitaxel, when only a low percentage of cells were in mitosis, was not more effective in inducing apoptosis than in tumors not treated with paclitaxel. However, when radiation was given 9 or 24 h after paclitaxel, when many cells were in mitosis, there was a significant increase in radiation-induced apoptosis.

Mitotically arrested cells were rapidly destroyed by radiation, most of them by apoptosis. When delivered at the peak of mitotic arrest (9 h after paclitaxel), radiation induced apoptosis so that the percentage of apoptotic cells was higher than the sum of the percentages of apoptotic cells induced by radiation and paclitaxel separately. The percentage of apoptotic cells in tumors treated with radiation 24 h after paclitaxel was equal to the sum of the percentages of apoptotic cells produced by individual treatments. Thus, based on the data at 9 h, it is possible that paclitaxel enhances tumor radioresponse by rendering tumor cells more susceptible to apoptotic death induced by radiation. Overall, these results do not explain the larger increase in tumor radioresponse as the time interval between paclitaxel administration and radiation is prolonged.

An alternative explanation is that treatment with paclitaxel results in reoxygenation of hypoxic tumor cells, a reoxygenation that increases with time. About one third of total tumor cell population becomes mitotically arrested within 9 h after paclitaxel administration, and the majority of these cells die by apoptosis (Fig. 3*B*) or other modes of cell death (8). The dead cells are rapidly removed from the tumor so that at 24 h after paclitaxel, the MCA-4 tumor was histologically depopulated. It is logical to anticipate that this removal of dead cells should result in tumor reoxygenation, which then makes hypoxic tumor cells 2–3 times more sensitive to radiation (9). Since about 30% of cells in 8-mm MCA-4 tumors are hypoxic (15) in untreated air-breathing mice, their reoxygenation would considerably increase tumor radioresponse.

The degree of tumor radiopotentiation by paclitaxel was radiation dose dependent within the range of doses we used, *i.e.,* 9 to 21 Gy, (Fig. 2). Although at present we have no explanation for this, two possibilities may account for the relation. One possibility is the TBE, *i.e.,* tumors in tissues damaged by irradiation grow slower (16). MCA-4 exhibits TBE after about 10 Gy, but the effect is fully expressed after doses between 15 and 20 Gy (16). The slopes of the tumor growth curves after 15 and 21 Gy in paclitaxel-treated mice were somewhat shallower than those after radiation only (Fig. 1), suggesting that paclitaxel might have increased TBE. The other possibility is that paclitaxel enhanced the radiosensitivity of hypoxic cells, the effect of which would be expressed more as the dose of irradiation is increased because tumor response with high radiation doses is dominated by the response of hypoxic cells. Since there is an obvious trend toward lower radioenhancement with a decrease in the radiation dose, it is essential, if clinical application of paclitaxel plus radiotherapy is to be successful, that we determine whether this trend extends down to the range of radiation doses commonly used in clinical practice and whether paclitaxel enhances tumor response to fractionated irradiation.

In conclusion, our data demonstrated that paclitaxel is a potent augmenter of the MCA-4 murine tumor radioresponse *in vivo* when given to mice 1 to 24 h before tumor irradiation. The radiopotentiating activity of paclitaxel increased as the time between paclitaxel administration and radiation delivery was increased, and it also increased with the increase in the radiation dose. We consider destruction of mitotically arrested cells, increase in apoptosis, and tumor reoxygenation to be possible mechanisms for the paclitaxel-induced potentiation of tumor radioresponse.

ACKNOWLEDGMENTS

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