Role of Reoxygenation in Induction of Enhancement of Tumor Radioresponse by Paclitaxel

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ABSTRACT

We reported previously (L. Milas et al., Cancer Res., 54: 3506–3510, 1994) that paclitaxel greatly enhances the response of a murine mammary carcinoma to subsequent irradiation and hypothesized that the enhanced radioreponse was mediated by tumor cell reoxygenation caused by treatment with paclitaxel. Because paclitaxel induced massive tumor cell destruction by apoptosis, it was reasoned that as apoptotic cells were removed from the tumor more hypoxic cells would have access to oxygen, be reoxygenated, and, thus, become more sensitive to radiation. The present study tested this hypothesis by assessing the effect of 60 or 40 mg/kg paclitaxel on radioreponse of an 8-mm MCA-4 tumor irradiated under air-breathing or hypoxic conditions 9, 24, 48, or 72 h after paclitaxel administration. If the hypothesis was correct, paclitaxel would enhance tumor radioreponse more under air breathing than under hypoxic conditions, and the enhancement would increase as the time between paclitaxel administration and tumor irradiation increased within a few days after paclitaxel treatment but only when radiation was given under air-breathing conditions.

The effect of the treatments was determined by tumor growth delay and the radiation dose required to control 50% of the tumors (TCD50). Paclitaxel greatly enhanced tumor radioreponse under air-breathing (and not hypoxic) conditions, increasing tumor growth delay, and reducing TCD50. These effects increased as the time interval between paclitaxel administration and tumor irradiation increased within the observation period of 72 h after paclitaxel treatment. The enhancement factors for tumor growth delay ranged from 1.19 at 9 h to 1.86 at 48 h and for TCD50 from 1.16 at 9 h to 1.47 at 72 h after paclitaxel. Direct measurements of tumor pO2 showed a median value in untreated tumors of 6.2 mmHg, which increased to 10.5 mmHg at 24 h and to 31.2 mmHg at 48 h after paclitaxel administration. Overall, these results show that paclitaxel is a potent enhancer of tumor radioreponse and that its effect is mediated by reoxygenation of hypoxic tumor cells.

INTRODUCTION

Paclitaxel is a chemotherapeutic agent possessing potent antitumor activity against a variety of common cancers in man (1–3) and experimental animals (1, 4, 5). Although the exact mechanism of its cytotoxic activity is unknown, it appears to be related to microtubule assembly. Paclitaxel increases microtubular assembly but inhibits its disassembly. Paclitaxel can also enhance tumor response to radiation in vivo (16). Paclitaxel, administered to mice 1, 9, or 24 h before tumor irradiation, enhanced tumor radioreponse, the degree of which increased as the time interval between administration of paclitaxel and tumor irradiation was lengthened. Contrary to expectation, this enhancement did not correlate with the extent of mitotic arrest caused by paclitaxel because histological analysis revealed the percentage of arrested cells to be about 4% at 1 h, 35% at 9 h, and 12% at 24 h after paclitaxel administration (5, 16). These results implied that mitotic arrest was not the dominant mechanism of paclitaxel-induced enhancement of the in vivo tumor radioreponse. Because most mitotically arrested cells rapidly died, mainly by apoptosis, and were subsequently removed, we hypothesized that this cell loss and removal resulted in reoxygenation of hypoxic tumor cells that survived paclitaxel treatment (16). It is a well-established radiobiological phenomenon that oxygenated cells are 2.5–3 times more sensitive to radiation than are hypoxic cells (20, 21). It was shown previously that a significant reoxygenation occurred in murine mammary carcinomas within 12 hours after exposure to single doses of ionizing irradiation (22).

The experiments described here tested the above hypothesis, i.e., that tumor reoxygenation is a mechanism of paclitaxel-induced enhancement of in vivo tumor radioreponse. The basic premise was that if tumor reoxygenation occurred, the paclitaxel-induced enhancement of tumor radioreponse, measured by both tumor growth delay and tumor cure rate, would increase as the time between paclitaxel administration and tumor irradiation increased but only when irradiation was delivered under air-breathing and not under hypoxic conditions. To correlate these observations with tumor oxygenation status, pO2 was assessed by direct measurements in the tumor.

MATERIALS AND METHODS

Mice and Tumors. We used inbred male C3Hf/Kam mice, bred and maintained in our own specific pathogen-free mouse colony. They were 3–4 months old at the beginning of the experiments and were housed 3–5/cage. The tumor used was a nonimmunogenic mammary carcinoma, designated MCA-4, syngeneic to this strain of mice. When used for these experiments, the tumor was in its 4th isotransplant generation. Solitary tumors were generated in the muscles of the right thigh of mice by the inoculation of 5 X 105 viable tumor cells, syngeneic to this strain of mice. When used for these experiments, the tumor was in its 4th isotransplant generation. Solitary tumors were generated in the muscles of the right thigh of mice by the inoculation of 5 X 105 viable tumor cells. Tumor cell suspensions were prepared by enzymatic digestion of nonnecrotic tumor tissue (23); cell viability was in the range of 80–85% as determined by trypan blue exclusion and phase microscopy.

Paclitaxel. Paclitaxel (Bristol-Myers Squibb Co., Wallingford, CT; and Baker Norton Pharmaceuticals, Inc., Miami, FL) 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 (20 or 30 mg/ml) was further diluted 1:4 (by volume) with sterile physiological solution within 15 min of injection. The paclitaxel solution was then injected i.v. at a dose of 40 or 60 mg/kg body weight. These two
doses have a similar antitumor activity against MCA-4 tumor, and they are much below LD_{50} because they cause no mouse mortality (5).

Irradiation Procedures. Tumors were exposed to single doses of 15–82 Gy γ-radiation when they grew to 8 mm in diameter. Local irradiation to the tumor was performed with a small animal irradiator with 2 parallel-opposed 137Cs sources at a dose rate of 7 Gy/min. During irradiation the mice were immobilized on a jig, and the tumor was centered in the circular irradiation field, which was 3 cm in diameter. The tumors were irradiated under air-breathing (nonclamped tumors) or under hypoxic (clamped tumor) conditions. In the latter case, a clamp was placed across the base of the tumor-bearing leg to occlude circulation to the tumor for 2 min before and during irradiation. The mice were anesthetized before clamping with 0.07 mg/g body weight Nembutal (sodium pentobarbital). The mice whose tumors were irradiated under air conditions were not under anesthesia during tumor irradiation.

The antitumor effect of the treatments was expressed by tumor growth delay, which was the end point used in our initial study on paclitaxel-induced potentiation of tumor radioresistance (16), and by TCD_{50} values. The TCD_{50} value is the end point of tumor radioresponsive and is defined as a radiation dose that yields local tumor control in 50% of animals. This assay enabled determination of the effect of paclitaxel on hypoxic cells. To determine tumor growth delay, tumor growth was determined at 2–3-day intervals by measuring three orthogonal tumor diameters with vernier calipers until tumors reached at least 14 mm in diameter. The groups consisted of 5-11 mice each.

Determination of Hypoxic Cell Fraction in Tumors. The hypoxic cell fractions in tumors treated under air-breathing conditions were estimated by fitting a mathematical model to the combined radiation-dose response data from all TCD_{50} assays (see “Results”; Table 2). This model, developed by Susan Tucker, Ph.D. (Department of Biomathematics, M. D. Anderson Cancer Center) allows calculations of hypoxic cell fractions and their 95% confidence limits. The assumptions underlying the model were:

(a) The relationship between radiation dose and tumor control is given by the Poisson model: Probability (cure) = exp(-λ), where λ is the average number of surviving clonogens/tumor after treatment. A = s × N_{0}, where N_{0} is the initial number of tumor clonogens and s is the surviving fraction.

(b) s is a weighted average of the surviving fractions of oxic and hypoxic cells: s = h × s_{h} + (1 - h) × s_{o}, where h is the hypoxic fraction and s_{h} and s_{o} are the surviving fractions for the hypoxic and oxygenated cells, respectively. The hypoxic fraction is assumed to be 1.0 under clamped conditions and is a parameter to be estimated otherwise.

(c) The cell survival curve for fully oxygenated cells is described by a log-linear model: ln(s_{o}) = ln(K) - D/D_{o}, where K is the extrapolation number.

(d) Oxygen has a dose-modifying effect, i.e., the cell-survival curve for hypoxic cells has the form ln(s_{h}) = ln(K) - D/\text{OER} × D_{o}.

(e) A 40 mg/kg paclitaxel dose causes tumor cell killing, as evidenced by the displacement of the dose-response curves for paclitaxel plus radiation under clamped conditions to the left of the curve for radiation alone.

(f) Administration of paclitaxel has no effect on either the OER or on the radiation D_{o} value. The latter assumption was tested by analyzing the dose-response data obtained under clamped conditions; a model with different D_{o}s for paclitaxel plus radiation versus radiation alone did not fit the data significantly better than a model with a common D_{o} value (P < 0.001).

(g) Paclitaxel given before irradiation of tumors in air changes the hypoxic fraction (h) by an amount that depends on the time elapsed between administration of paclitaxel and radiation.

The tumor-cure model resulting from these assumptions has nine unknown parameters: a constant representing the product of the initial N_{0} and K; the cell survival level after a paclitaxel dose of 40 mg/kg; the D_{o} for oxygenated cells; the OER; and five parameters representing the hypoxic fractions for tumors treated with radiation alone or with paclitaxel given 9, 24, 48, or 72 h before irradiation, respectively. The model was fitted to the dose-response data using maximum likelihood analysis, and the confidence intervals for the parameter estimates were obtained using standard maximum likelihood techniques.

Measurements of Tumor Oxygen Status. Tumor pO_{2} was measured using a Polarographic pO_{2} Histograph (Eppendorf, Hamburg, Germany). The electrodes consist of a gold cathode with an insulating glass sheath and a silver-silver chloride anode; the cathode tip is recessed and covered by an oxygen-permeable membrane. To take the measurement, the skin overlying 8-mm tumors was cut and the cathode was placed through the cut to the surface of the tumor. The electrode was then advanced automatically through the tumor, each forward step followed by a short backward step to avoid artifacts in pO_{2} measurements from the compression of the tissue by the electrode. The pO_{2} measurements were taken in several parts of the tumor, and 20–50 measurements were taken for each individual tumor. The pO_{2} values were expressed as a median value.

RESULTS

Mice bearing 8-mm tumors were given i.v. injections of paclitaxel at a dose of 60 mg/kg, and 9, 24, or 48 h later their tumors were irradiated with 15, 21, or 27 Gy γ-radiation given under air-breathing or hypoxic conditions. The controls were tumor-bearing mice untreated or treated with paclitaxel or local tumor irradiation only. The effect of these treatments was assessed by tumor growth delay. Radiation alone and paclitaxel alone greatly delayed tumor growth but when combined their effect was more than additive. This more-than-additive effect, however, occurred only when tumor irradiation was performed under air-breathing conditions. Table 1 shows tumor growth delay values for groups in which a 21-Gy radiation dose was given. Clearly, paclitaxel-induced enhancement of radiosresponse under air-breathing conditions was higher as the time interval between paclitaxel administration and tumor irradiation became longer.

The extent of paclitaxel-induced enhancement of tumor radio-

### Table 1: Effect of paclitaxel on radioresponse of MCA-4 tumor irradiated under air-breathing or hypoxic conditions: influence of time interval between paclitaxel administration and radiation delivery

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time in days required to grow from 8-12 mm</th>
<th>Absolute growth delay</th>
<th>Time in days required to grow from 8-12 mm</th>
<th>Absolute growth delay</th>
</tr>
</thead>
<tbody>
<tr>
<td>No treatment</td>
<td>5.1 ± 0.2²</td>
<td>6.0 ± 0.4</td>
<td>13.9 ± 0.6</td>
<td>16.2 ± 0.6</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>11.9 ± 0.3</td>
<td>21.0 ± 0.6</td>
<td>27.8 ± 0.8</td>
<td>32.1 ± 1.0</td>
</tr>
<tr>
<td>21 Gy</td>
<td>13.6 ± 0.6</td>
<td>19.0 ± 1.6</td>
<td>22.5 ± 0.8</td>
<td>25.2 ± 0.8</td>
</tr>
<tr>
<td>Paclitaxel + 21 Gy (9 h)</td>
<td>24.1 ± 1.6</td>
<td>17.6 ± 0.7</td>
<td>18.6 ± 0.5</td>
<td>20.6 ± 0.5</td>
</tr>
<tr>
<td>Paclitaxel + 21 Gy (24 h)</td>
<td>27.6 ± 0.8</td>
<td>19.0 ± 0.6</td>
<td>10.3 ± 0.4</td>
<td>11.9 ± 0.4</td>
</tr>
<tr>
<td>Paclitaxel + 21 Gy (48 h)</td>
<td>37.2 ± 1.0</td>
<td>13.9 ± 0.6</td>
<td>13.6 ± 0.6</td>
<td>15.3 ± 0.6</td>
</tr>
</tbody>
</table>

² Mice bearing 8-mm tumors in the right thighs were given 60 mg/kg paclitaxel i.v. or 21 Gy local tumor irradiation. When the two agents were combined, irradiation was given 9, 24, or 48 h after paclitaxel. Groups consisted of 5–11 mice each.

³ Absolute tumor growth delay caused by radiation, paclitaxel, or both agents is defined as the time (in days) tumors required to reach 12 mm from the time of treatment initiation minus the time in days untreated tumors required to grow from 8 to 12 mm.

4 Mean ± SE.
expressed as EF times the TCDsn for paclitaxel plus radiation. The models were fitted to data using maximum likelihood analysis, and 95% confidence intervals for the parameters were obtained using standard likelihood techniques. Numbers in parentheses are 95% confidence intervals.

response can be seen in Fig. 1, which plots NTGD as a function of radiation dose given either under air-breathing or hypoxic conditions. NTGD increased with the increase in radiation dose delivered either under air-breathing or hypoxic conditions; hypoxia, however, made tumors less radiosensitive. Treatment of mice with paclitaxel significantly increased the effect of local tumor irradiation given 9, 24, or 48 h later under air-breathing conditions (Fig. 1A). This enhancement of radiation effect was greater as the time interval between paclitaxel administration and tumor irradiation was longer within the time period used here. To determine EFs, we first constructed tumor growth delay regression lines and then calculated EF by dividing the radiation dose that produced NTGD of 12 days in mice treated by paclitaxel alone. For treatment details, see Table 1. Irradiation only under air-breathing (O) or hypoxic conditions (●); paclitaxel plus irradiation under air-breathing conditions 9 h (△), 24 h (○) or 48 h (●) later. Bars, SE.

The influence of tumor oxygenation status on the ability of paclitaxel to increase tumor radiosensitivity was also tested using tumor cure as the treatment end point (TCD50 assay). Mice bearing 8-mm tumors were given i.v. injections of paclitaxel at a dose of 40 mg/kg, and 9, 24, 48, or 72 h later, their tumors were exposed to single-graded doses of radiation delivered under air-breathing or hypoxic conditions. Radiation doses ranged from 33 to 82 Gy. The TCD50 values at 120 days after irradiation are shown in Table 2. For tumors irradiated under air-breathing conditions, paclitaxel significantly reduced TCD50 values. A control TCD50 value of 70.2 (CI, 68.1–72.3) Gy was reduced to 60.6 (CI, 58.8–62.4) Gy, 54.9 (CI, 52.1–57.6) Gy, 50.0 (CI, 47.3–52.7) Gy, and 47.6 (CI, 44.0–51.2) Gy when the drug was given 9, 24, 48, and 72 h before irradiation, respectively. Thus, like the growth-delay end point, paclitaxel increased tumor radiocurability more as the time interval between paclitaxel administration and tumor irradiation was increased. The EFs were 1.16, 1.29, 1.41, and 1.47 when paclitaxel was given 9, 24, 48, and 72 h before irradiation. Compared to the effect of paclitaxel on tumor radiocurability under air-breathing conditions the effect of paclitaxel on radiocurability of tumors irradiated under hypoxic conditions was minimal (Table 2). The drug reduced the TCD50 of hypoxic tumors, which was 76.2 (CI, 74.3–78.1) Gy, by only 2–5 Gy or less than a factor of 1.1. The effect did not significantly depend on the time interval between paclitaxel administration and tumor irradiation.

These observations are consistent with our hypothesis that tumor reoxygenation is an underlying mechanism of paclitaxel-induced enhancement of the MCA-4 radiosensitivity. We were able to determine the magnitude of the reduction in the fraction of hypoxic cells in 8-mm tumors caused by paclitaxel. Untreated tumors contained 32.1% (CI, 14.7–52.7) hypoxic cells, a value which was reduced to 14.5% (CI, 6.9–24.2), 3.7% (CI, 1.4–7.1), 1.8% (CI, 0.6–3.6), and 1.2% (CI, 0.2–3.1) at 9, 24, 48, and 72 h after treatment with paclitaxel, respectively.

Using the Eppendorf O2 histogram we were able to directly measure a change in oxygenation of 8-mm MCA-4 tumors treated with paclitaxel (60 mg/kg). The O2 measurements were performed at 9, 24, or 48 h after paclitaxel administration, at the times corresponding to radiation treatments. During the 48-h period after paclitaxel administration there was no significant change in tumor size. The O2 histograms in Fig. 2 show the relative frequency of oxygen pressure values in mmHg of untreated and treated tumors. The median O2 value for untreated tumors was 6.8 mmHg, ranging between 0 and 32 mmHg. This value remained unchanged 9 h after paclitaxel administration when the median was 6.6 mmHg (range, 0–35 mmHg) but...
increased to 10.5 mmHg (range, 0–55 mmHg) and to 31.2 mmHg (range, 0–55 mmHg) at 24 and 48 h, respectively, after paclitaxel. The increase in median pO₂ was associated with a decrease in the percentage of pO₂ measurements <2.5 mmHg. These values were: 27% in untreated tumors, and 28, 22, and 5% at 9, 24, and 48 h after paclitaxel treatment, respectively. Thus, these findings revealed that an increase in pO₂ in tumors was caused by paclitaxel, that it started taking place some time between 9 and 24 h after paclitaxel, and that it increased as the time between paclitaxel administration and tumor irradiation increased. In general, these data confirmed the radiobiological findings of paclitaxel-induced reoxygenation by both tumor growth delay and TCD₅₀ assays.

**DISCUSSION**

Tumor reoxygenation was a dominant mechanism by which paclitaxel enhanced radioreponse of the murine mammary carcinoma MCA-4. Paclitaxel-induced changes in tumor oxygenation were determined by using two different end points of tumor radioreponse, tumor growth delay and tumor cure, and by direct measurement of tumor oxygen status. It was postulated that if reoxygenation occurred as a result of paclitaxel administration, tumor radioreponse would be increased more when irradiation was given under air-breathing rather than under hypoxic conditions. The results obtained by both tumor growth delay (Fig. 1) and TCD₅₀ (Table 2) assays supported this contention.

The curves plotted in Fig. 1 show that paclitaxel greatly enhanced the radiation-induced growth delay of tumors irradiated under air-breathing conditions as manifested by the reduction of TCD₅₀ values, which decreased further as the time interval between paclitaxel injection and tumor irradiation increased. Here, the EFs were somewhat lower than those for growth delay, being 1.16, 1.29, 1.41, and 1.47 when paclitaxel was given 9, 24, 48, and 72 h before irradiation, respectively. In contrast, paclitaxel given 9, 24, or 48 h before irradiation reduced TCD₅₀ values of tumors irradiated under hypoxic conditions by only 2–5 Gy, which can be wholly accounted for by the number of clonogenic cells killed by paclitaxel itself. These experiments showed that paclitaxel reduced radiobiological hypoxia in tumors, a major cause of tumor cell resistance to radiation, and that the induced reoxygenation increased as the time between administration of paclitaxel and tumor irradiation increased within the observation period of 3 days. Using the TCD₅₀ assay, it was possible to quantify the reduction in hypoxic cell fraction by paclitaxel. The percentage of hypoxic cells was reduced from 32.1% in untreated tumors to 14.5, 3.7, 1.8, and 1.2% at 9, 24, 48, and 72 h after paclitaxel administration, respectively.

The tumor reoxygenation can be explained on the basis of extensive cell loss in tumors caused by paclitaxel. As reported previously (5, 16), MCA-4 tumors treated with this drug undergo mitotic arrest, after which the arrested cells die by apoptosis or other modes of cell death. Apoptosis begins at about 6 h after paclitaxel administration, increases to its maximum at about 12 h, and remains at that level for about 1–2 days (5, 16). The destruction of tumor cells took place primarily in microregions where cells actively proliferated, implying that cell loss was mainly due to killing of oxic cells. This loss of cells could result in tumor reoxygenation by a number of mechanisms, including shortening the distance between surviving cells and blood vessels, reducing the overall oxygen consumption as fewer cells require less oxygen, and increasing blood delivery to tumor microregions. As more cells are removed from the tumor within the 2–3-day period after paclitaxel administration, it is logical to expect that the degree of reoxygenation would also increase, and that is, in fact, what was observed.

Although the paclitaxel-induced enhancement of tumor radioreponse was demonstrated by both tumor growth delay and TCD₅₀, the enhancement was greater when tumor growth delay was the treatment end point. The reasons for this are at present unclear. Reoxygenation in paclitaxel-treated tumors observed by the above radiobiological determinants was in general confirmed by direct measurements of pO₂ in treated tumors (Fig. 2). The Eppendorf probe showed no change in pO₂ at 9 h after paclitaxel administration. However, at 24 h, pO₂ in paclitaxel-treated tumors was almost twice as high (10.5 mmHg) and at 48 h, five times as high (31.2 mmHg) as in untreated tumors (6.2 mmHg). Why radiobiological measurements revealed some degree of reoxygenation at 9 h after paclitaxel, as suggested by the paclitaxel-induced enhancement of tumor radioreponse under oxic but not hypoxic conditions (Fig. 1), whereas pO₂ measurements did not, is unclear. A likely possibility is that pO₂ probes are not as sensitive as radiobiological end points; pO₂ is measured in only small microareas (involving only a tiny fraction of the total number of tumor cells) within a tumor, whereas the radiobiological end points reflect a change of oxygenation in all tumor cells, including clonogenic cells.

There is already significant evidence that paclitaxel potentiates in vitro cell radiosensitivity through accumulation of cells in the G₂-M cycle (8, 11, 12, 14, 15). Commonly, the cell lines tested were also susceptible to the cytotoxic actions of paclitaxel. However, some cell lines could not be radiosensitized in spite of the fact that they responded to the drug by mitotic arrest and cytotoxicity (14, 17). The cells of MCA-4 tumor appear to belong to this latter group; at least the radiosensitization was undetectable by the...
in vivo assays used here. It is an important question, however, whether tumor reoxygenation is the dominant mechanism in other tumors in which paclitaxel induces significant cell destruction. If it does dominate, it could have a strong positive impact on tumor radiotherapy because tumors, but not normal tissues, commonly contain a large percentage of hypoxic cells. We observed recently that paclitaxel did not enhance radioresponse of mouse jejenum but rather protected it, although the drug induced significant mitotic arrest of cells in jejunal crypts that soon died by apoptosis (24). This jejunal study (24), the experiments with MCA-4 described earlier (16), and the results presented here clearly show that paclitaxel may have a high potential for increasing the therapeutic ratio of radiotherapy. Another important aspect warranting further investigation is whether paclitaxel is an effective enhancer of tumor radioreponse when combined with fractionated clinically relevant radiation doses such as 2 Gy/fraction.

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