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TUMOR REOXYGENATION AS A MECHANISM OF TAXOL-INDUCED ENHANCEMENT OF TUMOR RADIORESPONSE

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Paclitaxel is a novel chemotherapeutic agent that arrests cells in the radiosensitive G_2 and M phases of the cell cycle and as such may act as a specific cell cycle radiosensitizer. We recently reported that paclitaxel induces mitotic arrest in the MCA-4 murine mammary carcinoma and enhances radioresponse of this tumor. However, the greatest enhancement was observed not when radiation was given at the time of peak mitotic arrest, which was 9 h after paclitaxel administration, but when it was given 24 h after paclitaxel. This implied the involvement of other mechanisms in radiosensitization; we hypothesized that tumor reoxygenation was a likely mechanism based on the observed massive loss of mitotically arrested cells at 24 h. The present study shows that paclitaxel greatly enhanced MCA-4 tumor radioresponse when radiation was given under air-breathing conditions (DMF = 1.74), but not when it was performed under hypoxic conditions. This observation supports the hypothesis of tumor reoxygenation as a mechanism of enhancement of tumor radioresponse. That reoxygenation occurred in tumors treated with paclitaxel 24 h earlier was confirmed by direct measurements of pO_2 values, using the Eppendorf pO_2 histograph. Median pO_2 values increased from 6.2 mmHg in untreated tumors to 10.0 mmHg in tumors treated with paclitaxel. These observations emphasize the importance of timing of paclitaxel administration in relation to radiation treatment.

Paclitaxel is a potent chemotherapeutic agent derived from the bark of the western yew, Taxus brevifolia. The drug is effective against different types of malignant tumors, as tested both in experimental animal tumor models and in clinical trials (1). Paclitaxel poisons the mitotic spindle by promoting assembly of microtubules and by preventing microtubule depolymerization (2). Consequently, the transit of cells through mitosis is blocked so that most of the affected cells become arrested in the G_2 and M phases of the cell cycle (2, 3). The arrested cells may die by a number of mechanisms, including apoptosis (4, 5), micronucleus formation (6), and cytolysis (4). However, a proportion of mitotically arrested cells eventually recover and complete the mitotic process.

The ability of paclitaxel to arrest cells in the G_2/M compartment of the cell cycle served as a basic rationale for testing paclitaxel as a cell cycle radiosensitizer because the G_2 and M phases are the most radiosensitive of all cell cycle phases (3). Initial studies on the combination of paclitaxel and ionizing radiation were performed in vitro and showed that paclitaxel can greatly enhance radiation cell kill through the accumulation of cells in the G_2 and M phases (3, 7). Subsequent studies in vitro using several human tumor cell lines have shown that the induction of a G_2/M block may not be a sufficient condition for radiosensitization by paclitaxel; while all paclitaxel-treated cell lines exhibited a G_2/M arrest they were not all radiosensitized (8, 9). These studies (9) have further shown that paclitaxel radiosensitized cells mainly by enhancing the α-component of the radiation damage.

Recently, we demonstrated that paclitaxel can enhance tumor radioresponse in vivo (5). Mice bearing an 8-mm mammary carcinoma, designated MCA-4, were treated

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with a single i.v. injection of paclitaxel and 1, 9 or 24 h later with graded single doses of irradiation to the tumor. Paclitaxel enhanced tumor radioresponse by a range of factors between 1.2 and 2.5. In contrast to what might have been predicted, the maximum level of sensitization was seen at 24 h rather than at 9 h, when the peak of mitotic arrest occurred. Thus, mechanisms other than, or in addition to, the mitotic block were clearly involved in the potentiation of tumor radioresponse, and we hypothesized that tumor reoxygenation was a likely mechanism. Our hypothesis was based on the observation that the paclitaxel-treated cells in MCA-4 tumors undergo massive apoptotic deletion beginning several hours after mitotic arrest reaching a plateau between 12 and 48 h after paclitaxel treatment (4). This cytoreduction should theoretically improve the oxygenation of formerly hypoxic viable cells, thus increasing their radiosensitivity 2–3 fold (10).

We are currently investigating the role of reoxygenation in paclitaxel-induced enhancement of tumor radioresponse, using both radiobiological endpoints, such as growth delay when radiation is delivered under air-breathing or hypoxic conditions and direct measurements of tumor pO_2. We here describe our initial findings.

Material and Methods

Mice. Three-month-old C3Hf/Kam male mice, bred and maintained in our specific-pathogen-free mouse colony, were used.

Paclitaxel. Paclitaxel (Bristol-Meyers Squibb Co., Wallingford, CT: Batch 80635592B, Lot 906) was initially dissolved in absolute ethanol with an equal volume of cremaphor (Sigma Chemical Co., St. Louis, MO), sonicated for 30 min, and stored at 4°C. Immediately before injection, this stock solution (30 mg/ml) was further diluted 1:4 with physiological saline. The final paclitaxel solution was then injected i.v. at a dose of 60 mg/kg body weight.

Tumor. Experiments were performed using fourth generation isotransplants of the MCA-4 tumor, a non-immunogenic spontaneous mammary carcinoma syngenic to C3Hf/Kam mice. Single-cell suspensions were prepared by trypsin digestion of tumor tissue (11). Tumors were generated by injecting 5×10^5 viable tumor cells into the right thigh of mice.

Irradiations. The tumor-bearing legs were locally irradiated under either air-breathing or hypoxic conditions using a dual-source ^{137}Cs gamma-ray unit at a dose rate of 6.9 Gy/min. Air-breathing mice were unanesthetized but restrained in a specially designed jig, and the tumor was centered in the 3-cm-diameter circular irradiation field. Tumor hypoxia was induced by clamping the proximal tumor-bearing thigh for 2 min prior to and during irradiation; these mice were anesthetized with sodium Nembutal (0.07 mg/g body weight).

Response of tumors to paclitaxel and irradiation. When tumors grew to 8 mm in diameter, they were treated with either paclitaxel, a single dose of 21 Gy, or paclitaxel plus irradiation with the drug being given 24 h before 21 Gy. The antitumor effect of the treatments was expressed by tumor growth delay. Tumor size was determined by measuring three orthogonal diameters at 2-day intervals with a vernier caliper. Regression and regrowth of the tumors was followed until tumors reached approximately 14 mm. Groups consisted of 5 mice each.

Tumor oxygen measurement. Tumor oxygenation was measured using the pO_2-Histograph 6650 (Eppendorf, Hamburg, Germany), which directly measures interstitial pO_2 using a dynamic polarographic micro-eletrode. Unanesthetized air-breathing mice were immobilized in a custom-made jig. A 1–2 mm diameter piece of skin was excised over the tumor, and the electrode tip introduced through the tumor capsule under direct vision. The machine was set to advance the electrode through the tumor, measuring pO_2 at 0.4 mm step intervals (advancing 0.7 mm each time with a 0.3 mm backstroke to relieve pressure, after which the reading is taken); the path length was limited to 7 mm. The control group consisted of 4 mice from which a total of 118 pO_2 measurements were recorded. Measurements were also made 24 h after paclitaxel treatment in three mice from which a total of 64 measurements were recorded.

Results

Mice bearing 8-mm MCA-4 were given paclitaxel, 60 mg/kg i.v., and 24 h later their tumors were irradiated with 21 Gy γ-radiation under air-breathing or hypoxic conditions. Groups of tumor-bearing mice that received paclitaxel or 21 Gy local tumor irradiation under air-breathing or hypoxic conditions or remained untreated served as the controls. The effect of these treatments was expressed as tumor growth delay, i. e. time in days tumors needed to grow from 8 to 12 mm, and is shown in the Table.

Both paclitaxel and 21 Gy were effective in delaying tumor growth: paclitaxel delayed it for 7.1 days and irradiation delivered under air-breathing conditions delayed it for 8.5 days. The combined paclitaxel plus tumor irradiation treatment delayed tumor growth more than the additive effects of the individual treatments. In this group, tumor growth delay (NTGD) normalized to the delay of paclitaxel alone was 14.8 days, for a dose-modification factor of 1.74. In contrast, irradiation delivered under hypoxic conditions was similarly effective in mice treated with paclitaxel (NTGD = 7.2 days) and in mice not treated with paclitaxel (NTGD = 7.0 days).

Tumors in mice, sized 8 mm, that received no paclitaxel and tumors in mice that received 60 mg/kg paclitaxel i.v. 24 h earlier were analyzed for oxygen status using the Eppendorf pO_2 histograph. During the 24 h period after

Table

Effect of paclitaxel on radioresponse of MCA-4 tumor irradiated under air-breathing or hypoxic conditions, and on tumor oxygenation status

Group	Treatment[a]	Time in days for tumors to grow from 8 to 12 mm	Normalized tumor growth delay (days)	DMF[e]	Tumor oxygenation[f]	
					Median pO_2 in mmHg	% of measurements with pO_2 values less than 10 mmHg
1	None	4.3 ± 0.4[b]			6.2	64
2	Paclitaxel (60 mg/kg)	11.4 ± 0.5			10.0[g]	50
3	21 Gy (air-breathing)	12.8 ± 1.0	8.5[c]			
4	Paclitaxel + 21 Gy (air-breathing)	26.2 ± 1.2	14.8[d]	1.74		
5	21 Gy (hypoxia)	11.3 ± 0.4	7.0[c]			
6	Paclitaxel + 21 Gy	18.6 ± 0.5	7.2[d]	1.03		

[a] Mice bearing 8 mm tumors were given i.v. 60 mg/kg paclitaxel, or 21 Gy local tumor irradiation under air-breathing or hypoxic (tumor clamp) conditions, or both treatments where paclitaxel preceded irradiation by 24 h. Groups consisted of 5 mice each.
[b] Mean ± S.E.
[c] Obtained by substracting the value of 4.3 (control) from the values of the corresponding treatment groups.
[d] Obtained by substracting the value of 11.4 (paclitaxel only group) from the corresponding combination treatment groups.
[e] DMF = Dose modification factors. DMF of 1.74 was obtained by dividing normalized tumor growth delay in group 4 by that in group 3. DMF of 1.03 was obtained by dividing normalized growth delay in group 6 by that in group 5.
[f] Measured by the Eppendorf pO_2 histograph (see Material and Methods). Four control mice and 3 mice treated i.v. with 60 mg/kg paclitaxel 24 h earlier bearing 8 mm tumors were assayed. There were totals of 118 and 64 pO_2 measurements in control and paclitaxel-treated mice respectively.
[g] $p < 0.02$ compared to the control value of 6.2 mmHg.

paclitaxel administration there was no significant change in mean tumor diameter. The results of pO_2 measurements are shown in the Table. The median pO_2 in control mice was 6.2 mmHg, and 64% of the total of 118 measurements were below 10 mmHg. The paclitaxel-treated tumors were significantly more oxygenated: the median pO_2 value was 10.0 mmHg ($p < 0.02$), with only 50% of the total of 64 measurements being below 10 mmHg.

Discussion

The results presented here show that paclitaxel treatment of mice bearing 8-mm MCA-4 tumor greatly enhances, by a factor of 1.74, tumor response to radiation given 1 day later. The degree of enhancement was similar to that in a study reported by us that assessed the dependence of paclitaxel-induced enhancement of tumor radioresponse on radiation dose and on the time interval between administration of paclitaxel and radiation (5). In that study, the enhancement was greater when paclitaxel was given 24 h before radiation than when it was given 9 h before, although the opposite was expected inasmuch as the peak accumulation of mitotically arrested cells occurred 9 h after paclitaxel. In spite of that expectation the significant tumor cell loss by apoptosis and lytic cell death 24 h after paclitaxel (4, 5), made it reasonable to ascribe the greater enhancement at 24 h to tumor reoxygenation.

If tumor reoxygenation was a dominant mechanism of paclitaxel-induced enhancement of tumor radioresponse, the enhancement should be greater for tumors irradiated under air-breathing than under hypoxic conditions. The results shown in the Table support this hypothesis. While tumor radioresponse under air-breathing conditions was enhanced by a factor of 1.74, the radioresponse of tumors irradiated under hypoxic conditions was not enhanced at all. That reoxygenation occurred in tumors treated with paclitaxel 24 h earlier was confirmed by direct measurements of pO_2 values. The median pO_2 value increased from 6.2 mmHg in untreated tumors to 10 mmHg in tumors treated with paclitaxel.

Tumor reoxygenation can be explained on the basis of large tumor cell loss due to paclitaxel cytotoxicity. As we reported earlier, most of the mitotically arrested cells die by apoptosis or other modes of cell death, which starts after several hours and peaks between 12 h and 2 days after paclitaxel administration (4). This loss of cells is predominantly in the proliferative cell compartment, which is commonly located in the well-oxygenated areas of the tumor, and could lead to tumor reoxygenation by a number of means. By eliminating oxygenated cells, more oxygen becomes available to surviving cells. Also, cell loss may lower the extracapillary pressure on intratumor microvessels, reopen closed capillaries, and increase blood supply to surviving tumor cells. Furthermore, tumor cells may actively migrate from previ-

ously hypoxic microregions to regions closer to blood vessels.

These observations could have important implications for the therapeutic strategy of combining paclitaxel and radiation clinically, emphasizing the importance of timing of paclitaxel administration in relation to radiation treatment. Currently, experiments are underway to assess the kinetics and magnitude of tumor reoxygenation in mice treated with this drug and to establish in more detail the degree of enhancement of tumor radioresponse related to the process of reoxygenation.

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