Optimization of Perfluorochemical Levels with Radiation Therapy in Mice

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ABSTRACT

We have examined the effects of a wide range of levels of Therox, a perfluorochemical emulsion containing bis-perfluorobutylene ethylene (F44E) with carbogen breathing on the tumor growth delay of the Lewis lung carcinoma produced by single dose radiation and fractionated radiation. The enhancement in tumor growth delay with single dose radiation therapy increased as the dose of F44E was increased from 1.2 g/kg (0.03 ml) to 4 g/kg (0.1 ml). As the dose was increased further from 6 g/kg (0.15 ml) to 8 g/kg (0.2 ml) and then to 12 g/kg (0.3 ml), there was a progressive decrease in the tumor growth delay observed. The dose of 4 g/kg was the optimal F44E level with single dose radiation therapy, giving a dose modifying factor of 2.4 ± 0.2. This was true whether administered as a 48% (v/v) emulsion in 0.1 ml or as a 16% (v/v) emulsion in 0.3 ml. When the injection volume was varied from 0.1 ml to 0.4 ml at the 4 g/kg or 6 g/kg dose, thereby varying the emulsion concentration from 48% (v/v) to 12% (v/v) or 18% (v/v), the results tended to indicate that the volume of injection may be more important than the emulsion concentration, i.e., an injection volume of 0.2 ml produced the greatest tumor growth delay for both doses, and the emulsion concentration of 0.2 ml and 6 g/kg of F44E is 36% (v/v). Administering any dose of the emulsion with carbogen for 1 h prior to and during the radiation fraction on Day 1 only of a daily fractionated radiation protocol (3 Gy/fraction × 5 days) had very little effect on tumor growth delay compared to radiation and daily carbogen breathing. When F44E was administered on treatment Days 1, 3, and 5 with carbogen breathing, there was an increased effect on tumor growth delay which reached a maximum at 4 g/kg (0.1 ml) of 10.0 ± 1.2 days compared with 6.7 ± 1.0 days for radiation with daily carbogen breathing. However, when the F44E emulsion was administered every day with fractionated radiation and carbogen breathing, there was a marked enhancement in tumor growth delay observed across the entire dosage range from 4.2 g/kg to 12 g/kg. The F44E dose response curve was very broad so that there was no significant difference in tumor growth delay observed (12.6 ± 1.5 days maximum) from a dose of 2 g/kg (0.05 ml) to a dose of 8 g/kg (0.2 ml). When F44E in the dosage range from 2 g/kg to 8 g/kg in a constant volume of 0.2 ml was administered in various schedules with daily fractionated radiation with carbogen breathing for 1 h prior to and during each fraction, the dose of 4 g/kg administered daily, again, produced the largest enhancement in tumor growth delay, 15.2 ± 1.4 days. Dose and injection volume appear to be the most important variables in achieving optimal tumor response with PFC emulsions.

INTRODUCTION

Persuasive evidence exists that hypoxia protects cells against radiation cytotoxicity (1–4). Hypoxic cells in solid murine tumors have been shown to limit the responses of these neoplasms to treatment with ionizing radiation delivered as a single fraction, and the hypoxic cells surviving large doses of irradiation are capable of either reestablishing the tumor in situ or producing tumors in other animals upon transplantation (5–7). Hypoxic regions have been detected in many animal tumors using O2-microelectrodes (8–11). Evidence for the occurrence of hypoxia in human tumors has been provided by analysis of local control rates in patients with low hemoglobin levels (12) and more recently by 18O2-positron emission tomography in brain tumors (13) and O2-microelectrode measurements in lymph node metastases from squamous cell carcinoma of the head and neck (14).

Several methods have been developed in an effort to overcome the problem of hypoxia. These include the use of hyperbaric oxygen (15), nitroimidazole radiosensitizers (16–20), high linear energy transfer radiations (21), hypoxic cell selective cytotoxic agents (22), hyperthermia (23), and oxygen-carrying perfluorochemical emulsions (24, 25). The oxygen-carrying perfluorochemical emulsion Fluosol-DA has been shown to be effective in enhancing the effects of radiation therapy in a wide variety of solid animal tumors (9, 10, 24–32), and currently a Phase III clinical trial in head and neck cancer comparing standard radiation treatment with or without Fluosol-DA/carbogen breathing is ongoing (33).

PFCs, which are dense, highly water insoluble organic liquids, are excellent solvents for nonpolar gases such as oxygen (34, 35). These materials are essentially metabolically inert, and PFCs with molecular weights in the range of 450,000–550,000 have vapor pressures which allow them to be excreted by expiration. Emulsions of small particle size prepared from PFCs are biocompatible and can carry significant amounts of oxygen when exposed to atmospheres with high partial pressures of oxygen. Fluosol-DA, the only PFC emulsion which has been tested extensively in the United States, contains 10% PFC by volume. Recently, more concentrated PFC emulsions which maintain small particle size and suitable vapor pressure properties for use in animals have become available. A more concentrated emulsion allows several questions concerning the application of these materials with radiation therapy to be addressed which could not have been approached previously with the more dilute emulsions.

MATERIALS AND METHODS

Materials. The perfluorochemical emulsion, Therox (E. I. Du Pont de Nemours & Co., Chemicals and Pigments Dept., Deepwater, NJ), containing 48% (v/v) [33% (w/v)] F44E and egg yolk lecithin as the emulsifier in an isotonic buffer was used as the PFC source. The particle size of the emulsion is 0.25 μm. The half-life of this emulsion in circulation is about 2.5 h and the dwell time of this PFC in tissues is about 7 days (34, 35).

Tumor Growth Delay Experiments. Lewis lung tumor (36–38) was carried out in male C57BL/6J mice (The Jackson Laboratories, Bar Harbor, ME). For tumor growth delay experiments, 2 × 104 tumor cells prepared from a brei of several stock tumors were implanted i.m. into the gastrocnemius muscles of mice. The Lewis lung tumor was grown in 8- to 10-week-old male C57BL/6J mice. When the tumors were approximately 50 mm3 in volume (about 1 week after tumor cell implantation), various volumes of the undiluted F44E emulsion or various dilutions of the undiluted emulsion made with phosphate buffered saline were administered by tail vein injection. The animals were then placed in circulating carbogen (95% O2/5% CO2) chambers. One hour later, the animals, while breathing carbogen, were treated with a single dose of 137Cs γ-rays of 10, 20, or 30 Gy or with multiple 3-Gy

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fractons to the tumor-bearing limb (Gamma Cell 40, Atomic Energy of Canada, Ltd.; dose rate, 0.88 Gy/min). In the fractionated radiation experiments, the tumors received a 3-Gy fraction daily for 5 days. The shield portion of the animal received less than 2% of the delivered dose. The animals were anesthetized (Nembutal) during the radiation treatment. Tumor size was followed by thrice weekly measurements. The experimental end point was the number of days posttumor cell implantation for the tumors to reach a volume of 500 mm³ (~10 times the initial treatment volume) (39). Untreated tumors reach 500 mm³ in approximately 14 days.

Data Analysis. Data from the tumor growth delay experiments were analyzed using a computer program written in BASIC for the Apple II+ microcomputer. The program first derives the best-fit curve for each individual set of tumor volume data and then calculates the median, mean, and standard error for each experimental group. The day on which the tumor reached 500 mm³ and the median, mean, and standard error are then derived. A second program provides statistical comparisons between any number of groups using Student's t test and deriving degrees of freedom and P values. Each experimental group had seven mice, and each experiment was repeated at least twice; therefore, the minimum number of tumors examined at each point was 14 (25).

RESULTS

The PFC emulsion, Therox, itself had no effect on the growth or growth delay produced by X-rays when animals breathed air. Fig. 1 shows the tumor growth delay produced by a range of F44E doses with carbogen breathing and single dose radiation therapy. The enhancement in tumor growth delay increased as the dose of F44E was increased from 1.2 g/kg (0.03 ml) to 4 g/kg (0.1 ml). The dose-modifying factor produced by 1.2 g/kg with carbogen was 1.5 ± 0.1 and that produced by 4 g/kg with carbogen was 2.4 ± 0.2. As the F44E dose was increased further from 6 g/kg (0.15 ml) to 8 g/kg (0.2 ml) and then to 12 g/kg (0.3 ml), there was a progressive decrease in the tumor growth delay enhancement produced. The dose-modifying factors were 1.6 ± 0.1, 1.5 ± 0.1, and 1.3 ± 0.1 with 6 g/kg, 8 g/kg, and 12 g/kg, respectively. Therefore, the 4 g/kg dosage of PFC was the optimal PFC level tested with single dose radiation therapy.

In the experiment described in Fig. 1, the undiluted 48% (v/v) emulsion was used, so that the volume administered varied. Fig. 2 shows the tumor growth delay produced by a single 20-Gy radiation fraction and various doses of F44E but administered in a constant 0.3-ml volume plus carbogen breathing. These studies demonstrated that with a single large dose of radiation the optimal level of F44E was again 4 g/kg, whether it was administered as a 48% (v/v) emulsion in 0.1 ml or as a 16% (v/v) emulsion in 0.3 ml. In addition, for other F44E dosages studied, the degree of tumor growth delay observed was dependent on dose and independent of the volume administered.

Two levels of F44E, 4 g/kg and 6 g/kg, were selected to further examine the question of emulsion concentration (and therefore volume) as a variable in the therapeutic efficacy. As shown in Fig. 3, as the injection volume was varied from 0.1 ml to 0.4 ml at the 4 g/kg dose, thereby varying the emulsion concentration from 48% (v/v) to 12% (v/v), the tumor growth delay of the Lewis lung carcinoma increased from 11.2 ± 0.8 days at 0.1 ml to 13.5 ± 1.2 days at 0.2 ml, then decreased to 11.3 ± 0.8 days at 0.3 ml and to 8.4 ± 1.0 days at 0.4 ml. When the F44E dose was 6 g/kg and the injection volume was varied from 0.15 ml to 0.4 ml, with the emulsion concentration varying from 48% (v/v) to 18% (v/v), the results shown in the lower line in Fig. 3 were obtained. The tumor growth delay increased from 8.2 ± 0.9 days at 0.15 ml to 10.3 ± 1.3 days at 0.2 ml, then decreased to 8.9 ± 1.1 days at 0.25 and 8.1 ± 1.2 days and 7.9 ± 0.8 days at 0.3 ml and 0.4 ml, respectively. These results indicate that volume of injection may be more important than emulsion concentration within the 4 g/kg to 6 g/kg dosage range.

In the clinic, radiation is usually administered on a daily fractionated schedule. We, therefore, examined the effect of

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Fig. 1. Growth delay of the Lewis lung carcinoma produced by single dose radiation therapy with various levels of perfluorochemical and carbogen breathing for 1 h prior to and during X-ray delivery. The treatment groups were, indicating PFC dose (volume of injection): 12 g/kg (0.3 ml) (A); 8 g/kg (0.2 ml) (B); 6 g/kg (0.15 ml) (C); 4 g/kg (0.1 ml) (D); 1.2 g/kg (0.03 ml) (E); and no PFC (F). Points, mean growth delay; bars, SE.

Fig. 2. Growth delay of the Lewis lung carcinoma produced by a single dose of 20 Gy of X-rays preceded by various doses of PFC delivered i.v. in a volume of 0.3 ml with carbogen breathing for 1 h prior to and during X-ray delivery. PFC delivered in a volume of 0.3 ml (F); PFC delivered as the undiluted emulsion (48% v/v) (C). Points, mean growth delay; bars, SE.

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VARIABLE VOLUME/CONSTANT PFC LEVEL
X-RAY DOSE 20 GRAY

![Graph showing growth delay of the Lewis lung carcinoma produced by a single dose of 20 Gy of X-rays preceded by a PFC dose of 4 g/kg (●) or 6 g/kg (○) delivered intravenously (i.v.) in various volumes with carbogen breathing for 1 h prior to and during X-ray delivery. Points, mean growth delay; bars, SE.]

Fig. 3. Growth delay of the Lewis lung carcinoma produced by a single dose of 20 Gy of X-rays preceded by a PFC dose of 4 g/kg (●) or 6 g/kg (○) delivered i.v. in various volumes with carbogen breathing for 1 h prior to and during X-ray delivery. Points, mean growth delay; bars, SE.

FRACTIONATED X-RAYS 5X3 GRAY

![Graph showing growth delay of the Lewis lung carcinoma produced by fractionated radiation treatment (daily for 5 days with 3 Gy/fraction) and various levels of PFC delivered in a constant volume of 0.2 ml with carbogen breathing for 1 h prior to and during X-ray delivery. Points, mean growth delay; bars, SE.]

Fig. 4. Growth delay of the Lewis lung carcinoma produced by fractionated radiation treatment (daily for 5 days with 3 Gy/fraction) and various levels of PFC (undiluted emulsion; 48% v/v) with carbogen breathing for 1 h prior to and during X-ray delivery. PFC was administered i.v. on three different schedules: treatment Day 1 only (●); treatment Days 1, 3, and 5 (○); and treatment Days 1–5 (■). Points, mean growth delay; bars, SE.

DISCUSSION

It has been well established that administration of a biologically compatible perfluorochemical emulsion with carbogen or oxygen breathing prior to and during radiation therapy enhances the effect of radiation in murine solid tumors (9, 10, 24–32). The most widely studied emulsion is Fluosol-DA. The PFCs in Fluosol-DA are perfluorodecalin and perfluorotripropylamine. The range of PFC doses which we have examined previously with Fluosol-DA were from 0.4 to 4.8 g/kg in injection volumes of 0.05 to 0.6 ml (25). The optimal dose of this emulsion plus carbogen breathing on tumor growth delay produced by fractionated radiation (3 Gy daily × 5). Since the half-life of the Therox emulsion in circulation was 2.5 h, most of the circulating F44E was lost in 24 h. As shown in Fig. 4, administering any dose of the F44E emulsion with carbogen breathing for 1 h prior to and during the radiation fraction on Day 1 only had very little effect on tumor growth delay compared to radiation and daily carbogen breathing. When the emulsion was administered on treatment Days 1, 3, and 5 with carbogen breathing, there was an increased effect on tumor growth delay which reached a maximum at 4 g/kg (0.1 ml) of 10.0 ± 1.2 days as compared with 6.7 ± 1.0 days for radiation with daily carbogen breathing. However, when the F44E emulsion was administered every day with fractionated radiation and carbogen breathing, there was a marked enhancement in tumor growth delay observed across the entire dosage range, from 1.2 g/kg to 12 g/kg. The PFC dose-response curve was very broad so that there was no significant difference in tumor growth delay observed with 2 g/kg (0.05 ml) to 8 g/kg (0.2 ml). The maximal effect of 12.6 ± 1.5 days of tumor growth delay was again observed with 4 g/kg (0.1 ml) of F44E.

In Fig. 5, F44E in the dosage range from 2 g/kg to 8 g/kg in a constant volume of 0.2 ml was administered in various schedules with daily fractionated radiation with carbogen breathing for 1 h prior to and during each fraction. Administering the PFC emulsion on treatment Day 1 with daily carbogen breathing produced no significant effect on tumor growth delay compared to fractionated radiation with carbogen breathing. When the PFC emulsion in a volume of 0.2 ml was administered on treatment Days 1, 3, and 5 with daily carbogen breathing, there was an enhancement in tumor growth delay compared to radiation and carbogen breathing which reached a maximum of 11.6 ± 1.3 days with 4 g/kg of F44E. Daily administration of the PFC emulsion with fractionated radiation and carbogen breathing led to the greatest enhancement in tumor growth delay. There was no significant difference between the effects of 2 g/kg of F44E and 4 g/kg of F44E; however, 8 g/kg of F44E was less effective. The dose of 4 g/kg in 0.2 ml, again, produced the largest enhancement, with a tumor growth delay of 15.2 ± 1.4 days which was significantly greater than the tumor growth delay of 12.6 ± 1.5 days produced by 4 g/kg in 0.1 ml of injection volume.

CONSTANT VOLUME/VARIABLE PFC LEVEL
FRACTIONATED X-RAYS 5X3 GRAY

![Graph showing growth delay of the Lewis lung carcinoma produced by fractionated radiation treatment (daily for 5 days with 3 Gy/fraction) and various levels of PFC delivered in a constant volume of 0.2 ml with carbogen breathing for 1 h prior to and during X-ray delivery. Points, mean growth delay; bars, SE.]

Fig. 5. Growth delay of the Lewis lung carcinoma produced by fractionated radiation treatment (daily for 5 days with 3 Gy/fraction) and various levels of PFC delivered in a constant volume of 0.2 ml with carbogen breathing for 1 h prior to and during X-ray delivery. PFC was administered i.v. on three different schedules: treatment Day 1 only (●); treatment Days 1, 3, and 5 (○); and treatment Days 1–5 (■). Points, mean growth delay; bars, SE.
Fluosol-DA was found to be 2.4 g/kg in 0.3 ml; however, with single dose radiation, this dose was not significantly different from 1.6 g/kg in 0.2 ml (25). In the current study, we administered PFC doses between 1.2 and 12 g/kg to mice in injection volumes ranging from 0.03 to 0.3 ml. It appears that the optimal dosage of PFC in the Lewis lung carcinoma model system is 4 g/kg and the optimal volume of injection is 0.2 ml. To deliver 4 g/kg of PFC with Fluosol-DA requires an injection volume of 0.5 ml which is, in itself, stressful to the animals. An injection volume of 0.2 ml, however, is approximately 7% of the vascular volume of 8- to 10-week-old mice and should not result in significant fluid overload.

In terms of the tumor growth delay produced by either PFC emulsion and carbogen added to single dose radiation, the delay caused by Fluosol-DA consistently falls on the PFC dose-dependent effect line for the experimental emulsion used in the current study. This suggests that the character of the PFC in these materials is less important than the level in circulation. It appears that the beneficial effects of PFC with carbogen breathing on tumor growth delay are greatest with PFC doses from 2 to 6 g/kg and that higher levels are less beneficial, perhaps due to a decrease in the off-loading of oxygen from red blood cells in the presence of high concentrations of oxygenated PFC emulsions (40, 41). Alternatively, higher volumes of PFC emulsions are associated with transiently lower systemic blood pressure and hemodilution in rodent models (25, 40, 41). Both conditions may paradoxically decrease oxygen delivery by hemoglobin.

An increase in tumor blood flow may also contribute to the increase in tumor oxygenation by PFC emulsions and the subsequent increase in tumor growth delay produced by these materials in combination with radiation therapy. Klubes et al. (42) and Higara et al. (43) examined the effect of Fluosol-DA on tumor and normal tissue blood flow in animals which were either completely or partially blood/PFC emulsion-exchanged. They found an increase in blood flow in the cerebrum and intracranially implanted Walker 256 tumors in the completely PFC emulsion-exchanged animal, but no significant change in blood flow in either a s.c.-implanted Walker 256 tumor or in the muscle or skin. However, using laser doppler flow methodology, Song noted an increase in blood flow in s.c.-implanted RIF-1 tumors when animals with normal blood volumes were administered 0.3 ml of Fluosol-DA.

Recently, preliminary studies have appeared with other experimental PFC emulsions (44-46). Two of these, F66E and PF60, are concentrated emulsions (20-40% v/v PFC), similar to the F44E emulsion used in the current studies. The dose of PFC used to examine both toxicity (44) and therapeutic efficacy (45) in these studies was approximately 6–12 g/kg of PFC. The authors found a small enhancement in tumor cell killing by radiation in the presence of the F66E emulsion with carbogen breathing, compared to carbogen breathing and radiation alone. These results are consistent with those which we have obtained with a dose of 12 g/kg of F44E. Biaglow and Goodman (46) obtained more positive results with a perfluorocapramante-based PFC emulsion at a dose of about 3 g/kg in an injection volume of 0.3 ml. It appears, therefore, that within the range of chemical structures of PFCs with biocompatible vapor pressures and tissue dwell times, the influence of these materials with carbogen breathing prior to and during radiation delivery on treatment outcome is most dependent on PFC dose and to a lesser degree on the volume administered.

Fluosol-DA with carbogen breathing has been shown to enhance the outcome of radiation therapy in animal tumors by tumor growth delay assay (9, 10, 24–28), tumor cell survival assay (24, 27, 31) and TCD50 assay (29). Fluosol-DA with oxygen breathing has undergone a successful Phase I/II clinical trial with radiation therapy in head and neck cancer, and a Phase III clinical trial is now underway in that disease as well as several other Phase I/II trials with radiation in other disease sites (33). Development of the best PFC emulsion for use in conjunction with radiation therapy will require achieving a balance between maintaining a therapeutically effective PFC level in circulation and the rate of clearance of the PFC from normal tissues. It is likely that in humans there is an optimal level of oxygenation for radiation therapy and that ideally the dose of PFC used clinically should be adjusted, depending upon the patient, to perhaps some overall hematocrit/fluorocrit to reach that optimal level of oxygenation.

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