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Advances in Brief

Radiosensitization of Hypoxic Tumor Cells by Dodecafluoropentane: A Gas-Phase Perfluorochemical Emulsion[1]

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Abstract

One method to make hypoxic, radioresistant cells more radiation sensitive has been to increase the oxygen carrying capacity of normal blood using liquid perfluorochemical emulsions combined with breathing high pO2 gases. We investigated the ability of dodecafluoropentane (DDFP) to sensitize the moderately radiation-resistant Morris 7777 hepatoma based on our previous inability to modify the radiation response of this tumor. DDFP is used in very small quantities compared with perfluorchemicals reported previously. Rats under isoflurane anesthesia were administered EF5 3 h before irradiation to monitor the pretreatment level of tissue hypoxia. At −40 min, DDFP was administered i.v. at 3.5 ml/kg over 30 min. At −10 min, the rats were either continued with air (for controls) or switched to carbogen. The tumors were then irradiated and processed for evaluation of radiation response. Tumor-cell survival for DDFP treatment with air-breathing animals was not significantly different from controls treated without DDFP. Carbogen alone provided minimal sensitization. DDFP plus carbogen caused dramatic radiosensitization, and the radiation response of cells from these tumors was the same as a completely aerobic radiation response. DDFP plus carbogen appears to completely reverse the hypoxic cell radioresistance in this tumor model.

Introduction

Hypoxic cells are known to be resistant to radiation and other forms of therapy. Their presence has been very well established in animal tumor models using a number of techniques as well as direct measures of radiation resistance. Similarly, hypoxic cells in human tumors have been measured. The therapy resistance of these tumors has been correlated with direct pO2 measurements, using needle electrodes (for examples, see Ref. 1) and very recently with the presence of endogenous markers of hypoxia such as hypoxia-inducible factor 1α, vascular endothelial growth factor, and carbonic anhydrase IX (2–4). Hypoxic cells are not confined to specific tumor types, and their fractional abundance is not correlated with stage, grade, or size within a specific type (1). Thus, many attempts have been made to sensitize hypoxic cells to radiation or to kill them directly. Eliminating hypoxic tumor cells is a very broad topic that has been studied in great detail for several decades. Several general approaches have been followed (reviewed in Ref. 5). One approach is the use of agents that increase the oxygen carrying capacity of blood (6). An example is the use of PFCs[4] with carbogen breathing. This approach has several positive attributes, including the ability to increase both the oxygen carrying capacity and the pO2 of oxygen delivery to tissue. To maximize such benefits, a substantial replacement of PFC for blood must take place (*e.g.*, 20%; reviewed in Ref. 7). Because the half-life of the PFCs is too short to allow one PFC treatment to carry over to multiple radiation treatments, PFC use in patients is clearly limited by the extent and frequency of blood replacement (8). DDFP (Perflenapent, ISN name) has been formulated as a stable emulsion by Sonus Pharmaceuticals, Bothell, WA. It was originally developed as an ultrasound contrast agent because it changes from liquid to gas at just above room temperature (28°C). Therefore, at body temperature it forms submicron gas bubbles, which remain in suspension. The sizes of the gas microbubbles are determined by their surface tension and the partial pressure of gases inside the microbubble. At first glance, it is counter-intuitive that a gas could sequester other gases. However, the solubility of nonpolar gases in DDFP is so high that the bubbles absorb nitrogen and oxygen. Mathematical models suggest DDFP should be roughly 100 times more efficient at gas transport (on the basis of total drug administered) than liquid PFCs (9). This article addresses the question of whether DDFP can radiosensitize tumors when the animals are allowed to breathe a high-oxygen content gas. Our results show that the Q7 hepatoma is sensitized to the maximum extent possible (radiation resistance equivalent to aerobic cells) when administered at the extremely low DDFP level of 0.7 ml/kg. Thus, DDFP may be the first PFC emulsion practical and safe for use in multifraction clinical regimens.

Materials and Methods

The University of Pennsylvania Institutional Animal Care and Use Committee approved all of the animal protocols.

Cell Cultures and Tumor Models. Q7 hepatoma cells were obtained from the American Type Culture Collection (Manassas, VA). They were maintained in exponential growth by transfers at 3.5-day intervals with culture conditions as described previously (10). Tumor cell dissociation was based on methods described previously (10).

Female Buffalo rats (Harlan Sprague Dawley, Indianapolis, IA) were used for most studies. Passage "zero" tumors were created by injecting 1 million Q7 cells s.c. into the thigh region. In most experiments, tumors were studied at a size of ~0.4 g, typically requiring 12–20 days of growth. Because tumors grow more rapidly in male rats and have a higher "take" rate than in females, in some experiments male rats were used. In these rats, later passage tumors were initiated by allowing the cells from one tumor to be passaged once *in vitro* followed by reinoculation into recipient animals. In this way, passage 1–5 tumors were studied over a size range of 0.3–3 g. For the radiation sensitization studies, rats were maintained on a 37°C warm water circulating heating pad, anesthesia-induced with 2.5% isoflurane, and maintained using continuous inhalant anesthesia consisting of air with approximately 1.75–2.5% isoflurane.

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[4] The abbreviations used are: PFC, perfluorochemical; DDFP, dodecafluoropentane; Q7, Morris 7777.

The percentage of isoflurane was adjusted to maintain a normal respiration rate of 48–56 breaths/min. At the time of tumor removal, the isoflurane was increased to 2.5% to provide a surgical level of analgesia.

Drugs and Irradiation. EF5 [2-(2-nitro-1H-imidazol-1-yl)-*N*-(2,2,3,3,3-pentafluoro-propyl)acetamide] was synthesized by contractors for the National Cancer Institute, Bethesda, MD. Its use in animal studies has been described previously (10).

DDFP was prepared as an emulsion (liquid particle size ~200 nm diameter) by the Sonus Pharmaceutical Company. The precise composition of the emulsion is proprietary. Because the emulsion has a substantial vapor pressure at room temperature, it was stored at 4°C in a sealed vial with minimal headspace. The suggested method of administration (infusion over 30 min of 0.75 ml/kg; *i.e.*, 5 μl/min for a 200-g rat) was too low for accurate monitoring, so we diluted the emulsion 1 part emulsion plus 4 parts cold physiological saline, and infused at a 5-fold higher rate. The diluted emulsion was kept ice cold, but the infusion pump and tubing were at ~23°C so the mixture immediately warmed to room temperature at such low flow rates. Flow rates were measured before the actual infusion by assessing total weight collected from the infusion line over 30 min. Additionally, immediately preceding infusion of the DDFP, the patency of the tail catheter was checked. The infusion was started ~140 min after the bolus EF5 administration. Control animals received physiological saline at the same total infusion rate. With one exception (see below) this rate was constant to within +/− 5% for all of the experiments. Rats that developed catheter patency problems before DDFP infusion were assigned to the control group rather than attempting a second catheterization just before tumor treatment. Therefore, rather than wasting animals, some rats in the control group received no infusion. No physiological effects of such small infusions of saline were expected nor have been noted.

At the end of the infusion, the inhalant gas was either maintained as air or switched to carbogen (95% O2, 5% CO2) for 7–10 min and the tumors irradiated with 12 Gy during carbogen breathing. Irradiation was performed with an orthovoltage X-ray unit operated at 225 kV and 13 mA with a 0.2-mm copper filter. The dose rate was 3.5 Gy/min. At the completion of the irradiation, the animals were returned to air breathing, the tumors removed, and the animals euthanized. Tumors were prepared for *in vivo* to *in vitro* plating efficiency assay as described previously (10). We found that most dye-including cells lyse during the centrifugation so that dye-excluding cells comprise >75% of the population. Cells were then diluted to levels appropriate for plating, and colony formation was allowed for 14–17 days.

Assay of EF5 Cellular Binding *in Vitro*. To determine maximum binding levels of Q7 tumor cells, *in vitro* studies were performed as has been described previously in detail (11). Cellular EF5 binding was assessed using two methods: (*a*) by covalent binding of radioactive drug into acid insoluble cell lysates (data not shown); (*b*) by flow cytometric analysis of antibody-stained cells (11).

Flow Cytometry. Cells obtained from enzyme dissociation of whole tumors or from oxygen chambers were prepared for flow cytometric analysis by fixing, rinsing, blocking against nonspecific binding, and staining as described previously (12). Cells were subjected to flow cytometric analyses within 5 days, although the fluorescent dye signal has been found to be stable for several weeks (13).

Analyses of Plating Efficiency and Flow Cytometric Data. The plating efficiency of tumor cells was calculated based on Coulter counts modified by the fraction of dye-excluding cells as determined by hemocytometer. All of the survival levels are plotted as absolute values (*i.e.*, not modified by plating efficiency at zero Gy; Fig. 3).

Flow cytometric analyses of EF5 binding were based on the calculation of mean EF5 binding for cells stained with Cy5-labeled antibody minus mean value of the same cells stained with competed antibody (Cy5 antibody mixed with 0.5 mM EF5; Ref. 13). The flow cytometric instrument (FACScalibur; Becton Dickinson, Mountain View, CA) uses a second laser operating in the red (635 nm) to monitor Cy5. Sensitivity was set to obtain a constant signal using cell standards; V79 Chinese hamster cells were exposed to 400 μM × Hr of EF5 under conditions of severe hypoxia (<0.005% O2; Ref. 13).

Thiol Analyses. Biopsy specimens for thiol analysis were rapidly removed from the anesthetized animal before the tumor or other organs were excised. Sample preparation for thiol analysis involves a simple homogenization and precipitation of macromolecules, and has been described previously (14).

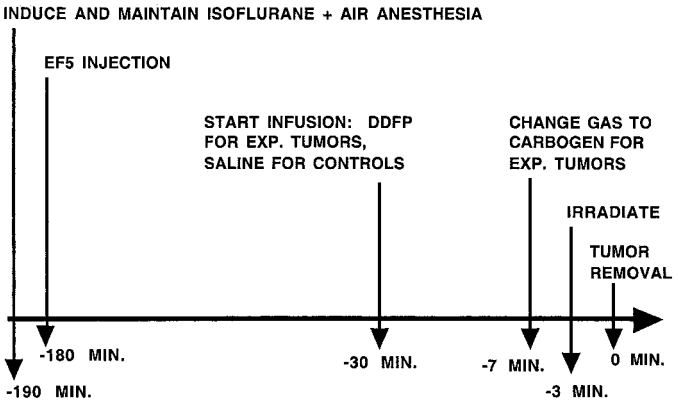

Fig. 1. Timing of experimental protocol.

Statistics. Similar treatment groups were compared by one-tailed *t* test (assuming that the treatment would only cause sensitization) and *P*s calculated using standard methods. The presence or absence of a relationship between mean EF5 binding and cell survival at 12 Gy was tested using linear regression.

Results

The experimental protocol is summarized in Fig. 1. Note that the animals were anesthetized with isoflurane with body core temperature maintained near 37°C throughout the entire experimental procedure. Additionally, the animals were only subjected to carbogen for the last few min of the 3-h EF5 drug exposure at the time of irradiation.

The Q7 hepatoma was quite radiosensitive when grown as described, although we have found that this tumor/host is predisposed to transient resistance using injectable anesthetics such as ketamine/xylazine.[5] Because the tumor cell was originally derived from a female rat (15), initial experiments were done with female Buffalo rats and passage 0 tumors with mass limited to 0.4 +/− 0.15 g. Under these conditions, there was a highly significant relationship between median EF5 binding and survival after 12 Gy for cells from tumors irradiated *in situ* (Figs. 2 and 3). Limited data demonstrate that carbogen breathing alone produced surviving fractions in the low range of normal. Similarly, DDFP infusion in air-breathing rats had no impact on this relationship. In dramatic contrast, DDFP with switch to carbogen for 7 min before and during irradiation produced highly radiosensitive tumors. EF5 binding was not different for this group of tumors (Fig. 3), confirming that endogenously sensitive (oxic) tumors had not been chosen by chance. The radiation sensitivity of this group of tumors was not statistically different from that of cells irradiated under aerobic conditions after disaggregation from an unirradiated tumor or for cells grown in culture (Table 1).

We wanted to test the radiation sensitizing ability of DDFP + carbogen in more radioresistant tumors. In studies published previously of 9L glioma tumors grown in Fischer rats, we reported that later passage tumors had increased radiation resistance (10). Therefore, in male rats we investigated a larger range of tumor size (up to 3 g) with passage numbers from 0 to 5. The relationship between mean EF5 binding and endogenous radiation response was not statistically significant. This was at least partly caused by the larger variability of necrosis with the larger tumor volumes and the observation that the zero Gy radiation dose plating efficiency also became 2–3-fold lower and more variable. Nevertheless, the DDFP-treated, carbogen-breathing animals maintained their much lower radiation resistance than the non-DDFP-treated animals (Table 1).

[5] S. M. Evans. Ketamine xylazine anesthesia causes hypoxia and radiation resistance in rat morris 7777 hepatomas, manuscript in preparation.

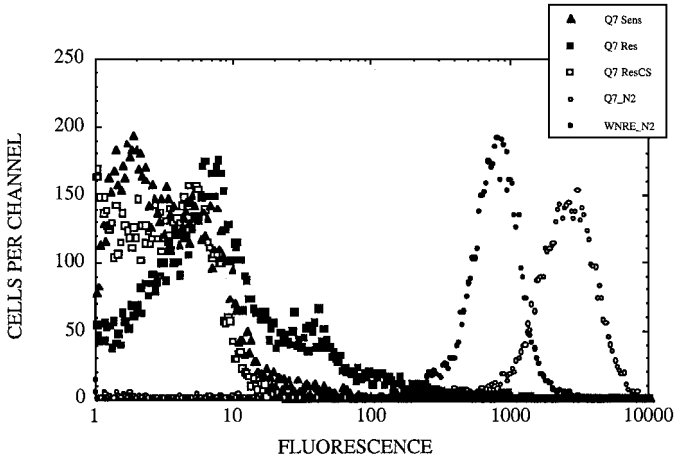

Fig. 2. Flow cytometric analysis of EF5 binding in moderately hypoxic and minimally hypoxic Q7 hepatoma, compared with tumor cells incubated *in vitro* under conditions of severe hypoxia. ●, positive control, WNRE cells incubated in severe hypoxia + EF5; ○, Q7 cells incubated *in vitro* under conditions of severe hypoxia; ■, cells from relatively radioresistant (hypoxic) tumor; ▲, cells from relatively radiosensitive (oxic) tumor; □, cells from resistant tumor, competed stain.

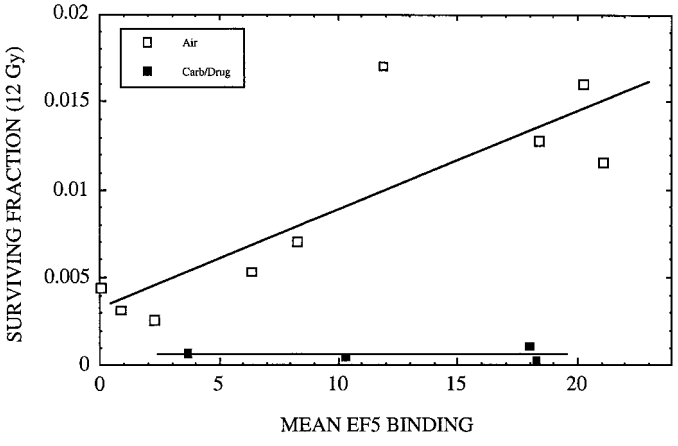

Fig. 3. Relationship between mean EF5 binding and surviving fraction of cells from individual Q7 hepatoma tumors irradiated with 12 Gy *in vivo* then plated for colony formation *in vitro*. Surviving fraction of cells from unirradiated tumors was 0.17 ± 0.005.

Of all of the DDFP + carbogen tumors analyzed, only one had a response that was at the lower range of the control animals. This was traced to a leak in the DDFP infusion line (within the rollers of the pump), which was initially unobserved preceding the experiment.

We have reported previously that nonprotein thiol values can modify radiation response of intermediately hypoxic tumor cells (16). Therefore, to assess the role of nonprotein thiols in the endogenous changes in radiation response, thiol values were measured in the tumors studied herein. We found that the variable radiation response was not likely to have been caused by tumor nonprotein thiol values because the average glutathione and cysteine content of tumors treated with DDFP + carbogen were not statistically different from untreated tumors (Table 1).

Discussion

Over the past several years, our rodent-model tumor data have leant extensive support to the idea that tumor cells at intermediate oxygen concentrations pose an important therapeutic limitation (17). This idea is inherently logical from a physiological standpoint and was first suggested in 1972 when Tannock (18) noted that it would be very difficult to discriminate experimentally between a classical hypoxic fraction [binary; The "hypoxic fraction" has been defined previously by modeling a tumor as two cell populations, one with aerobic response (Fa) and one with anoxic response (Fn). Thus, the hypoxic fraction is described as (Fn)/(Fa + Fn)] *versus* a distribution of pO2 values. Wouters and Brown (19) have discussed recently the therapeutic consequence of tumors maintaining a population of cells at intermediate oxygen concentrations throughout multidose therapy. It was suggested several years ago that a possible reason for the superior cytotoxic properties of tirapazamine *versus* nitro-based hypoxic cell cytotoxins *in vivo* was that the former was effective over a much broader oxygen concentration range than the latter (20). There are many biological and clinical ramifications of cells at intermediate oxygen concentrations. Perhaps the most clinically relevant of these is that electron-affinic compounds other than oxygen (*e.g.,* 2-nitroimidazoles) would be at a disadvantage as radiosensitizers except at concentrations well beyond the toxicity-limited range. This additionally implies that finding a new method to improve tumor tissue oxygenation is paramount. As mentioned in the introduction, PFC emulsions have certain advantages over other modifiers of the oxygen-carrying capacity of blood (*e.g.,* stabilized hemoglobin preparations and transfusion), because this added capacity is effective at all of the oxygen concentrations. Indeed, the hemoglobin concentration in whole blood is already so high that any practical use of PFC requires the simultaneous use of high oxygen concentration (7). This, in turn, increases the oxygen gradient between blood and (hypoxic) tissue, the force driving oxygen diffusion.

The results reported herein illustrate the utility of this method using a PFC emulsion based on the exciting new concept of forming stabilized microscopic bubbles of PFC gas at physiological temperature. The subsequent absorption of uncharged gases such as oxygen into the bubbles causes them to swell but not to lose stability. The efficiency of this process may allow multiple injections of the low volumes required for efficacy in multitreatment therapy. Furthermore, the lungs are the primary sites of fluorocarbon elimination. Thus, the reticuloendothelial system, lung and liver toxicities seen from previous liquid PFC emulsions may be minimized by this drug because it can be cleared in the vapor phase (data on file, Sonus Pharmaceuticals).

We selected a tumor model for these studies that, in our experience, was refractory to other methods of radiosensitization such as carbogen plus nicotinamide or misonidazole. EF5 binding data suggest that the reason for this is related to the lack of severely hypoxic tumor cells. Because we achieved the maximum possible radiosensitivity, our optimization strategies for volume and timing will be performed in a more radioresistant tumor model; such experiments are planned. The DDFP bubbles, like former liquid PFC emulsions, serve as high-capacity oxygen carriers but, unlike all of the previous formulations, do so at much lower concentrations. Thus, DDFP may be the first PFC emulsion practical and safe for use in multifraction clinical regimens.

Table 1 *Summary of all results*

There is no statistical difference between the first three radiation groups. For the DDFP plus carbogen group, survival is significantly reduced ($P = 0.01$). The mean EF5 binding signal, as assessed by flow cytometry, was similar for all groups, although that for the group given 12 Gy plus carbogen was the highest (most hypoxic). Note that all responses are given as absolute survival (*i.e.* not corrected for plating efficiency). Thus, the response of cells grown and maintained in tissue culture to 12 Gy would be substantially higher, because the plating efficiency is >0.75 (*vs.* 0.13 for cells isolated from tumors).

Condition	*n*	PE ± SD	Mean FlCyt ± SD	GSH (mM)	CySH (mM)
No radiation	7	0.13 ± 0.05	18.2 ± 11.6	5.0 ± 1.8	1.6 ± 0.5
Air, 12 Gy	20	0.0066 ± 0.0048	17.9 ± 14.3	4.0 ± 1.4	0.9 ± 0.5
Carbogen, 12 Gy	3	0.0042 ± 0.0004	37.5 ± 13.5	6.0 ± 0.3	1.7 ± 0.3
Air/DDFP, 12 Gy	3	0.0045 ± 0.0012	9.6 ± 10.5	5.8 ± 0.5	0.8 ± 0.2
Carbogen/DDFP, 12 Gy	7	0.0006 ± 0.0003	14.5 ± 7.3	4.5 ± 0.4	0.6 ± 0.2
Tumor cells, 12 Gy		0.001 ± 0.0003			

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