Increase in pO₂ and Radiosensitivity of Tumors by Fluosol-DA (20%) and Carbogen

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ABSTRACT

The potential usefulness of i.v. injection of perfluorochemicals and breathing carbogen (95% O₂ and 5% CO₂) to improve the radiation-induced control of tumors was investigated. When C3H mice, bearing RIF-1 tumors in the legs, were given i.v. injections of Fluosol-DA (20%) at 12 ml/kg, and allowed to breathe carbogen for 1 h before and during a single dose of X-irradiation, the curability of tumors increased by a dose modification factor of 1.47 ± 0.03 (SE). Such a treatment also increased the radiation-induced skin damage by a factor of 1.15 ± 0.12, resulting in a therapeutic gain of 1.28 ± 0.04. Measurement of intratumor pO₂ by oxygen microelectrodes demonstrated small increases in pO₂ when the animals breathed carbogen, and marked increases in pO₂ when Fluosol-DA (20%) was injected into the animals and the animals breathed carbogen. It was concluded that i.v. injection of Fluosol-DA (20%) followed by carbogen breathing significantly improved the oxygen supply to hypoxic cells in the RIF-1 tumors and thus increased the control of tumors by radiation.

INTRODUCTION

Minimization of hypoxic protection in radiotherapy has been of continuous interest in the radiotherapy community during the last several decades. Consequently, a number of different methods have been proposed, tested on experimental animal tumors, and clinical usefulness of some of these have been assessed (1-9). Unfortunately, however, none of them have been proven to be clinically effective or useful, and the hypoxic protection still remains as one of the major limiting factors for the complete control of malignant tumors by radiotherapy. Recently, interesting observations have been presented by several groups of investigators, suggesting that perfluorochemicals in combination with breathing of a mixture of 95% O₂ and 5% CO₂ (carbogen) or pure O₂ may improve tumor oxygenation and thus increase the radioresponse of tumors (10-17). Based on these encouraging results with rodent tumor models, Phase I clinical trials of this approach have recently been initiated in several medical centers, including our own institute (18).

The radiosensitizing effect of i.v. injected perfluorochemicals have been attributed to their ability to dissolve large amounts of oxygen in the lung and to release the oxygen in the tissues, resulting in reoxygenation of hypoxic tumor cells (10, 11, 17). In addition, there has been a suggestion that nonspecific immune reactions might be triggered by perfluorochemical preparations, leading to an improved control of tumors by radiation, at least in the mouse tumor system (19). Undoubtedly, a better understanding of the mechanism as well as factors involved in the use of perfluorochemicals in conjunction with carbogen or oxygen breathing is needed for the effective application of this potentially useful approach in clinical radiotherapy of human tumors.

We previously reported our preliminary observation on the effect of Fluosol-DA (20%), a preparation of perfluorochemicals, on the radioreponse of RIF-1 tumors of mice with the use of growth delay as the end point (15-17). In the present report, we describe our further investigations into the effect of Fluosol-DA (20%) injection and carbogen breathing on the radiation-induced cure of RIF-1 tumors, as well as skin damage in C3H mice. In addition, for a better understanding of the mechanism by which Fluosol-DA (20%) radiosensitizes tumors, we measured the change in tumor pO₂ using oxygen microelectrodes in the tumors upon injection of Fluosol-DA (20%), with or without carbogen breathing.

MATERIALS AND METHODS

Tumor and Animals. The RIF-1 tumor syngeneic to C3H mice was used. Female C3H mice were obtained from The Jackson Laboratory (Bar Harbor, ME) when they were about 7 weeks old, and were provided with standard laboratory chow and water ad libitum. The characteristics of the RIF-1 tumor have been previously described (20), and we have been using this tumor for various radiobiological and hyperthermia experiments (21). The early generation cells are being stored in liquid nitrogen in our laboratory. The frozen stock cells are thawed and cultured in RPMI 1640, supplemented with 10% bovine serum and antibiotics. The cells are subcultured every 4-6 days, and new cells are activated every 2-3 months from the frozen stock. For the present study, the cells in exponential growth phase were harvested from cultures by trypsin treatment, washed, and 1 × 10⁶ cells in 0.05 ml medium were injected s.c. into the thighs of 8- to 10-week-old female C3H mice. When the tumors grew to 7-8 mm in diameter, about 12 days after the inoculation, the mice were randomly divided and used for various studies. Mice without tumors were also used to study the radiation effect on the leg skin.

Fluosol-DA (20%) and Carbogen Treatments. Fluosol-DA (20%) (Alphatherapeutic Corp., Los Angeles, CA 90032) is an emulsified preparation of a mixture of perfluorochemicals consisting of 14% (w/v) perfluorodecalin, 6% of perfluorotripropylamine, and other components used as emulsifiers and to adjust osmotic pressure in Krebs-Ringer's bicarbonate solution (22, 23). The emulsion was oxygenated by slowly stirring with a magnetic stirrer under carbogen atmosphere in small Erlenmeyer flasks for 30 min. The oxygenated Fluosol-DA (20%) was injected into the mice through tail veins at a dose of 12 ml/kg, the mice were placed in a Plexiglas tank (30 x 15 x 20 cm), and the tank was continuously gassed with carbogen. After exposure to carbogen atmosphere for 45 min, the mice were anesthetized by i.p. injection of sodium pentobarbitol at 50 mg/kg, and kept in the carbogen atmosphere for another 15 min. The mice were then quickly mounted in radiation jigs, and the head of each mouse was covered with a small plastic cone which was connected to a carbogen tank with tubing. The animals were allowed to breathe carbogen supplied through the cone for 10 min while the legs were positioned and taped for irradiation. The tumor-bearing legs or normal legs were then irradiated with graded doses of X-rays in a single exposure. Other groups of mice did not receive Fluosol-DA (20%), but breathed carbogen before and during X-irradiation. The control group received neither Fluosol-DA (20%) injection nor carbogen treatment, but X-irradiation only to the legs.

X-Irradiation. The legs with or without tumors were irradiated with various doses of X-rays in a single exposure. The radiation source was...
tumors. A reference glass electrode with outside tip diameter of about 50-60 μm were constructed, as previously described by other investigators (25, 26). Briefly, commercially available glass tubing with diameters of 1.5 mm were pulled to 50-60 μm in outside diameter with a vertical pipet puller. Melted Wood's metal was sucked into the capillaries, leaving a recess of 40-50 μm, and the Wood's metal was plated with 10-μm-thick gold on the side facing the recess. The current-voltage curve for each electrode was obtained and the best polarizing volt was chosen. The electrodes were calibrated by immersing the electrodes in saline saturated with gas of different PO2, e.g., 0, 12, and 100%. The PO2 measurement becomes erratic when the electrode tip is contaminated with protein upon repeated use. Thus, we calibrated the electrodes before and after each measurement of tissue PO2, and discarded the electrodes when the measured PO2 under identical conditions shifted by more than 2%.

Mice bearing tumor in the leg were anesthetized with Inactin (sodium salt of ethyl-(1-methylpropyl)malonylthiourea; Andrew Lockwood Associates, East Lansing, MI) (180 mg/kg), and taped on a holder in a Faraday cage. Mice foreheads were placed in a small plastic cone which was connected to a carbogen tank with tubing. Parts of the skin covering the exposed tumor surface was moistened with drops of saline. An oxygen microelectrode, calibrated as described above, was mounted on a micromanipulator, and carefully inserted and advanced into the capillaries, leaving a recess of 40-50 μm, and the Wood's metal was plated with 10-μm-thick gold on the side facing the recess. The current-voltage curve for each electrode was obtained and the best polarizing volt was chosen. The electrodes were calibrated by immersing the electrodes in saline saturated with gas of different PO2, e.g., 0, 12, and 100%. The PO2 measurement becomes erratic when the electrode tip is contaminated with protein upon repeated use. Thus, we calibrated the electrodes before and after each measurement of tissue PO2, and discarded the electrodes when the measured PO2 under identical conditions shifted by more than 2%.

RESULTS

The effects of X-irradiation alone, X-irradiation after carbogen breathing, and X-irradiation after i.v. injection of Fluosol-DA (20%) plus carbogen breathing on the cure of tumors are summarized in Fig. 1. The percentage of tumors judged to be cured 120 days after the treatments was plotted as a function of radiation dose given to the tumors. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. 

Table 1: Radiation effect on RIF-1 tumor and skin

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>Skin reaction</th>
<th>Growth delay of 20 days</th>
<th>TCD50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbogen</td>
<td>1.13 ± 0.08</td>
<td>1.22 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Carbogen + Fluosol-DA (20%)</td>
<td>1.15 ± 0.12</td>
<td>1.70 ± 0.08</td>
<td></td>
</tr>
</tbody>
</table>

* See Ref. 9.  
† Therapeutic gain.

Fig. 1. Local tumor control as a function of X-irradiation dose; the TCD50 and 95% confidence intervals were obtained by probit analysis. For each experimental group, 7-14 mice were used.

Fig. 2. Skin reaction in C3H mice during 0-40 days postirradiation as a function of X-irradiation dose. Points, average of 7-14 mice are shown for each skin reaction; bars, SE.

The effects of X-irradiation alone, X-irradiation after carbogen breathing, and X-irradiation after i.v. injection of Fluosol-DA (20%) plus carbogen breathing on the cure of tumors are summarized in Fig. 1. The percentage of tumors judged to be cured 120 days after the treatments was plotted as a function of radiation dose given to the tumors. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads. The results are summarized in Table 1. The TCD50 of control tumors which received X-rays only, calculated by probit analysis, was 5487 rads with 95% confidence interval of 4980-6108 rads.

Skin Damage. The average skin reactions during the first 40 days postirradiation are shown as a function of X-irradiation dose in Fig. 2. An i.v. injection of Fluosol-DA (20%) at 12 ml/kg and carbogen breathing for 1 h before and during X-irradiation of normal legs slightly increased the skin damage by irradiation. Carbogen breathing without Fluosol-DA (20%) injection also increased the radiation-induced skin damage. The dose required...
INCREASE IN pO2 AND RADIOSENSITIVITY OF TUMORS

Fig. 3. Changes in tumor pO2, measured with microelectrodes, by carbon breathing alone, or by carbon breathing with i.v. injection of Fluosol-DA (20%).

A total of 47 tumors were used for the determination of changes in pO2 by carbon breathing without Fluosol-DA (20%) injection. The pO2 in the same site was then determined after the animals were given injections of Fluosol-DA (20%) and allowed to breathe carbon. Injection of Fluosol-DA (20%) was unsuccessful in 8 cases. Thus, only 39 determinations are shown for the carbon plus Fluosol group (B).

to induce an average skin reaction of 2.0, assessed with linear regression analysis, was 4015 rads with 95% confidence interval of 3839–4248 rads for the control, while it was 3565 (3136–4089) rads and 3490 (3034–4109) rads for the carbon alone and carbon plus Fluosol-DA (20%) group, respectively. The dose modification factor for the skin reaction of 2.0, therefore, was 1.13 ± 0.08 for the carbon only group, and 1.15 ± 0.12 for the carbon plus Fluosol-DA (20%) group (Table 1).

Tumor pO2. The distribution of pO2 in RIF-1 tumor, measured with microelectrode, varied considerably in the same tumor and among the different tumors. Detailed assessment of change in tumor pO2 by carbon breathing or carbon breathing in conjunction with Fluosol-DA (20%) injection is being conducted in our laboratory, and we are only reporting our preliminary results here. Fig. 3 shows the change in pO2 in RIF-1 tumors by carbon breathing alone, and that by carbon breathing in conjunction with Fluosol-DA (20%) injection. It is shown that the majority of pO2 values in the tumors before carbon breathing was lower than 10 mm Hg. When the animals were allowed to breathe carbon, the pO2 in the tumors occasionally increased significantly (Fig. 3A). A significant increase in pO2 was noticed in 13 of 47 measurements (27.7%). In fact, the highest pO2 detected before the carbon breathing was about 30–35 mm Hg, and the highest pO2 during carbon breathing we observed was about 100 mm Hg. When the animals were given injections of oxygenated Fluosol-DA (20%) at 12 ml/kg and breathed carbon, the magnitude and frequency of the increase in pO2 was more marked as compared with that by carbon breathing alone (Fig. 3B). The highest pO2 in the tumors of mice which received Fluosol-DA (20%) injection together with carbon breathing treatment was as high as 230 mm Hg. Of 39 measurements, a significant increase in pO2 occurred in 29 cases (74.4%).

Fig. 4. A segment of original graph showing changes in pO2 in RIF-1 tumor during air breathing and carbon breathing, before and after i.v. injection of Fluosol-DA (20%). The animal was given injections of Fluosol-DA (20%) during the interval between the two carbogen breathing periods.

DISCUSSION
It was demonstrated in the present study that an i.v. injection of Fluosol-DA (20%) in conjunction with carbon breathing significantly increases the control of RIF-1 tumor of C3H mice.
and after an i.v. injection of Fluosol-DA (20%). The changes in pO2 in killing tumors during air breathing and carbogen breathing, before and after an i.v. injection of Fluosol-DA (20%). Injection of Fluosol-DA (20%) was done while the animal was breathing carbogen.

by radiation (Fig. 1). Although this conclusion is in general agreement with the results of our previous study with RIF-1 tumors, in which growth delay of tumors was used as the end point (17), there appears to be some variance in the magnitude of the efficacy of Fluosol-DA (20%) injection and carbogen breathing to enhance the radiation-induced tumor control and the radiation-induced delay of tumor growth. As shown in Table 1, the dose modification factor for TCD50 obtained in the present study was 1.47 ± 0.03, while the dose modification factor for the growth delay was 1.96 ± 0.09 (Table 1). It has been suggested that the growth delay of tumors after irradiation mainly reflects the damage in the proliferating cell population, which are well or moderately oxygenated cells, whereas the tumor curability is mainly dependent on the radiation damage in the uncycling and yet viable cells in severely hypoxic regions in the tumors. The greater dose modification factor for growth delay relative to that for TCD50 we observed may be interpreted to mean that the moderately hypoxic cells are more readily reoxygenated, as compared with severely hypoxic cells in the RIF-1 tumors when the host mice are given injections of Fluosol-DA (20%) and allowed to breathe carbogen for 1 h.

The radiation-induced skin damage was also found to increase with dose modification factors of 1.13 ± 0.08 and 1.15 ± 0.12 when the animals were treated with carbogen breathing alone or Fluosol-DA (20%) injection and carbogen breathing, respectively (Fig. 2; Table 1). It is well-known fact that the mouse skin is somewhat radiobiologically hypoxic, particularly when the animals were anesthetized. Indeed, the mouse skin has been reported to be radiosensitized by misonidazole and similar hypoxic cell radiosensitizers (27). In light of these facts, it is not surprising that carbogen breathing or Fluosol-DA (20%) injection combined with carbogen breathing sensitized the mouse skin to radiation, albeit the sensitization was small.

The therapeutic gain obtained in this study with the RIF-1 tumors for a single radiation exposure was 1.28 ± 0.04, whereas our preliminary study with the SCK tumor indicated that the therapeutic gain for the complete control of SCK tumor is about 2.0. Larger therapeutic gains than we obtained in the present study were also reported by other investigators (11-13). The different degrees of hypoxia in the tumors may account, at least in part, for the variance in the therapeutic gain by Fluosol-DA (20%) injection and carbogen treatment in different tumors. The

Fig. 5. A segment of original graph showing the changes in pO2 in RIF-1 tumor during air breathing and carbogen breathing, before and after an i.v. injection of Fluosol-DA (20%).
therapeutic gain in clinical radiation therapy with fractionated radiation may be smaller than that observed with a single radiation dose because of reoxygenation of tumor cells during the course of fractionated radiation. Nevertheless, it appears that further investigation on the potential usefulness of perfluorochemicals to increase tumor pO2, and thus to sensitize human tumors to radiotherapy, is warranted.

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