Changes in Radiation Sensitization Induced by Fluosol-DA as Measured by $^{31}$P Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT

Numerous agents have been studied in attempts to sensitize radioreistant hypoxic tumor cells. We have investigated the effect of Fluosol-DA plus carbogen (95% oxygen and 5% CO$_2$) on the sensitivity of a radioreistant mammary carcinoma in C$_5$H/He mice and also on tumor metabolism by $^{31}$P nuclear magnetic resonance spectroscopy. Statistically significant increases in phosphocreatine/P$_r$, were noted for small- (150-350 mm$^3$) and medium- (351-650 mm$^3$) sized tumors treated with Fluosol-DA plus carbogen. Small tumors were shown to undergo significant radiosensitization in the presence of Fluosol-DA plus carbogen and medium-sized tumors showed a lesser degree of radiosensitization. Large tumors (>900 mm$^3$) showed no effect. Fluosol-DA or carbogen alone had no effects on animals with any tumor volume, as monitored by significant changes in radiosensitivity or nuclear magnetic resonance parameters. An approximately linear relationship was found between the decrease in the tissue for radiation dose which yields 50% tumor control and the increase in phosphocreatine/P$_r$, with a correlation of $r = -0.93$. $^{31}$P nuclear magnetic resonance spectroscopy may be useful for monitoring changes in radiosensitivity induced by agents which alter tumor oxygenation and subsequent metabolic status.

INTRODUCTION

Tumor hypoxia is probably the major cause of resistance to radiation (1) and an important factor in local treatment failure. Hypoxic cells are radioreistant relative to well oxygenated cells and are potentially clonogenic when reoxygenated after therapy. Numerous agents have been tried to enhance tumor oxygenation, including Fluosol-DA in conjunction with oxygen, which has been shown to enhance radiation response of in vivo tumor models (2-4). Radiosensitization induced by Fluosol-DA plus oxygen is thought to be due to enhanced oxygen delivery to hypoxic regions by the small (0.2-$\mu$m) emulsion particles of Fluosol-DA. Delivery of oxygen to these hypoxic regions could induce an alteration in tumor metabolism.

Clinical trials of Fluosol-DA plus oxygen to date have been limited but are encouraging (5, 6). Recently, Sagai et al. (7) have shown that Fluosol-DA plus carbogen only radiosensitizes certain tumors. A technique to identify tumors which could be radiosensitized by Fluosol-DA plus carbogen would be useful for selection of patients for radiosensitization treatments in conjunction with radiation therapy.

$^{31}$P NMR spectroscopy has been used to monitor tumor metabolism and its alteration in response to various treatments (8-13). Previous studies have related changes in $^{31}$P NMR spectra to alterations in tumor hypoxia. Okunieff et al. (14) have noted an increase in tumor P$_c$/P$_r$, with inspiration of 100% oxygen. Steen, et al. (15) have related anesthetic-induced changes in P$_c$ to alterations in tumor oxyhemoglobin. Rofstad et al. (13) found linear correlations between hemoglobin oxygen saturation and bioenergetic status, as measured by $^{31}$P NMR, in three of four tumor models studied. $^{31}$P NMR spectroscopy may be useful as a noninvasive probe to monitor tumor metabolism and subsequent changes induced by enhanced delivery of oxygen to hypoxic regions of the tumor secondary to treatment with Fluosol-DA plus carbogen.

For this study, the effect of Fluosol-DA and carbogen on tumor metabolism was investigated with $^{31}$P NMR spectroscopy, using a murine MCa tumor model. This tumor has been shown to have a relatively low P$_c$/P$_r$, value, as measured by in vivo $^{31}$P NMR spectroscopy (16). The tumor is radioreistant, has been shown to have a high hypoxic cell fraction (70-40%) and to undergo reoxygenation after radiation (17), and therefore was considered to be an appropriate model to study the effect of reoxygenation on tumor metabolism.

MATERIALS AND METHODS

Nuclear Magnetic Resonance. The $^{31}$P NMR spectra were obtained with a NT-300 wide-bore magnet operating at 121.5 MHz (General Electric NMR, Fremont, CA). Experimental parameters included a spectral width of $\pm 12,000$ Hz, recycle delay time of 2 s, 60-degree tip angle, and 256 or 512 averaged free induction decays. Using these acquisition parameters, the spectra are partially saturated, particularly the P$_c$ and P$_r$ resonances, leading to a decrease in their relative peak area. Four-turn solenoid coils were used to obtain the spectra. A range of solenoid coils was available for this study, and the size of the coil was chosen to fit closely around the tumor without compressing it. The distal aspect of the foot dorsum was chosen as the site of tumor inoculation, to minimize spectral contamination from adjacent tissues (9). The $B_0$ field was shimmed prior to spectral acquisition, using the $^1$H water signal from the tumor. Spectra were analyzed using a 25-Hz exponential multiplication filter followed by Fourier transformation. Spectral peak areas were estimated by fitting the spectra to a series of Lorentzian peaks, using a program (GEMCAP) available on the spectrometer, after fitting the baseline to a third-order polynomial (using standard General Electric software). Tumor pH was estimated from the chemical shift of P$_c$, relative to that of P$_r$ (18). Because the exact intracellular concentrations of substances which could affect the P$_c$-P$_r$ chemical shift are not known, the derived pH-chemical shift curve has an uncertainty of $\pm 0.1$ pH units (19).

This study focused primarily on tumors in the volume range of 150-1200 mm$^3$. A total of 25 animals were studied before and after treatment with Fluosol-DA and carbogen. Control $^{31}$P NMR studies before and after treatment with Fluosol-DA alone (16 mice) and carbogen alone (15 mice) were also done. A summary of the tumor volumes of the different cohorts is presented in Table 1.

Fluosol-DA (20%) (Alpha Therapeutics Corp., Los Angeles, CA) was stored frozen and made up fresh prior to each study. Fluosol-DA was equilibrated with carbogen by bubbling the gas mixture through the emulsion for 15 min. The animals were inserted unanesthetized into the NMR probe in a vertical upright position. After obtaining a
baseline spectrum, the animal was treated with either Fluosol-DA, carbogen, or Fluosol-DA plus carbogen. Animals treated with Fluosol-DA plus carbogen were given injections i.v. in the retro-orbital sinus, at a dose of 0.25 ml (10 ml/kg), and were then placed in a carbogen environment chamber for approximately 30 min. They were then re-studied by NMR spectroscopy, with continuous delivery of carbogen through a small inlet on the top of a plastic cylinder which surrounded the upper 50% of the animal. The interval between removal of the animals from the carbogen chamber and insertion into the plastic cylinder in the NMR probe (in the carbogen environment) was typically about 30 sec. The animals were in a carbogen environment for a total of approximately 45 min prior to the beginning of spectral acquisition. Animals treated with carbogen alone were treated in a similar manner but did not receive Fluosol-DA. For the experiments in which the animals received only Fluosol-DA, the drug was bubbled for 15 min with carbogen. The animals were given injections of the Fluosol-DA while breathing air and were mounted in the NMR probe with room air delivered instead of carbogen.

Tumor Transplantation and Irradiation. The MCa tumors were removed aseptically from tumor-bearing animals by previously described techniques (20, 21). Briefly, a single-cell suspension was prepared from a solid tumor by teasing and abrasion against stainless steel mesh immersed in iced minimal essential medium (Earle’s balanced salt solution) containing 2% heparin. Cell suspensions were agitated constantly by a magnetic spin bar. A tumor inoculum of 0.025 to 0.04 ml (approximately 10^5 cells) was injected s.c. into the dorsum of the foot while breathing air and were mounted in the NMR probe with room temperature and carbogen. The animals were given injections of the Fluosol-DA plus carbogen in a carbogen environment.* Mean ± SD.

RESULTS

Table 1 summarizes the pretreatment spectral characteristics of the animals. The tumor volumes of the three drug groups (Fluosol-DA, carbogen, and Fluosol-DA plus carbogen) do not differ significantly. Similarly, the NMR parameters PCr/Pi, NTP/Pi, and pH have similar pretreatment values for all three groups (Fluosol-DA, carbogen, and Fluosol-DA plus carbogen) in the comparable volume range. Small but not significant differences in pH [as measured by a Scheffé test for multiple comparisons (22)] are present between the large tumor volume cohorts.

Fig. 1, bottom, shows a spectrum of an approximately 400-mm³ tumor from an unanesthetized animal. The spectrum is qualitatively similar to previously published spectra from murine tumors, with peak assignments noted in the legend. Fig. 1,

![Fig. 1. Bottom, 31P NMR spectrum of an approximately 400-mm³ tumor from an unanesthetized animal. The spectrum is qualitatively similar to previously published spectra from murine tumors, with peak assignments noted in the legend. Fig. 1,](image-url)
top, shows a spectrum obtained on the same animal during treatment with Fluosol-DA plus carbogen. Qualitatively, there is a relative increase in PCr.

The change in PCr/Pi, after treatment with Fluosol-DA plus carbogen, as measured by 31P NMR spectroscopy, has an approximately linear dependence \( (r = -0.47, P < 0.02) \) on tumor volume in the range of 150 to 1650 mm\(^3\). Fig. 2 shows a plot of \( \Delta \text{PCr/Pi} \) versus tumor volume, where the measurements are grouped into size categories of 200 mm\(^3\) (200–399 mm\(^3\), 400–599 mm\(^3\), 900–1099 mm\(^3\), and >1100 mm\(^3\)), showing a decrease in \( \Delta \text{PCr/Pi} \) with increasing tumor volume. An increase in PCr/Pi, after treatment with Fluosol-DA and carbogen is observed in tumors between 150 and about 650 mm\(^3\) (Table 1). The greatest effect is noted on small tumors (150–350 mm\(^3\)), a smaller effect for medium-sized tumors (351–650 mm\(^3\)), and virtually no effect on large tumors (>900 mm\(^3\)). Fourteen of 17 tumors in the volume range of 150–650 mm\(^3\) showed an increase in PCr/Pi, after treatment with Fluosol-DA plus carbogen. If one ignores increases of less than 25%, 10 of 12 animals with tumor volumes less than 450 mm\(^3\) had increases in PCr/Pi, of greater than 25%. However, only 6 of 13 animals with tumor volumes more than 450 mm\(^3\) had an increase in PCr/Pi, of greater than 25%. This difference in response between tumors smaller and larger than 450 mm\(^3\) tended towards significance \( (x^2 = 3.75, P = 0.06) \). When the carbogen was shut off and room air administered, a decrease in PCr/Pi was observed (data not shown). A similar plot for NTP/Pi showed that there was no relationship between tumor volume and change in NTP/Pi after treatment with Fluosol-DA plus carbogen. The correlation coefficient for this plot was 0. Similarly, there was no relationship between tumor volume and changes in pH after treatment with Fluosol-DA and carbogen. The correlation coefficient for this plot was virtually zero (0.04).

Similar studies were done on two control cohorts of tumor-bearing mice treated with either Fluosol-DA or carbogen. PCr/Pi did not change due to treatment with either Fluosol-DA alone or carbogen alone.

Table 1 summarizes the changes observed after treatment with Fluosol-DA plus carbogen, Fluosol-DA, or carbogen alone for the three different tumor volume ranges studied. To analyze whether the changes observed after treatment with Fluosol-DA and carbogen had a significant effect upon tumor metabolism, as observed by 31P NMR spectroscopy, an analysis of variance with repeated measures was used (22). Scheffé multiple comparison tests were calculated to compare the NMR measurements before and after treatment. There were significant increases in PCr/Pi after treatment with Fluosol-DA plus carbogen in the small \( (F = 15.12, P < 0.001) \) and in the medium \( (F = 8.86, P < 0.01) \) tumor volume cohorts. The largest tumors had no statistically significant changes in PCr/Pi. No significant changes were noted for NTP/Pi or pH for any of the tumor volume groups. Mice bearing tumors showed no significant changes in PCr/Pi, NTP/Pi, or pH after treatment with either Fluosol-DA or carbogen alone.

In Table 2 the results of TCD\(_{50}\) measurements made on parallel cohorts of mice bearing s.c. grown (foot dorsum) tumors are presented. Dose-modification factors due to Fluosol-DA plus carbogen (the ratio of TCD\(_{50}\) values without drug and with drug) were 1.36 and 1.17 for the small and medium tumor volumes, respectively. No substantial increase in radiosensitivity was noted in the large tumor volume group. Modest dose-modification factors values were measured with carbogen alone in the small (1.08) and medium (1.11) tumor volumes. No radiosensitization with carbogen was observed in the large tumor volume. No effect was observed for animals treated by Fluosol-DA emulsion alone.

Fig. 3 is a graphical presentation of the change in TCD\(_{50}\) \( (\Delta \text{TCD}_{50}) \) plotted against the change in PCr/Pi, for each of the nine groups in Table 1. There is an excellent fit to a linear plot \( (r = -0.93, P < 0.001) \), indicating that, for small degrees of radiosensitization (dose modification factor \( \leq 1.36 \)), there is an excellent correlation between radiosensitization and alterations in PCr/Pi. Thus, in this tumor model, the radiobiological sen-

![Figure 2](image-url)  
**Fig. 2.** Changes in PCr/Pi, after treatment with Fluosol-DA plus carbogen are plotted against tumor volume. The data are grouped into size categories of 200 mm\(^3\) (200–400 mm\(^3\), 400–600 mm\(^3\), 900–1100 mm\(^3\), and >1100 mm\(^3\)). Changes in PCr/Pi, that occur after treatment decrease as tumor volume increases.

![Figure 3](image-url)  
**Fig. 3.** Plot of the change in TCD\(_{50}\) after treatment against the associated change in PCr/Pi. Correlation coefficient \( (r) = -0.93 \) \( (P < 0.001) \), indicating that changes in PCr/Pi, are related to changes in tumor radiosensitivity in this tumor model.
sitivity enhancement parallels changes in PCr/Pi, detected non-invasively by 31P NMR.

**DISCUSSION**

The proposed mechanism of radiosensitization with Fluosol-DA and carbogen is via increased oxygen delivery to the tumor. A possible mechanism whereby Fluosol-DA acts as a radiosensitizer is due to the smaller (0.2-µm) size of the emulsion particles compared to RBCs (7-µm); therefore, the drug can deliver oxygen by traversing small tortuous capillaries, whereas RBCs cannot. The results of this study demonstrate that concomitant treatment with Fluosol-DA and carbogen can potentially enhance delivery of oxygen to hypoxic regions of the tumor, with a resultant change in cellular oxygen status and metabolism, as was detected noninvasively by NMR spectroscopy. In a previous study (16), it was hypothesized that the hypoxic murine MCa tumor (17) may undergo significant oxidative phosphorylation but had relatively low PCr/Pi ratios because of limitations of oxygen delivery. Thus, if treatment with Fluosol-DA plus carbogen did increase oxygen delivery to hypoxic areas, an increase in the energy level, as manifested by relative changes in PCr or NTP, would be expected.

In this study, the PCr/Pi ratio, as measured by 31P NMR spectroscopy, most closely predicted the change in tumor radiosensitivity due to treatment with Fluosol-DA plus carbogen. Previously, TCD50 and hypoxic cell fractions within a given tumor model were found to be related to PCr/Pi (9, 23). Okunieff et al. (9) related increases in hypoxic cell fraction to decreases in PCr/Pi. They also noted a linear relationship between NTP/Pi and mean tumor oxygen partial pressure, as measured by electrodes (24). Rofstad et al. (13) found that within a given cell line there was a linear correlation between (PCr plus NTPβ)/Pi and the fraction of vessels with a hemoglobin saturation of greater than 30%. However, they noted that comparisons between metabolite ratios of different tumor lines were of no use in predicting relative hemoglobin saturation. Mueller-Klieser (25) noted a positive correlation between hemoglobin saturation and ATP concentrations measured by bioluminescence.

Similarly, it has been noted previously that changes in PCr/Pi can be induced by agents that modify tumor oxygenation. Okunieff et al. (14) showed that, when 100% oxygen was administered to mice bearing FSAI and MCA IV tumors, there were prominent changes in PCr/Pi, although not in NTP/Pi, similar to the results noted in the current study for tumors between 150 and 350 mm³. Irradiation of a hypoxic tumor model has been associated with an increase in PCr/Pi, suggesting that in this tumor model delivery of oxygen is a limiting factor for energy production.

We estimate the precision of the measurement of PCr/Pi and NTP/Pi to be approximately 10–15% of the measured value, based on previous studies (23). Similar uncertainties are estimated from the data on the cohort of mice treated with Fluosol-DA alone (Table 1), where no NMR or radiobiological changes were found. Therefore, the probable cause of the scatter in the PCr/Pi values (Fig. 2) is due to tumor heterogeneity. As tumors grow, diffusion barriers for nutrient and oxygen delivery become more restrictive, with variation from animal to animal, resulting in variability of the NMR data. At very large tumor volumes (>4% of total body weight), a restricted nutrient supply is present in almost all tumors and, therefore, less variation is noted in the NMR data. This is suggested by the larger range of values of pretreatment PCr/Pi, for small (0.11–0.59) and medium (0.03–0.56) tumors compared to large (0.04–0.31) tumors. The smaller range of PCr/Pi values for large tumors occurs despite the much larger volume range (900–1650 mm³) of tumors in this cohort.

The relationship between tumor radiosensitization (ΔTCD50) and change in PCr/Pi indicates that rapid metabolic alterations can occur in response to tumor oxygen status. The data suggest that in this tumor model delivery of oxygen is a limiting factor for energy production. Further studies are necessary to determine whether increased energy production is due to a shift in the relative utilization of different metabolic pathways or an absolute increase of ongoing metabolic activity.

Fig. 3 suggests that it may be feasible within a particular tumor model to relate changes in tumor hypoxia and radioreistance to metabolic alterations measured noninvasively by 31P NMR spectroscopy, i.e., to relate metabolic and radiobiological hypoxia. Since the NMR data are a measurement of the “averaged” metabolic profile of the whole tumor, it might be somewhat unexpected to find that it correlated with changes in the hypoxic fraction or radiosensitivity of the tumor. It is possible that the metabolic alterations induced by Fluosol-DA plus carbogen are occurring primarily due to increased oxygen delivery to the hypoxic, oxygen diffusion-limited regions of the tumor. Thus, the changes in tumor metabolism detected by 31P NMR spectroscopy may arise primarily from the hypoxic fraction and not from well oxygenated regions, explaining the correlation between changes in TCD50 and PCr/Pi. Since the metabolic changes detected may be occurring in only a fraction of the tumor volume, a very large percentage increase in PCr/Pi would not be expected. An alternative mechanism of Fluosol-DA radiosensitization is increased tumor blood flow (27), which could lead to increased oxygen and nutrient delivery. This mechanism might also lead to alterations of tumor metabolism in nonhypoxic regions, which would affect the correlation between NMR-derived measurements and radiosensitization. Further studies are necessary to determine if, within other tumor model systems, changes in PCr/Pi, and radiosensitization are correlated.

It has previously been shown that, under conditions of higher oxygenation status such as induced by carbogen plus Fluosol-DA, the longitudinal relaxation time of Pi, is decreased (28). Since these experiments were done under conditions of partial saturation, the area of the Pi peak was relatively less saturated under oxygenated conditions, thereby increasing the area under the Pi resonance. Therefore, a larger change in PCr/Pi would...
likely have been observed if the experiment had been done under conditions of full relaxation.

The association between changes in PCr/Pi and enhanced tumor radiosensitization suggests that 31P NMR spectroscopy may be useful to monitor patients being treated with perfluorochemical radiation sensitizers, to determine if it is likely to be an efficacious therapy. The use of 31P NMR spectroscopy to study tumors in patients receiving antineoplastic therapy has been demonstrated (29–32). Future clinical studies may require an increase in the signal to noise ratio or relatively large volume elements for spectroscopic study, in order to minimize the error in estimation of peak area. The study of human tumors will be far more complex, because of tumor heterogeneity between patients or even within different volume elements of the same tumor. Potentially, it may be feasible to map tumor hypoxia and to determine the extent of local radiosensitization by observing changes in NMR measurements and, subsequently, to adjust treatments accordingly.

Tumor hypoxia may be “acute” (33, 34), due to blood vessel constriction and intermittent closure (perfusion limited), or “chronic,” a result of oxygen diffusion gradients caused by cells being distant from tumor vasculature. The apparent lack of radiosensitization observed when Fluosol-DA plus carbogen were administered to animals with large tumor volumes (>1000 mm3) and the modest changes seen in the NMR studies would suggest that Fluosol-DA would not influence hypoxia due to blood vessel constriction. Fluosol-DA plus carbogen might exert an effect in clinically hypoxic tumor regions caused by oxygen diffusion gradients, which might be a predominant cause of hypoxia in smaller tumors. If further studies in other animal tumor models corroborate the correlation between radio-sensitization and 31P NMR studies, the implications for clinical trials of Fluosol-DA would be to require re-evaluation of the effects of chemotherapy. Cancer Res., 45: 2929–2933, 1985.


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