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Improving Oxygenation in the Murine Tumors by a perfluorochemical Emulsion (Fluosl-DA 20%)

Intae Lee, Ph.D., Gwi E. Kim, M.D.* and Chang W. Song, Ph.D.

Department of Therapeutic Radiology, University of Minnesota Medical School, Harvard Street at East River Road. Box 494 UMHC Minneapolis, MN 55455, U.S.A.

**Department of Radiation Oncology, Yonsei University Medical College, Yonsei Cancer Center, Seoul, Korea*

In the present study, a perfluorochemical emulsion (Fluosol-DA 20%) did not alter D_0 and D_q values on cell survival curve , indicating that the lack of a direct effect of Fluosol-DA 20% on cellular radiosensitivity ***in vitro***. The effect of Fluosol-DA 20% injection in combination with carbogen breathing was determined on the hypoxic cell fraction in SCK tumors. The hypoxic cell fraction in control SCK tumors was 0.39. This value decreased to 0.05 when the mice were i.v. injected with 12 ml/kg of Fluosol-DA 20% in a carbogen atomosphere. The measured mean and median PO_2 values with a microelectrode in the control tumors was 9 mmHg and 4 mmHg, respectively. The treatment of the SCK tumors in the host mice with injected Fluosol-DA 20% in combination with carbogen breathing increased the mean and median PO_2 values to 67 mmHg and 62 mmHg, respectively. Using carbogen breathing alone caused a moderate increase of tumor PO_2. But Fluosol-DA 20% injection alone caused little change PO_2 in the tumor. It was concluded that the combination of Fluosol-DA injection and carbogen breathing is an effective means to improve oxygenation of tumors.

Key Words: A Perfluorochemical Emulsion, Fluosol-DA 20%, Intratumor pO_2, Hypoxic cell fraction, Carbogen breathing, SCK tumors

INTRODUCTION

The radiation-induced growth delay and cure of murine tumors can be markedly enhanced when the host animals are i.v. injected with Fluosol-DA 20% in a carbogen atomsphere before and during irradiation of the tumors[1,2~4]. The treatment of C_3H mice bearing RIF-1 tumors with Fluosol-DA 20% in combination with carbogen markedly increased the pO_2 in the tumors[3~5]. In the present study, the changes in hypoxic cell fraction in addition to the PO_2 in SCK tumor of A/J mice were measured. This study used the more hypoxic SCK tumors in order to better understand the mechanism and factors responsible for the radiosensitizing effect of Fluosol-DA 20% and carbogen breathing.

MATERIALS AND METHOD

1. Tumors

The SCK tumor cells were grown *in vitro* with the RPMI 1640 medium supplemented with 10% calf serum. The cells were harvested during exponential growth phase by treatment with 0.25% trypsin solution. About 1×10^5 viable cells, suspended in 0.05 ml medium, were injected subcutaneously into the right thigh of 8 to 10-week-old A/J mice. Tumors size in the host mice in range of 100-130 mm^3 were used for the experiment.

2. Perfluorochemical Emulsions (Fluosol-DA 20%)

Fluosol-DA 20% (Alpha Therapeutic Corporation, Los Angeles, CA, USA) is an emulsified perfluorochemical preparation consists of perfluorodecalin (14 g/100 ml) and perfluoropropylamine (6 g/100 ml) in Krebs' Ringer bicarbonate solution. Other components of the preparation are Pluronic F-68 and yolk phospholipids as emulsifiers, and glycerol as a stabilizer[1].

3. ***In Vitro*** Survival Studies

The SCK cells in the exponential growth phase *in vitro* were dispersed to single cells by treatment with 0.25% trypsin solution for 10 min. at 37℃. Specific numbers of cell were cultured in the flask and kept in a humid incubator (95% air and 5% CO_2) at 37℃. The cells were maintained under these conditions for 4 hr. Fluosol-DA was then added to each culture flask at a dose of 15 ml/liter in a RPMI culture media. The cells were incubated for another hour. Untreated cells as well as treated

with Fluosol-DA were then X-irradiated, and incubated for 1 week. Only colonies containing 50 or more cells were counted in each experiment. Radiation survival curves for both Fluosol-DA treated and untreated cells were constructed. D_0 was calculated from the slope of the best-fit line of regression by the least square method.

4. Determination of Hypoxic Cell Fraction in Tumors

Tumor-bearing mice were randomly divided into 4 groups and used for the following studies. Group 1: the host mice were asphyxiated with N_2, and then the tumors were X-irradiated. Group 2: the host mice breathed normal air alone, or air in combination with 12 ml/kg of Fluosol-DA, and then the tumors were irradiated. Group 3: the host mice were kept in a carbogen atomosphere for 1 hr. before and during irradiation of tumors. Group 4: the host mice were injected with 12 ml/kg of Fluosol-DA and kept in a carbogen atmosphere for 1 hr. before and during irradiation of the tumors. For the irradiation of Group 2~4 tumors, the host mice were anesthetized with pentobarbital at 50 mg/kg, and placed in a circular Plexiglas jug.

The tumors were irradiated with various doses of X-rays in a single exposure. The radiation source was a G.E.. Maxima 220 X-ray unit and the physical factors were 220 kVp, 15 mA, and filtration with 1.0 mm Al and 0.25 mm Cu. The FSD was 40 cm. Tumors were excised soon after irradation and dispersed to single cells. The clonogenecity of the recovered cells were determined using *in vitro* culture and the surviving fraction of tumor cells was calculated. By comparing the terminal portion of the survival curve, the hypoxic cell fraction in the tumors of each group was determined.

5. Measurement of PO_2

Polarographic measurement of tumor pO_2 was done using recessed tip microelectrode with diameters of 40~50 μm. The electrodes were constructed in our laboratory, as described previously[4,5]. The current-voltage curve for each electrodes ranged from −0.6 to −0.7 volts. The electrodes were calibrated by immersing them in a series of 0.9% saline solution saturated with 100% N_2, 10% O_2 plus 90% N_2 or 20% O_2 plus 80% N_2. The O_2 electrodes were recalibrated before and after each measurement of tissue PO_2. The mice were anesthetized with an i.p. injection of 180 mg/kg Inactin (sodium salt ethyl-(1-methyl-propyl)-malonyl-thio-urea-) (BYK Gulden Konstanz, West Germany) and placed on an electrically insulated holder in a Faraday cage. The tail vein was cannulated with a 30-gauge needle, which was connected to a 1 ml syringe containing Fluosol-DA. The skin over the tumor was carefully removed using a pair of fine scissors and a scalpel. The exposed tumor surface was immediately covered with gauze moistened with 0.9% saline. An aforementioned oxygen electrode was carefully inserted and advanced into the tumors with the use of a micromanupulator. A reference electrode was also inserted into the periphery of the tumor. The mice were injected with Fluosol-DA through the tail vein and breathed carbogen throguh a plastic cone-shaped mask placed over the head. The sequence of the carbogen breathing before and during the dosage of the Fluosol-DA injection were varied. The electrical current generated from the sensor electrode was continuously monitored and recorded with a chart recorder.

RESULTS

In order to elucidate whether Fluosol-DA directly modifies the radioresponse of tumor cells, SCK tumor cells *in vitro* were exposed to X-irradiation in

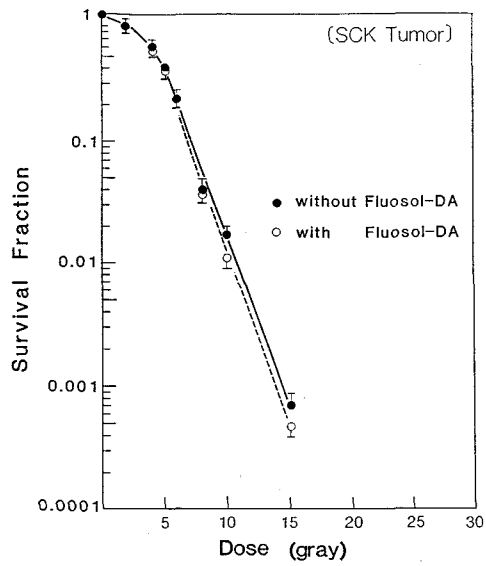

Fig. 1. Survival curves of SCK tumor cells following exposure to X-irradiation *in vitro with* (open circles) and without (closed circles) the administration of Fluosol-DA 20% *in vitro* before 1 hr. of graded doses of X-irradiation. (points = mean values from five experiment; bars = standard errors)

the presence of Fluosol-DA. Fig. 1 shows the effect of Fluosol-DA on the radioresponse of SCK tumor cells *in vitro*. The plating efficiency of control SCK tumor cells was not significantly different ($p>0.1$) than that of cells treated with Fluosol-DA (60+1% (. S.E.)). The cell survival, analyzed by linear regression and tested by Student's t-test ($p>0.1$), were shown to have identical D_0 values ($D_0=1.4$ Gy) as well as D_q values ($d_q=3.8$ Gy), indicating the lack of a direct effect of Fluosol-DA on cellular radiosensitivity *in vitro*.

The percent survival of SCK tumor cells after irradiation of the tumors *in vivo* determined by using *in vitro* cloning methods is shown in Fig. 2. The terminal portion of the survival curves for the tumor cells of mice treated with both Fluosol-DA and carbogen breathing as well as those treated with carbogen alone were parallel with the survival curve of the tumor cells of the air breathing mice

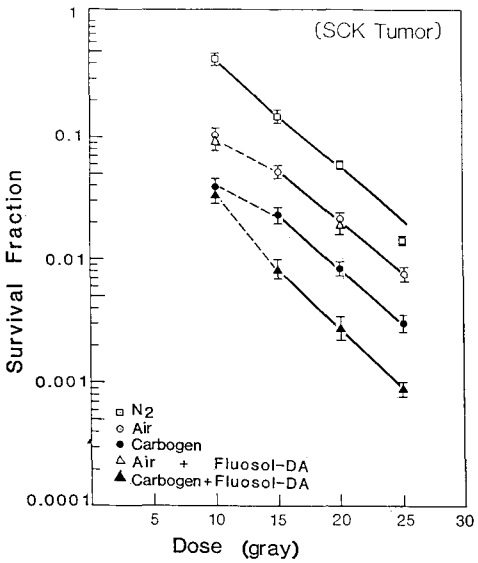

Fig. 2. Survival curves of SCK tumors cells X-irradiated *in vivo*. The animal breathed air alone (open circles) or air in combination with an i.v. injection of Fluosol-DA 20% (open triangles). Before and during irradation of tumors, the host animals breathed carbogen (closed circes), or carbogen in combination with an i.v. injetion of Fluosol-DA 20% (close triangles). The other group was asphyxiated by N_2 (open squares) prior to X-irradiation. Lines between 15 Gy and 25 Gy were determined by linear regression. (point=mean values from about 30 tumors; bars=standard errors)

($p>0.05$ in all cases). This suggested that the treatment of mice with Fluosol-DA and carbogen breathing changed the hypoxic fraction in the tumors without alteration of the slopes. A comparison of the terminal portion of the survival curves demonstrated that the hypoxic cell fraction in the SCK tumors of air breathing mice was about 0.39. When the animals breathed carbogen, the hypoxic cell fraction in the tumors decreased to 0.15. Treatment of mice with Fluosol-DA injection and carbogen breathing further decreased the hypoxic cell fraction in the tumors to about 0.05. The hypoxic cell fractions of mice treated with different protocols (for example; air alone, carbogen alone, or Fluosol-DA plus carbogen, etc.) were significantly different from that of mice asphyxiated with N_2 ($p<0.01$ in all cases). Fig. 3 is a segment of PO_2 measurement taken in a SCK tumor. The PO_2 before treament was 0 mmHg. Upon termination of carbogen breathing, the PO_2 rose to 35 mmHg in 3 ~4 min. When 12 ml/kg of Fluosol-DA was i.v. injected and the animal breathed carbogen, the PO_2 increased to 70~75 mmHg. Upon termination of carbogen breathing, the PO_2 values immediately dropped. By carefully advancing the electrode, it was possible to mearsure PO_2 values at 2~3 places along each track. Changes in PO_2 values in SCK tumors under various conditions are shown in Talbe 1. It can be seen that more than 50% of the PO_2 measured were less than 5 mmHg in the tumors of air-breathing mice. The mean and

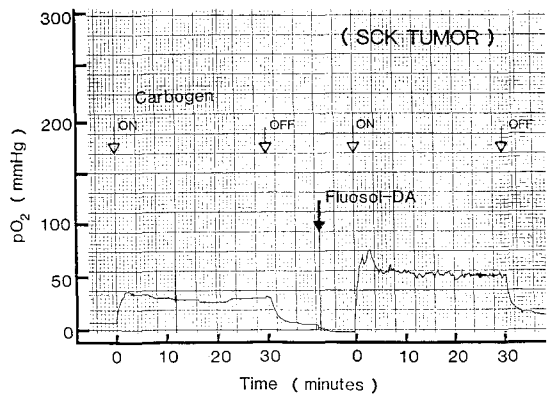

Fig. 3. Segment of actual PO_2 recording in an SCK tumor. The host A/J mouse breathed carbogen in combination with Fluosol-DA 20% injection at a dose 12 ml/kg., Carbogen breathing alone increased intratumor PO_2, and the addition of Fluosol-DA 20% injection futher increased the intratumor PO_2.

Table 1. Intratumor PO_2 Values (mmHg) in SCK Murine Tumors Under Various Conditions

	Mean	Median	Mode
Air Breathing N=344	9.4 (8.1 – 10.7)*	3.8	0 – 5 (55%)**
Carbogen Breathing N=246	32.2 (27.5 – 36.8)*	19.8	0 – 5 (31%)**
Fluosol-DA + Air Breathing N=346	10.0 (8.6 – 11.4)*	6.1	0 – 5 (39%)**
Fluosol-DA + Carbogen Breathing N=227	67.5 (61.4 – 73.6)*	62.3	over 90 (30%)**

* 95% Confidence interval
** percentage from total numbers of measurements
N numbers of measurements

median PO_2 values in the control tumors were 9 mmHg and 4 mmHg, respectively. Carbogen breathing significantly increased the tumor PO_2, but Fluosol-DA injection alone had little effect on the tumor PO_2. When the mice were injected with Fluosol-DA and kept in a carbogen atmosphere, the mean and median PO_2 increased to 67 mmHg and 62 mmHg, respectively.

DISCUSSION

Preclinical as well as clinical studies demonstrated that Fluosol-DA injections in combination with breathing oxygen enriched gas (carbogen, hyperbaric oxygen) significantly enhanced the response of tumors to radiation[1~4,6]. The results obtained in the present study unequivocally demonstrated that an i.v. injection of Fluosol-DA in combination with carbogen breathing markedly improves the oxygenation of tumors and thereby decreased the hypoxic cell fraction in the tumors. It has been known that perfluorochemicals, such as Fluosol-DA, dissolve an extraordinary large amount of oxygen. The solubility of O_2 in perfluorochemicals is dependent on environmental PO_2 so that the O_2 dissolved in perfluorochemicals is easily and quickly released when the environmental PO_2 is decreased. Apparently, the i.v. injected Fluosol-DA dissolved a large quantity of oxygen in the lung when the hosts breathe carbogen and releases oxygen in the hypoxic area in tumors. The use of hyperbaric oxygen instead of carbogen breathing to increase the amount of O_2 transported by Fluosol-DA has been proposed by other investigators[7]. Indications are that, in addition to the above mentioned mechanism, there are other mechanisms by whcih Fluosol-DA injection plus carbogen breathing might increase the tumor PO_2. Our preliminary studies, along with recently developed laser Doppler flowmetry, demonstrated that i. v. injection of Fluosol-DA as well as carbogen breathing increases blood flow in tumors[8]. An increase in blood flow may facilitate the oxygen delivery by Fluosol-DA as well as by the host RBC and plasma. The mechanism of the increase in blood flow by Fluosol-DA is not clear, but it has been postulated that Fluosol-DA decreases blood viscosity and dilates blood vessels[7]. It has also been known that carbogen breathing causes vasodilation and increas blood flow[9].

in summary, Fluosol-DA did not alter the radiosensitivity of tumor cells as shown by our *in vitro* cell survival studies. The hypoxic cell fraction decreased from 0.39 to 0.05 by the treatment of i.v. injection of Fluosol-DA in combination with carbogen breathing. As a consequence, an i.v. injection of Fluosol-DA in combination with carbogen breathing significantly increases delivery of oxygen to tumors and oxygenates hypoxic cells within tumors. We conclude that Fluosol-DA injection in combination with carbogen breathing is an effective means to increase tumor oxygenation.

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= 국문초록 =

Carbogen 흡입하에서 Fluosol-DA 20%의 투여가 이식동물 종양의 산소분압에 미치는 영향

미네소타 의과대학 치료방사선과 방사선생물학부, 연세대학교 의과대학 치료방사선과학교실*

이인태 · 김귀언* · 송창원

SCK 종양세포를 이식받은 mice에 Fluosol-DA 20%를 정맥주사한 후 이 종양세포를 추출하여 in vitro 실험으로 측정해 본 방사선 체포생존곡선의 Do와 Dq의 값은 Fluosol-DA 20%를 투여하지 않은 대조군과 대동소이하여 Fluosol-DA 20%가 방사선 감수성 자체에 미치는 영향은 미미하다고 해석되었다. 또한 Fluosol-DA 20%만을 투여한 종양의 산소분압(PO_2)에는 별 변화가 없었으나 Fluosol-DA 20%를 Carbogen 흡입하에 투여시킨 종양에선 대조군의 산소분압 중앙값 4 mmHg가 62 mmHg로 10배이상 증가되어 reoxygenation effect를 직접 증명할 수 있었고 hypoxic cell fraction도 약 8배정도 감소됨을 규명하였다. 한편 Carbogen만을 단독으로 흡입시킨 종양의 경우에도 산소분압의 증가를 관찰할 수 있었으나 Fluosol-DA 20% 병용군보다는 산소분압상승 효과가 미약함을 관찰할 수 있었다.

따라서 Fluosol-DA 20%에 의한 방사선 반응의 향상은 방사선 감수성의 증가보다는 reoxygenation의 결과이며 reoxygenation 효과를 개선하기 위해서는 Fluosol-DA의 단독투여보다는 Carbogen 흡입하에서 Fluosol-DA 20%의 투여가 이상적이라는 결론을 얻었다.