Hypoxic Tumor Can be More Responsive to Fractionated Irradiation Combined with SR 4233 (Tirapazamine)

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= Abstract =

Hypothesis that hypoxic tumors should be more responsive to the addition of preferential hypoxic cell cytotoxin SR 4233 (tirapazamine) to fractionated irradiation was tested in the mouse SCCVII carcinoma and RIF-1 sarcoma. Model of hypoxic tumor was established using the tumor bed effect; tumors growing in the preirradiated tissue (preirradiated tumors) were more hypoxic than tumors growing in the unirradiated tissue (unirradiated tumors). When the tumors reached a mean volume of 100 mm^3, both unirradiated and preirradiated tumors were treated with a fractionated course of 6×2 Gy in 3 days or 8×2.5 Gy in 4 days with SR 4233 (0.08 mmol/kg /injection) given 30 minutes before each irradiation or without SR 4233. Compared to the unirradiated tumors, hypoxic preirradiated tumors were approximately 5 times more resistant to fractionated irradiation alone but were approximately 5 times more responsive to SR 4233. Addition of SR 4233 potentiated the effect of fractionated irradiation in both unirradiated and preirradiated tumors. Potentiation in the preirradiated tumors was morequal to or greater than that in the unirradiated tumors and seemed to be higher for more fractionated treatment. We confirm the hypothesis in a transplantable mouse tumor. Present results suggest that radioresistance of some hypoxic tumors can be overcome with hypoxic cytotoxin.

Key Words : Hypoxia, Tumor bed effect, SR 4233 (tirapazamine), Radiation

INTRODUCTION

The presence of hypoxic clonogenic cells ciritically influences the response of tumors to radiotherapy[1]. A variable proportion of hypoxic cells were confirmed to be present in almost all transplantable tumors in rodents[2,3]. There is also both direct and indirect evidence for the existence of hypoxic cells in a large proportion of human tumors,[4-7]

As a way of overcoming tumor hypoxia, bioreductive hypoxic cell toxins have been enthusiastically investigated. SR 4233 (3-amino-1, 2, 4-benzotriazine-1, 4-dioxide), one of the most promising bioreductive hypoxic cell toxin, preferentially kills hypoxic cells *in vivo*[8,9] and *in vitro*[10]. When SR 4233 was combined with radia-

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tion or some anticancer chemotherapeutic agents, the combined treatment resulted in more cell killing than anticipated from addition of two effects in some rodent tumors[11-13]. Thus the hypoxic cells in certain tumor now are no longer obstacle to the way for the local control of tumor but can be exploited if bioreductive radiotherapy could be applied.

At this moment when the phase I clinical trial of the drug is currently going on, we hypothesized that the tumors with higher fraction of hypoxic cells, such as tumors recurring after radiotherapy, should be more responsive to the combined treatment with SR 4233 and radiation than less hypoxic tumors. To test this hypothesis, we have measured clonogenicity after fractionated treatments in tumors growing in unirradiated tissue and tumors growing in preirradiated tissue, that is more hypoxic resulted from the tumor bed effect[14-15], using mouse SCCVII and RIF-1 tumors. If response of more hypoxic tumors to combined radiation and SR 4233 when compared to response to radiation alone is equal to or greater than of less hypoxic tumors, it might suggest, therefore, clinical usefulness of bioreductive radiotherapy in some hypoxic tumors if tumor hypoxia could be confirmend before treatment.

MATERIALS AND METHODS

1. Mice and Tumors

SCCVII carcinomas and RIF-1 sarcomas in female C3H/Km mice were used in these experiments. The tumors were maintained alternatively *in vivo* and *in vitro*. The mice were bred and held under defined flora condition in the Stanford Research Animal Facility and kept during experiments in the Stanford Radiation Biology Mouse Facility. Mice were 12-16 weeks old and weighed 25-35 g. The derivation of the cell lines and details of handling have been described[16-18]. Tumor cells were harvested from monolayer culture and 2×10^5 tumor cells in 0.05 ml of Waymouth's media with 15% fetal calf serum were inoculated in the lower back intradermally or thigh of right hindleg intramuscularly. Mice were anesthetized by intraperitoneal injection of pentobarbital sodium (67.5 mg/kg) before inoculation of tumor cells.

2. Tumor Models

Experiments were performed in tumors growing in preirradiated tissue ('preirradiated tumors') and tumors growing in unirradiated normal tissue ('unirradiated tumors'). Preirradiated tumors usually have higher fractions of hypoxic fraction than unirradiated tumors usually have higher fractions of hypoxic fraction than unirradiated tumors because of the tumor bed effect. The profiles of tumor hypoxia and details of producing these two types of tumor models with SCCVII and RIF-1 tumors have been described previously[19]. In brief the tumor beds (i.e., lower back) of the mice were irradiated (20 Gy in single fraction) 4 weeks before inoculation of tumor cells to establish the preirradiated tumors. For the irradiation of tumor bed, the unanesthetized mice were placed in individual lead jigs with a cut-out to enable the tumor bed to be irradiated without irradiation of the rest of the mouse. Irradiation was done using a 250 kVp X-ray apparatus at a dose rate of 1.69 Gy/min (Philips RT 250; 15 mA with 0.35 mm Cu filter, SSD of 31 cm). No specific treatments were applied to the tumor beds of the unirradiated tumors before the inoculation of tumor cells. Fractionated treatments were performed when volumes of back tumors reached about 100 mm^3. Volumes (V) of back tumors were calculated by an ellipsoid approximation using the 3 orthogonal diameters a, b, and c ($V=abc\pi/6$). Measured values were corrected for skin thickness. Hypoxic fractions (%), measured by paired survival curve method, of SCCVII tumors were 2.1% in tumors growing unirradiated back, and 18.1% in the tumors growing in preirradiated back. For the RIF-1 sarcomas the hypoxic fractions were 1.0% in tumors growing in irradiated back, and 13.6% in tumors growing in preirradiated back.

3. Drug

SR 4233 (tirapazamine, 3-amino-1, 2, 4-benzotriazine-1, 4-dioxide : MW = 179.95) was synthesized by Dr. Michael Tracy (SRI International, U.S.A.). The drug was freshly dissolved in physiological saline and injected intraperitoneally in a volume of 0.04 ml/g of body weight at a dose of 0.08 mmol/kg.

4. Irradiation

Unanesthetized mice were placed in individual lead jigs with a cut-out to enable the whole tumor to be irradiated without irradiation of the rest of the mouse. Irradiation conditions were the same as those for the tumor bed irradiation.

5. Fractionated Treatment

Four arms of treatments such as saline (0.04 ml/g) as control, tirapazamine alone, radiation alone, and combination of radiation and tirapazamine were delivered by two different schedules of fractionation ; 6 fractions in 3 days and 8 fractions in 4 days. In the arm of radiation combined with tirapazamine, the drug was injected 30 min before irradiation. Each fractionation schedule was twice-a-day treatment with an interval of 12 hr. The dose of tirapazamine per fraction was 0.08 mmol/kg regardless of fractionation schedule but radiation dose per fraction, in both cases when radiation was used alone or combined with tirapazamine, was 2.0 Gy for 6-fraction schedule and 2.5 Gy for 8-fraction schedule.

6. Evaluation of Tumor Response

Tumor response was evaluated by *in vivo/in vitro* clonogenic assay. Mice with back tumors, both the SCCVII and RIF-1, were sacrificed 12 hr after the last fractionated treatment, and their tumors were excised, weighed, chopped and disaggregated with an enzyme cocktail, and appropriate number of cells were then plated into culture dishes containing Waymouth's media with 15% fetal calf serum. Dishes were stained with crystal violet after 12-14 days of incubation. Three to six independently assayed tumor-bearing mice were used for each experiment. Cell yield per tumor was the total number of viable cells which excluded trypan blue dye in suspension. Relative clonogenic cells per tumor were calculated as the product of surviving fraction and cell yield per tumor after treatment relative to those obtained from saline-treated control tumors. The surviving fraction was the proportion of viable cells which subsequently form colonies relative to control tumors.

RESULTS

1. SCCVII Tumor

Results are plotted by the fractions in Fig. 1; 6 fractions in 3 days (Fig. 1A, B, and C) or 8 fractions in 4 days (Fig. 1D, E, and F). Cell yield per control tumor, which was not treated, is lower by 1 to 2 log in the preirradiated tumors compared to unirradiated tumors (Fig. 1A & D). For the unirradiated tumors, reduction in the cell yield is higher after irradiation combined with SR 4233 when compared to tumors treated with radiation of SR 4233 alone. For the preirradiated tumors, meanwhile, this is not the case. Although treatments reduce cell yields, differences in cell yields are not remarkable.

As for the surviving fractions or relative clonogenic cells per tumor (RCC/T), we obtained a typical pattern of oxic and hypoxic tumors as expected (Fig. 1B & E and Fig. 1C & F). Compared to the rather oxic unirradiated tumors, hypoxic preirradiated tumors are approximately 4-5 times more resistant to fractionated irradiation but are approximately 4-6 times more responsive to hypoxic cytotoxin SR 4233. Effect of fractionated irradiation is potentiated by the addition of SR 4233 in both preirradiated and unirradiated tumors.

For tumors growing in the unirradiated tissue, radiation alone (2.5 Gy × 8) produced a RCC/T of 3.27×10^{-3}. SR 4233 alone produced a RCC/T of 0.209. The combination of SR 4233 given 30 min

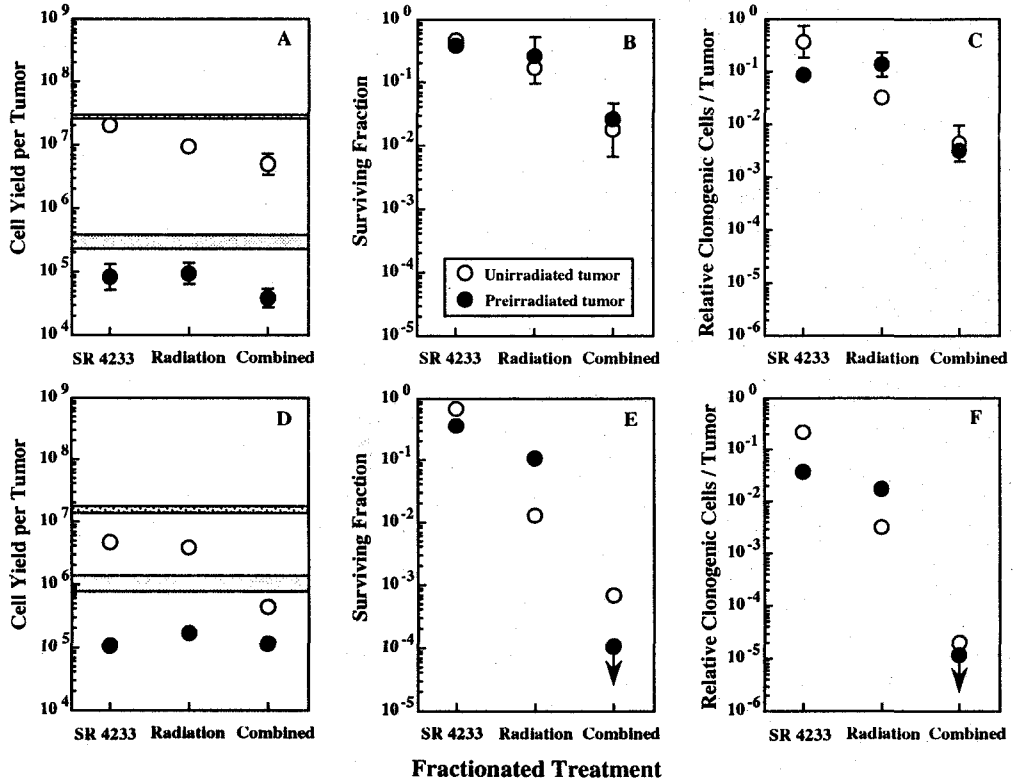

Fig. 1. (A, B, C) Results of a fractionated treatment (6 fractions/3 days) with radiation (2.0 Gy), SR 4233 (tirapazamine, 0.08 mmol/kg), and SR 4233 combined with radiation in the SCCVII mouse carcinoma. (D, E, F) Results of a fractionated treatment (8 fractions/4 days) with radiation (2.5 Gy), SR 4233 (tirapazamine, 0.08 mmol/kg), and SR 4233 combined with radiation in the SCCVII mouse carcinoma. Tumors were grown in unirradiated (open circles) or preirradiated (closed circles) back. In Panel A and D, dotted areas denote ranges of cell yield per tumor that were not received any treatment; areas with coarse dots for the tumors grown in unirradiated back or fine dots for the tumors grown in preirradiated back. Each point shows the mean of 3-6 independently assayed tumors($\pm$1 S.E.M).

before each fraction of irradiation produced a RCC/T of 1.95×10^{-5}. Thus RCC/T is approximately 170-fold tissue, meanwhile, radiation alone produced a RCC/T of 1.74×10^{-2}, SR 4233 did 3.64×10^{-2}. The combination of SR 4233 produced a RCC/T of 1.20×10^{-5}. So RCC/T is approximately 1,500-fold potentiated than that of radiation alone. Table 1 shows a comparison of potentiation factors based on relative clonogenic cells per tumor. Potentiation of radiation effect by the addition of SR 4233 is higher for the preirradiated tumors and for more fractionated treatment.

2. RIF-1 tumor

Results are plotted by the fractionations in Fig. 2; 6 fractions in 3 days (Fig. 2A, B, and C) or 8 fractions in 4 days(Fig. 2D, E, and F). Cell yield per control tumor is lower by 2 to 3 log in the preirradiated tumors compared to unirradiated tumors (Fig. 2A & D). For the unirradiated tumors, reduction in the cell yield is higher after irradiation combined with SR 4233 when compared to tumors treated with radiation or SR 4233 alone. For the preirradiated tumors, meanwhile, the cell yield of the control tumors are not

Table 1. Potentiation of effect of fractionated irradiation by combining# SR 4233 (tirapazamine ; 0.08 mmol/kg) with fractionated irradiation

Tumor type	Tumor site	Fractionation of irradiation	Tumor bed	Potentiation* factor
SCCVII	Back	6×2.0 Gy/3 days	Unirradiated	7.4
			Preirradiated	41.6
		8×2.5 Gy/4 days	Unirradiated	167.7
			Preirradiated	>1450.0
RIF-1	Back	6×2.0 Gy/3 days	Unirradiated	3.1
			Preirradiated	3.2
		8×2.5 Gy/4 days	Unirradiated	98.3
			Preirradiated	>153.2

SR 4233 (tirapazamine) was injected 30 min before each radiation dose.
* (Relative clonogenic cells per tumor after treatment with radiation alone)/(relative clonogenic cells per tumor after treatment with SR 4233 and radiation)

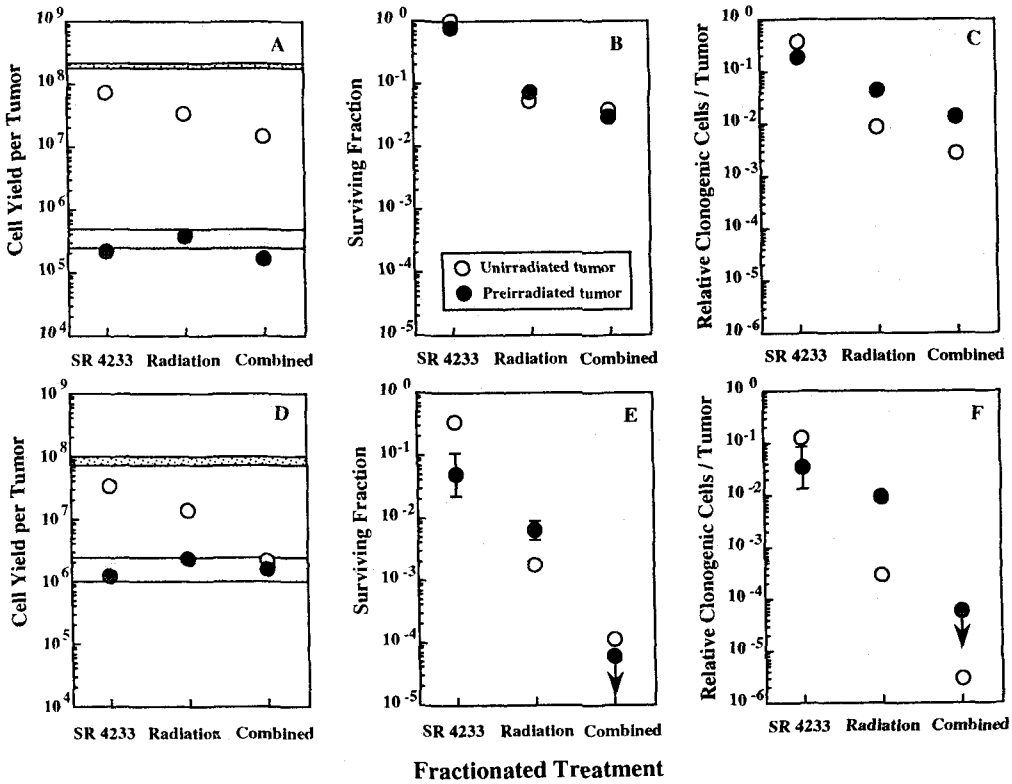

Fig. 2. (A, B, C) Results of a fractionated treatment (6 fractions/3 days) with radiation (2.0 Gy), SR 4233 (tirapazamine, 0.08 mmol/kg), and SR 4233 combined with radiation in the RIF-1 mouse sarcoma. (D, E, F) Results of a fractionated treatment (8 fractions/4 days) with radiation (2.5 Gy), SR 4233 (tirapazamine, 0.08 mmol/kg), and SR 4233 combined with radiation in the RIF-1 mouse sarcoma. Tumors were grown in unirradiated (open circles) or preirradiated (closed circles) back. In Panel A and D, dotted areas denote ranges of cell yield per tumor that were not received any treatment ; areas with coarse dots for the tumors grown in unirradiated back or fine dots for the tumors grown in preirradiated back. Each point shows the mean of 3-6 independently assayed tumors(±1 S.E.M.).

reduced after any treatments.

As for the surviving fractions or relative clonogenic cells per tumor (RCC/T), we obtained a typical pattern of oxic and hypoxic tumors as expected (Fig. 2B & E and Fig. 2C & F). Compared to the rather oxic unirradiated tumors, hypoxic preirradiated tumors are approximately 2-32 times more resistant to fractionated irradiation but are approximately 4-5 times more responsive to hypoxic cytotoxin SR 4233. Radiation effect is potentiated by the addition of SR 4233 in both preirradiated and unirradiated tumors.

For tumors growing in the unirradiated tissue, radiation alone (2.5 Gy×8) produced a RCC/T of 2.91×10^{-4}. SR 4233 alone produced a RCC/T of 1.127. The addition of SR 4233 to each fraction of irradiation produced a RCC/T of 2.96×10^{-6}. Thus RCC/T is approximately 100-fold potentiated than that of radiation alone. For tumors growing in preirradiated tissue, meanwhile, radiation alone produced a RCC/T of 9.30×10^{-5}. So RCC/T is approximately 150-fold potentiated than that of radiation alone. As shown in Table 1, potentiation in the preirradiated tumors is nearly equal to that in the unirradiated tumors with both fractionation schedules.

DISCUSSION

It has been well established that radiation response of transplantable mouse tumors is enhanced by combining agents which preferentially kills hypoxic cells to fractionated irradiation[20]. But the present series of experiments is the first demonstration that potentiation of radiation response of hypoxic tumors is equal to or greater than that of euoxic tumors of the similar size and the same histology. Not unexpectedly, the present data further show that the potentiation is greater with more fractionated irradiation. This finding is in line with the study that multifractionated combination regimen is more effective than combination of SR 4233 and single large dose of radiation[21]. If the fraction of hypoxic cells in the human solid tumor reestablish itself to the pretreatment level after each dose of SR 4233 as we demonstrated in SCCVII tumors (termed 'rehypoxiation') recently[8], it can be clinically implicated that hypoxic tumors are not radioresistant any more or can become more responsive than less hypoxic tumors by combining hypoxic cell cytotoxic agents to conventional fractionated radiotherapy. Although the size of SCCVII tumors growing in the unirradiated tissue in the present experiments has not been shown to be comprised largely of acutely hypoxic cells[22], It is not known whether this is also the case for the SCCVII tumors growing in the preirradiated tissue or not. If increased fraction of hypoxic cells of the preirradiated tumors are majorly chronically hypoxic cells instead of acutely hypoxic cells, other crucial process (than 'rehypoxiation') must exist to explain the present results of greater potentiation with more fractionated treatment. Study such as perfusion mismatch will answer this question.

A comment should be made for the reason why the preirradiated tumors have no greater potentiation than the unirradiated tumors by combining SR 4233 with fractionated irradiation in RIF-1 tumor. As already known, RIF-1 tumor has unique feature in terms of tumor oxygenation. First, there is no increase of radiobiological hypoxic fraction as tumor size increases from 100 mm^3 to 400 mm^3 contrary to other tumors[18,19,23]. Second, there is discrepancy between radiobiological hypoxic fraction and pO_2 or oxyhemoglobin saturation status[8,24], while there is correlation between these parameters in SCCVII tumor. The reason for this discrepancy in RIF-1 tumor is at present not known. The present results suggest that the difference in tumor oxygenation between SCCVII and RIF-1 tumors might result in the difference in potentiation of radiation response by combination of SR 4233 with fractionated irradiation.

In conclusion, it is expected that radioresistance of some human solid tumors which have high proportion of hypoxic cells can be overcome or can be more responsive than tumors

with good oxygenation by combining hypoxic cell cytotoxic agents with fractionated radiotherapy.

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=국문초록=

분할방사선조사와 SR 4233 병용에 의한 저산소분압 종양의 반응증강

서울대학교 의과대학 치료방사선과학교실, 스탠포드 의과대학 방사선종양학과

김 일 한 • J. 마틴 브라운

마우스 종양인 SCCVII과 RIF-1에서 선택적 저산소세포 치사제인 SR 4233 (tirapazamine)과 분할 방사선조사를 병용하면 저산소분획이 큰 종양에서 방사선 감수성이 더욱 증강될 것인가를 확인하기 위하여 본 연구를 실시하였다. 종양세포 이식이전에 방사선조사를 받은 정상조직에서 성장한 종양의 저산소세포 분획이 크다는 사실을 이용하여 저산소세포분획이 큰 종양 모델을 수립 사용하였다. 일정 용적의 ($100mm^3$) 종양에 대하여 1회 2 Gy를 1일 2회씩 6회 또는 1회 2.5 Gy를 1일 2회씩 8회 조사하는 등의 분할조사법을 사용하여 방사선조사를 시행하였다. SR 4233를 병용한 경우에는 매회 방사선조사 30분 전에 0.08 mmol/kg를 복강내 주사하였다. 정상조직에서 성장한 종양에 비하여 방사선 조사를 받은 조직에서는 성장한 종양은 분할 방사선조사에 대하여 약 5배의 내성을 보였으나 SR 4233 분할 치료에 대하여는 약 5배의 감수성을 나타내었다. 정상 종양 및 저산소 세포 분획이 큰 종양의 방사선 감수성은 공통적으로 SR 4233의 병용후 증강되었으며 저산소세포 분획이 큰 종양에서의 증강율이 정상종양에서의 증강율과 동일하거나 더욱 높았다. 따라서 선택적 저산소세포 치사제인 SR 4233과 분할 방사선조사를 병용할 경우 저산소분획이 큰 종양에서 치사효과가 더욱 증강된 사실을 확인하였다. 이러한 결과는 높은 저산소세포 분획에 의한 방사선내성을 보이는 종양중 일부는 저산소세포치사제와 방사선치료를 병용함으로써 국소적 종양관해 실패의 요인인 저산소 세포를 극복하는 임상 적용 가능성을 시사한다.

중심단어 : 저산소상태, SR 4233 (tirapazamine), 방사선, 종양기저조직효과